Mutated in colorectal cancer (MCC) : a novel centrosomal protein involved in intestinal homeostasis and disease

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**Mutated in Colorectal Cancer (MCC): A Novel Centrosomal
Protein Involved in Intestinal Homeostasis and Disease**

Lucian Brito Tomaz

SCHOOL OF BIOLOGICAL SCIENCES

2020

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Protein Involved in Intestinal Homeostasis and Disease**

Lucian Brito Tomaz

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2020

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Lucian Brito Tomaz

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Mutated in Colorectal Cancer (MCC) is a candidate tumor suppressor gene reported to be somatically mutated in the inherited colorectal cancer (CRC) syndrome Familial Adenomatous Polyposis. Additionally, *MCC* deletion and loss-of-heterozygosity have also been reported as a common event in human CRC. However, to date, more than 28 years since its discovery, the mechanisms by which *MCC* contributes to intestinal cancer development as well as its function during normal intestinal tissue homeostasis remain unknown. Here, I investigate the subcellular localization and function of the mouse *Mcc* homolog in the intestinal epithelium and the consequences of its loss *in vivo*. My studies reveal that *Mcc* expression is restricted to cycling cells within intestinal crypts. On a protein level, *Mcc* specifically localizes to the centrosome of proliferative cells. As cells undergo terminal differentiation, *Mcc* re-localizes from the centrosome to the apical membrane in enterocytes where it becomes a component of the Non-Centrosomal Microtubules Organizing Center (NMTOC), which is responsible for the establishment of apico-basal polarity and the maintenance of epithelial integrity. Redeployment of proteins from the centrosome to NMTOC sites has been previously reported in other epithelial tissues. However, the molecular mechanisms underlying this redistribution are poorly understood. My biochemical studies establish that phosphorylation of *Mcc* by Casein Kinases triggers the subcellular redeployment of centrosomal *Mcc* to the NMTOC. Consistent with a role in the establishment of apico-basal polarity, I additionally provide a comprehensive molecular and morphological characterization of previously unappreciated phenotypes in the adult intestine of *Mcc*-deficient mice as well as unexpected genetic interactions with the *Apc^{Min}* intestinal tumor model. Taken together, these findings reveal clinically relevant insights into the involvement of a novel centrosome component in intestinal tissue homeostasis and disease.

LIST OF ABBREVIATIONS

Å	Angstroms
AE	Absorptive enterocytes
AJ	Adheren Junctions
AMP	Antimicrobial Peptides
APC	<i>Adenomatous Polyposis Coli</i>
AT	Active transport
ATP	Adenosine triphosphate
BB	Brush border
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CBC	Crypt base columnar
CK1	Casein-kinase 1
CRC	Colorectal cancer
DLL	Delta-like
DP	Desmoplakin
E	Embryonic day
EGF	Epidermal growth factor
FAP	Familial adenomatous polyposis coli
FT	Facilitated transport
GC	Goblet cells
GI	Gastrointestinal
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IP	Immunoprecipitation
ISC	Intestinal stem cell
JAM	Junctional adhesion molecule
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAPK	Mitogen-activated protein kinase
MCC	Mutated in Colorectal Cancer
MeOH	Methanol
Min	Multiple intestinal neoplasia
MS	Mass spectrometry
MTOC	Microtubule-Organizing Center
MT	Microtubule
NCBI	National Center for Biotechnology Information
NMTOC	Non-Centrosomal Microtubules Organizing Center
NTC	Non-treated control
PBM	PDZ binding motif
PCM	Pericentriolar material
PFA	Paraformaldehyde
PFPE	Paraformaldehyde-Fixed Paraffin-Embedded
PKC	Protein kinase C
PT	Paracellular transport
RIN	RNA Integrity Number
RT-PCR	Reverse transcriptase-polymerase chain reaction
SA	Splice acceptor
SEM	Scanning Electron Microscopy
SIM	Structured Illumination Microscopy
TA	Transit-amplifying
TBE	Tris/Borate/EDTA
TEM	Transmission Electron Microscopy
TJ	Tight junctions
TW	Terminal web
WB	Western blot
WT	Wild-type

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1.1 THE INTESTINAL TRACT

The intestine is a vital component of the gastrointestinal (GI) tract, a system that plays essential roles in processing our daily intake of food for nutrient absorption. The intestine is a long tubular organ that starts from the stomach pyloric sphincter and extends distally to the anus and is divided into two segments: the small and large intestines. The large intestine is the most distal segment and it is further subdivided into the cecum, colon, rectum and anal canal. The small intestine is the largest portion measuring nearly 7 meters in humans and 35 centimeters in mice. The segmentation of the small intestine begins with the duodenum at the pyloric sphincter, followed by the jejunum as the middle section and, most distally, bordering the cecum is the ileum (Reed and Wickham, 2009).

The intestine is comprised of three tissue layers (**Figure 01**). The most external layer that wraps the intestinal tube is called muscularis externa, which is an innervated smooth muscle layer responsible for peristaltic activity in the intestine (MIXTER, 1915; Noah et al., 2011; Reed and Wickham, 2009). An intermediate tissue layer, termed the submucosa, separates the muscle layer from the most internal layer and is composed of a large amount of extracellular matrix harboring nerve fibers, lymphatic and blood vessels and various immune cells. Lastly, the mucosa, a monolayer of highly organized epithelial cells, covers the entire lumen of the intestinal tube. This monolayer of intestinal epithelial cells (IEC) develops small tubular invaginations into the submucosa layer, the crypts of Lieberkühn, where rapidly proliferating cells and a small population of adult stem cells reside. Towards the lumen, the small intestinal

epithelium expands into finger-like structures called villi. A villus contains terminally differentiated and specialized intestinal cells (**Figure 02A-C**) (Barker et al., 2012; Flier et al., 2009; Sancho et al., 2015). The large intestine, which starts at the cecum, is distally divided into three other segments: colon, rectum and anal canal.

Figure 01 - Layers of the intestinal tract. The Intestinal tract is constituted of 4 layers: The innermost layer is the **mucosa**, which contains a single cell layered epithelium responsible for mucus production, absorption and transport of nutrients into adjacent capillaries. Lymph vessels and immune cells are also often found in the mucosa. The **submucosa** is a tissue layer formed by loose connective tissue harboring larger nerves, blood and lymphatic vessels. Next is a layer of smooth muscle, the **muscularis externa**, responsible for peristalsis contractions that move luminal contents through the gut. The outermost layer enfolding the smooth muscle layer is the **serosa**.

The intestinal epithelium is morphologically distinct along the length of the intestinal tube to accommodate different functional requirements. For instance, the villi are longer in the duodenum to ensure maximum surface area and absorption of contents arriving from the stomach. As the intestinal tube extends distally, the villi become smaller. The colonic epithelium lacks villus structures and instead presents multiple elongated crypts with differentiated cells migrating upwards and forming a flat luminal surface with many mucus-secreting cells who facilitate movement of luminal contents (**Figure 02D-F**). The major function of the large intestine is the absorption of ions and water from digested contents (Clevers, 2013; Gehart and Clevers, 2019).

In the mouse, approximately 21 days after birth, intestinal development is fully completed, with the organ achieving its characteristic epithelial morphology. At this

point, the epithelial cells are highly organized to accomplish their main functional roles in maintaining tissue integrity and nutrient absorption throughout life (Barker et al., 2009; Gehart and Clevers, 2019).

1.1.1 The Intestinal Epithelium

The inner surface of the intestine is lined by a columnar epithelium composed of several functionally specialized cell types that are constantly being renewed by a small population of adult intestinal stem cells (ISC) (Barker et al., 2007). The intestinal epithelium has a semipermeable surface that functions as a barrier, both limiting the passage of potentially harmful microorganism antigens and toxins into the blood circulation and also selectively allowing the absorption of nutrients (Johansson et al., 2011). The integrity and permeability of the intestinal tissue are regulated by protein complexes linking adjacent epithelial cells through their apicolateral membranes. Disruption of intestinal epithelial integrity directly affects its barrier function and is often associated with diseases of the gastrointestinal (GI) tract (Camilleri et al., 2019).

The organization and dynamics of IEC as well as their specific roles in tissue homeostasis and regeneration have been extensively covered in a number of recent reviews (Beumer and Clevers, 2016; Leung et al., 2018; Leushacke and Barker, 2012, 2014).

Figure 02 - Intestinal epithelium organization. **A:** Scanning Electron Microscopy (SEM) image of the small intestine showing villus and crypt structures. Each villus is maintained by 6 to 10 crypts. **B:** Cellular organization of the small intestinal epithelium. Epithelial renewal occurs every 3 - 5 days. As crypt cells differentiate, they migrate upwards along the crypt-villus axis. Differentiated cells undergo apoptosis as they reach the villus tip and are shed off into the intestinal lumen. **C:** Cell types of the small intestinal epithelium. Crypt base columnar cells (CBC) (also referred to as LGR5+ cells) are active stem cells that generate +4 stem cells, and differentiate into Paneth cells and into highly proliferating progenitors transit amplifying (TA) cells. LGR5+ cells also have the potential to self-renew. TAs rapidly divide and give rise to specialized differentiated cell lineages such as absorptive enterocytes and the secretory cell lineage comprised of goblet cells, enteroendocrine cells, and Tuft cells. **D:** Architecture of the Colon Epithelium. SEM image of the colon showing elongated crypts and the absence of villus structures. **E:** Cellular organization of the colonic epithelium. Differentiated cells move up along the crypt axis and form a flat luminal epithelial bed. LGR5+ cells differentiate into TA cells that subsequently give rise to all the secretory and absorptive cell lineages. In the colon, enterocytes are shed off into the lumen after 5 – 7 days (*Adapted from Barker, 2014*).

Briefly, a single villus is maintained by approximately 6 to 10 crypts harboring cells that are constantly dividing and eventually terminally differentiating into the various intestinal cell lineages that funnel into the villus compartment. As differentiation takes place in the crypt compartment, terminally differentiated cells migrate upwards along the crypt-villus axis until they reach the villus tip and eventually undergo apoptosis and are shed off into the intestinal lumen (Barker, 2014; Bullen et al., 2006; Gehart and Clevers, 2019) (**Figure 02A-C**). Intestinal epithelial renewal is a constant process that results in complete intestinal cell replacement every three to five days. Therefore, intestinal crypt cells are highly proliferative, compensating for the high cell death rate and rapid renewal of the intestinal epithelium (Barker et al., 2007). Molecularly, this IEC turnover is principally regulated by WNT/ β -catenin signaling (Myant and Sansom, 2014; Peterson and Artis, 2014), which is discussed at length later in the Introduction.

A small population of multipotent ISC, termed Crypt Base Columnar (CBC) cells, is responsible for the constant replenishment of the intestinal epithelium. CBC cells are famously known as LGR5-positive (LGR5⁺) cells because the expression of *Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5)* specifically labels these cells, whose stem cell potential has been rigorously demonstrated by genetic *in vivo* lineage tracing (Barker et al., 2007). These cells are positioned at the bottom of the crypt compartment and they are intermixed by neighbouring crypt-differentiated Paneth cells that together establish the stem cell niche (or stem cell zone) that is limited at cell-position +4 at the base of the crypt (referring to the fourth cell position from the base of the crypt) (**Figure 03**).

Figure 03 - Crypt stem cell zone. Schematic representation of the intestinal stem cell niche at the crypt base. LGR5+ stem cells occupy positions +1 to +3 (green numbers) at the crypt base (intermingled by Paneth cells). +4 stem cells occupy the fourth position. Pericryptal stromal cells are located beneath the crypt epithelium. Along with Paneth cells, pericryptal stromal cells provide essential factors that regulate stem cell homeostasis and epithelial renewal. (*Adapted from Barker, 2014*).

During homeostasis, these cells are capable of self-renewal giving rise to other undifferentiated stem cells by symmetric division to ensure maintenance of the stem cell pool in the crypt base. The fate of LGR5+ cell is decided by neutral competition for occupancy of positions within the stem cell zone (Snippert et al., 2010). Because of the limited space in the stem cell zone, some of the daughter cells are pushed out of the zone reaching the position 5. Once a CBC cell is displaced from contact with an adjacent Paneth cell, it loses stemness and becomes a rapidly proliferating, transit-amplifying (TA) cell with a limited life span. Eventually, TA cells will produce all functionally differentiated cell lineages that migrate upwards to form the villus, with the exception of crypt-differentiated Paneth cells that instead migrate downwards to the stem cell niche (Clevers and Bevins, 2013). Upon tissue damage and loss of active CBC cells, intestinal tissue still has the potential to regenerate by using its “reserve” stem cells that are located at position +4 in the stem cell zone (Barker, 2014; Cheng and Leblond, 1974; Potten et al., 1974). The +4 cells are normally quiescent, but can become active in response to tissue damage as they are resistant to acute injuries. In

addition, the newly born TA progenitors display plasticity: they can revert into stem cells, if necessary (Spit et al., 2018).

TA cells move up the crypt-villus axis while committing towards terminally differentiated specialized epithelial cell types that are required for normal physiological functions and predominantly shape the villus compartment. Absorptive enterocytes are the most common cell type in the villus followed by secretory cell lineages such as goblet cells (GC), which are intermingled among enterocytes along the entire intestinal epithelium, but are more abundant distally in the large intestine (Beumer and Clevers, 2016; Blander, 2016). GCs are fundamentally involved in the secretion of a thick mucus lining that covers the epithelial luminal surface and acts as a barrier that limits interactions with commensal microorganisms and facilitates the passage of gut contents. Enteroendocrine cells also belong to the secretory cell lineage of the intestine. They, too, are intermingled among the absorptive enterocytes. However, they are a rare cell type in the intestine, making up less than 1 percent of total epithelial cells. They function mainly in secreting a wide range of local and systemic regulatory hormones including serotonin and secretin (Barker et al., 2008; Turner, 2009).

Tuft cells are rare, poorly defined secretory cells that can be found along the crypt-villus axis. They have been reported to play roles in detection and recognition of luminal bacterial antigens to initiate immune responses (van der Flier and Clevers, 2009). Next, terminally differentiated crypt Paneth cells are epithelial cells that originate from TA cells, but they are the only TA descendants that migrate back into the stem cell zone. They are easily recognizable histologically due to their prominent secretory granules (Clevers and Bevins, 2013; Sato et al., 2011). Paneth cells are well known to play important roles in maintaining the stem cell niche as well as secretion of antimicrobial peptides (AMP) into the crypt lumen such as lysozyme and defensins.

Inflammatory bowel disease (IBD) can directly affect Paneth cells resulting in a deficiency in AMP secretion (Clevers and Bevins, 2013; Sato et al., 2011). Finally, the microfold (M)-cells are found in lymphoid aggregates that underlie the intestinal epithelium, known as Peyer patches. Although the origin of M-cells is controversial, they are generally believed to originate from intestinal crypt stem cells (Beumer and Clevers, 2016; Noah et al., 2011). In the colon, the major cell populations are colonic enterocytes and GCs. Interestingly, the colonic epithelium does not present Paneth cells (**Figure 2E-F**) (Barker et al., 2012; Leung et al., 2018).

1.1.2 The Principal Signaling Pathways Regulating the Intestinal Epithelium.

The self-renewal of Lgr5⁺ CBC cells as well as their continuous generation of the terminally differentiated cells such as enterocytes that maintain intestinal function are processes controlled by several well-characterized signaling pathways, including Notch, Bone Morphogenetic Protein (BMP), Epidermal Growth factor (EGF), Hedgehog (Hh) and WNT/ β -catenin (Gehart and Clevers, 2019; Sancho et al., 2004). Within the intestinal stem cell niche and villus compartment, it has been demonstrated in recent years that these signaling pathways are activated and regulated by essential signals secreted by the subepithelial mesenchyme that lies in close contact with the basal surface of the intestinal epithelium (Greicius and Virshup, 2019; Greicius et al., 2018; Kabiri et al., 2014; Kurahashi et al., 2013). The mesenchymal cells can be generally subdivided based on the expression of several key marker genes, including *Platelet-derived growth factor receptor alpha (Pdgfr α)*, *Forkhead box L1 (FoxL1)* and *Gli family zinc finger 1 (Gli1)* (reviewed in Greicius and Virshup, 2019; Karlsson et al., 2000).

Molecular and functional diversity exists within the intestinal subepithelial mesenchyme. Lineage tracing experiments, for example, have identified a subpopulation of $\text{Pdgfr}\alpha^+$ mesenchymal cells that additionally expresses the cardinal myofibroblast gene *smooth muscle actin alpha 2 (Acta2)* and the vascular marker *Cd31*, therefore being termed $\text{Pdgfr}\alpha^+$ myofibroblasts (Kurahashi et al., 2013). $\text{Pdgfr}\alpha^+$ mesenchymal cells were further subdivided into telocytes, which are characterized cytologically by their long membranous protrusions and molecularly by their expression of *Cd34* but not *Cd31* (Kondo and Kaestner, 2019; Kurahashi et al., 2013; Vannucchi et al., 2013). Collectively, myofibroblasts and telocytes that reside within the stroma surrounding the crypt compartment are often referred to as pericryptal cells.

$\text{Pdgfr}\alpha^+$ mesenchymal cell progenitors have been shown to be required for the morphogenesis of the developing intestine (Karlsson et al., 2000). Importantly, several studies indicate that $\text{Pdgfr}\alpha^+$ mesenchymal cells are an essential source of WNT ligands and R-spondins (RSPOs), which is consistent with a role for the stroma in regulating the WNT/ β -catenin signaling pathway in the intestinal epithelium, ensuring homeostasis and tissue renewal (Greicius and Virshup, 2019; Greicius et al., 2018; Kabiri et al., 2014; Karlsson et al., 2000). $\text{Pdgfr}\alpha^+$ mesenchymal cells also contribute to the regulation of other signaling pathways in the intestine, depending on their location in the subepithelial mesenchyme. For instance, further up in the crypt compartment and along the villus, some mesenchymal cells secrete BMP ligands, while others produce BMP inhibitors, such as GREM1, thereby orchestrating the dynamics of the differentiation of ascending epithelial cells (Greicius and Virshup, 2019; Wang and Chen, 2018; Yang et al., 2017). Reciprocally, intestinal epithelial cells can regulate mesenchymal cells by secreting Hh ligands (Kolterud et al., 2009; Mao

et al., 2010). In addition, both pericryptal and crypt-differentiated Paneth cells secrete EGF and Transforming Growth Factor- α (TGF α), both ligands that interact with high affinity with the EGFR/ERBB1 cell surface receptor (Ebner and Derynck, 1991; Singh et al., 2016). EGFR/ERBB1 is enriched on LGR5⁺ cell membranes and the activation of the EGF signaling cascade induces cell proliferation (He et al., 2004; Sancho et al., 2004; Singh et al., 2016).

Paneth cells are a known source of Notch ligands such Delta-like1/4 (DLL1/DLL4) or Jagged (Pellegrinet et al., 2011). The Notch pathway regulates the fate of secretory and absorptive lineage cell progenitors in the crypts of the intestinal epithelium (Fortini, 2009; Kay et al., 2017). Crypt progenitor cells can express DLL1 at their membrane and thus determine the fate of adjacent cells by ‘lateral inhibition’, a process that is discussed later in this Chapter.

Paneth cells are an epithelial source of different WNT ligands including WNT3, WNT6, and WNT9B (Gehart and Clevers, 2019; Sato et al., 2011). Intestinal Lgr5⁺ cells have the potential to generate organoids when co-cultured with Paneth cells or supplied with exogenous WNT factors, indicating that mesenchymal cells/factors are not required to maintain intestinal stem cell niche *in vitro* (Sato et al., 2009, 2011). However, recent studies have demonstrated that intestinal epithelial cells provide a redundant source of WNT ligands that is dispensable for intestinal epithelium maintenance *in vivo*. For instance, no ostensible phenotypes were observed in the intestinal homeostasis upon deletion of Porcupine (*Porcn*) and Wntless (*Wls*), which are both crucial for WNT ligands secretion (Kabiri et al., 2014; Roman et al., 2014). Similarly, deletion of *Wnt3*, which is an important WNT ligand secreted by Paneth cells, resulted in no phenotypes in the mouse intestine (Farin et al., 2012). Furthermore, depletion of Paneth cells induced by deletion of specific genes such *Atoh1* and *Gfi1*

did not affected WNT signaling pathway activity (Durand et al., 2012; Kim et al., 2012; Shroyer et al., 2005). These studies provide compelling evidence that mesenchymal cells are the essential source of WNT ligands and RSPOs regulating stem cells niche and intestinal homeostasis *in vivo* (Degirmenci et al., 2018; Greicius et al., 2018; Kabiri et al., 2014; Karlsson et al., 2000; Shoshkes-Carmel et al., 2018).

1.1.3 WNT/ β -Catenin Signaling Pathway

The WNT/ β -catenin signaling pathway plays essential roles in the regulation of survival, differentiation and function of CBC cells in adult intestinal tissue homeostasis (Lustig and Behrens, 2003). In the intestine, the WNT ligand is secreted by pericryptal and Paneth cells. The WNT ligand specifically binds to Frizzled receptors at the cell membrane leading to the formation of a complex that also includes the co-receptors LRP5/6 (**Figure 04**). The assembly of this complex at the cell membrane turns the WNT/ β -catenin pathway to its 'on-state', in which Dishevelled (Dvl) is then activated by phosphorylation and displaces GSK-3 β from the APC/Axin/GSK-3 β 'destruction complex' that is responsible for degradation of the transcriptional co-regulator β -catenin (Daniels and Weis, 2005; Rubinfeld et al., 1993). Consequently, active WNT signaling results in accumulation of β -catenin. When stabilized in the cytoplasm, β -catenin is translocated into the nucleus by Rac1 where it binds to transcription factors (LEF/TCF) and activates the transcription of WNT target genes such as *CYCLIN-D1*, *c-MYC*, *AXIN-2*, *LGR5* and others (Haegebarth and Clevers, 2009; de Lau et al., 2014; Sancho et al., 2004). Without nuclear β -catenin, the Groucho/Transducin-Like Enhancer of Split (TLE) family of proteins binds to LEF/TCF resulting in transcriptional repression of WNT target genes (Daniels and Weis, 2005). β -catenin also functions

as a cell-cell adhesion adaptor that connects E-cadherin to the cell cytoskeleton at the lateral cell membrane (Valenta et al., 2012). In the absence of the WNT, cytoplasmic β -catenin is phosphorylated by Casein-kinase 1 epsilon (CK1 ϵ) and by the APC/Axin/GSK-3 β destruction complex, leading to its ubiquitination and proteasomal degradation. In addition, Frizzled (FZD) can be ubiquitinated by the E3 ubiquitin ligases (ZNRF3 and RNF43) leading to prompt endocytosis of the WNT-Frizzled-LRP5/6 membrane complex and its lysosomal degradation. On the other hand, ZNRF3 and RNF43 activity can be inhibited when RSPO binds to LGRs increasing the sensitivity of the cell receptor FZD to the WNT ligand. Therefore, RSPOs are potent enhancers of WNT pathway activation (Basu et al., 2016; Haegebarth and Clevers, 2009; de Lau et al., 2014; Niehrs, 2012).

If cultured in the presence of EGF, RSPO and Noggin, single LGR5⁺ stem cells have the potential to generate *ex vivo* intestinal organoids resembling the intestinal epithelial organization with crypt- and villus-like domains containing all major differentiated small intestinal cell lineages. In addition to EGF, RSPO, Noggin and WNT3A are needed for the *ex vivo* culture of colonic LGR5⁺ stem cell- derived organoids. Such stemness properties that characterize LGR5⁺ cells as highly active stem cells also make them prone to accumulating mutations that potentially underlie intestinal cancers (Leung et al., 2018; Sato et al., 2009).

Figure 04 - WNT/β-catenin signaling pathway. The conserved WNT/β-catenin signaling pathway is involved in the regulation of stem cell survival, renewal and cell fate decisions of the adult intestinal epithelium. (**WNT ON**) The WNT/β-catenin pathway is activated by the WNT ligand that binds to Frizzled (FZD) receptors, leading to the formation of a cell membrane complex with LRP5/6 and activation of Dishevelled (DVL). Activated DVL collapses the APC/Axin/GSK3β “destruction complex”. Stabilized β-catenin then translocates to the nucleus where it binds to LEF/TCF transcription factors to regulate the expression of WNT target genes. In the absence of the WNT ligand (**WNT OFF**), β-catenin is targeted for coordinated phosphorylation by CK1 and the APC/Axin/GSK3β complex resulting in its ubiquitination and degradation by proteasomes. With no β-catenin in the nucleus, Groucho interacts with LEF/TCF resulting in transcription repression of WNT target genes. In the absence of RSPO, Wnt signaling is further decreased through the action of the transmembrane E3 ligases RNF43/ZNRF3, which internalize and degrade the WNT receptors Frizzled and LRP5/6. The ZNRF3/RNF43 Wnt antagonist activity can be inhibited by RSPO binding to LGR5, which neutralizes the RNF43/ZNF3 E3 ligases (right panel) (*Adapted from Gehart and Clevers, 2019*).

1.1.4 Notch Signaling Pathway

In the intestine, the Notch pathway is a key player in modulating proliferation of stem cells, cell survival and maintenance of the stem cell pool. It has been reported that depletion of either WNT or Notch signals leads to loss of ISC (van Es et al., 2005; Korinek et al., 1998). Notch also plays a crucial role in differentiation of IEC by defining the fate of absorptive and secretory cell lineages (Fortini, 2009).

Paneth cells present Notch ligands DLL4 and DLL1 to the CBC cells. In the transit amplifying compartment, secretory progenitor cells express DLL1. Both Notch ligands and their cognate receptor (such as NOTCH1) are transmembrane proteins; therefore, activation of the Notch pathway requires direct cell-to-cell membrane contact (Guruharsha et al., 2012; Pellegrinet et al., 2011). The molecular regulation of the Notch pathway has been comprehensively covered in numerous reviews (Collu et al., 2014; Fortini, 2009; Gehart and Clevers, 2019; Guruharsha et al., 2012; Hori et al., 2013; Tsai et al., 2014; VanDussen et al., 2012). Therefore, it is briefly introduced in this chapter.

Notch is activated once a Notch ligand bind to its receptor at the cell membrane (**Figure 5A**). This binding results in sequential cleavage of the Notch receptor by ADAM10 and by the γ -secretase complex, which leads to the release and activation of the intracellular domain of the receptor (intracellular fragment of Notch (NICD)) (McGill and McGlade, 2003; Pellegrinet et al., 2011; Tsai et al., 2014). Active NICDs translocate into the nucleus and bind to RBPJ to regulate the expression of Notch targets, including the transcription factor genes *Hes* and *Hey*, which in turn regulate the expression of several genes controlling stem cell proliferation and differentiation into TA cells (Guruharsha et al., 2012; Kay et al., 2017).

Figure 05 - Notch and BMP signaling pathways. A: Notch signaling is activated in epithelial cells when Notch ligands (DLL1/4) bind to their receptors (e.g. NOTCH1). The receptor is subsequently cleaved by ADAM and the γ -secretase complex releasing its intracellular domain (NICD) into the cytoplasm. NICD translocates to the nucleus, where it binds RBPJ to activate Notch target genes (e.g. *HES1*). **B:** Activation of the BMP pathway occurs when BMP ligands bind to their type 2 BMPRII receptor and recruits BMPRI. This dimerization event leads to phosphorylation and subsequent dimerization of rSMADs. rSMADs forms a complex with cSMAD and translocate to the nucleus to regulate target gene expression. The BMP pathway can be inhibited by sequestration of BMPs through secreted BMP inhibitors (e.g. Chordin-like 1, Gremlin 1 or Gremlin 2) (Adapted from Gehart and Clevers, 2019).

One of the main Notch target genes is the transcription factor HES1 whose function is to repress the expression of ATOH1, another transcription factor. In the absence of ATOH1 expression, cells do not express Notch ligands. Therefore, a cell presenting Notch ligands such as DLL1 on its membrane induces Notch activation in all signal-receiving adjacent cells (Gehart and Clevers, 2019; Pellegrinet et al., 2011). Consequently, Notch activation in these cells reduces their ability to activate the Notch pathway on its neighbouring cells – a process called lateral inhibition.

Several *in vivo* studies have shown the consequences resulting from inhibition of Notch signals. For example, genetic deletion of *Adam10* or simultaneous deletion of the ligands *Dll1* and *Dll4* in mice caused loss of CBC stem cells (Pellegrinet et al.,

2011; Tsai et al., 2014; VanDussen et al., 2012), suggesting an essential role of Notch signaling in maintaining the stem cell pool.

1.1.5 BMP Signaling Pathway

BMP ligands belong to the TGF β (transforming growth factor beta) superfamily of secreted cytokines. In the intestinal epithelium, BMP2 and BMP4 are the main ligands secreted by mesenchymal cells along the crypt-villus axis (Sancho et al., 2004). Briefly, activation of BMP signaling occurs when BMP ligands bind to their type II (BMPRII) receptor triggering activation of the type I (BMPRI) receptor by phosphorylation (**Figure 5B**). BMPRI and BMPRII dimerize to form an activated receptor complex, which results in the phosphorylation of intracellular SMAD proteins (SMAD1/5/8) (Lowery and Rosen, 2017; Massagué, 2012; Wang and Chen, 2018). Activated SMAD1/5/8 heteromerize with the obligate cofactor SMAD protein SMAD4 and this cytoplasmic complex then translocates to the nucleus, associates with additional tissue-specific transcription cofactors to regulate the expression of BMP target genes (Qi et al., 2017). The WNT and BMP signaling pathways are finely regulated to maintain the balance between proliferation and differentiation of intestinal cells. Smooth muscle cells and myofibroblasts residing around the crypt base secrete proteins (Gremlin 1, Gremlin 2 or Chordin- like 1 and Noggin) that inhibit the binding of BMPs to their receptors at the stem cell zone, thereby generating higher BMP activity distal to the crypt base (Groppe et al., 2002; He et al., 2004; Lowery and Rosen, 2017; Wang and Chen, 2018).

BMP signaling is well known for regulating cell proliferation and differentiation during embryonic development and adult stem cell homeostasis (He et al., 2004;

Lowery and Rosen, 2017). Auclair et al. (2007), for example, showed that specifically eliminating BMP signaling genetically in the mouse intestinal epithelium results in failure of proliferation, terminal differentiation and maturation of the intestine secretory cell lineage.

1.1.6 Absorptive Enterocytes

The differentiation of intestinal cells into the absorptive lineage is a process modulated by Notch signals. The CBC cells that migrate out of the stem cell zone having an active Notch signaling due to their contact with DLL1 presented by adjacent Paneth cells, will differentiate into absorptive TA cells (Collu et al., 2014; Gehart and Clevers, 2019). These committed absorptive TA cells will undergo approximately 6-8 rounds of cell division. Once they migrate out of the transit amplifying compartment, these cells will mature and become terminally differentiated absorptive enterocytes (AE) (Guruharsha et al., 2012; Wang and Chen, 2018). AEs reside along the entire intestinal epithelium. They are the predominant differentiated cell type making up more than 80% of cells in the villi in the small intestine and on the surface of the colon epithelium, which in the colon are termed colonocytes. The final structural organization of AEs is achieved when these cells fully differentiate into a highly polarized columnar epithelial cell (Achler et al., 1989; Schneeberger et al., 2018).

The establishment of enterocyte cell polarity is a key component for determining their columnar shape and is essential for the normal function of the intestinal epithelium (Miron and Cristea, 2012; Overeem et al., 2016; Toya et al., 2016). Fundamentally, enterocyte polarization depends on a number of cellular structures and machineries, including the compartmentalization of the cell space into the

characteristic apical-basal membrane domains established by the rearrangement of the cytoskeleton; the assembly of microvilli at the apical domain; appropriate formation of lateral junctions with tight junctions distributed apically and desmosomes basolaterally (Shin et al., 2006); and finally, the appropriate localization of proteins to their specific intracellular sites. Several of these processes have been described in detail elsewhere (Assimakopoulos et al., 2011; Engevik and Goldenring, 2018; Schneeberger et al., 2018; Tonucci et al., 2018).

The actin cytoskeleton is fundamental to the organization of enterocyte polarity. Its structure is composed of short actin filaments forming a dense complex comprising several proteins (Apodaca, 2001; Massey-Harroche, 2000). Myosin Vb (MYO5B) and the t-SNARE syntaxin-3 (STX3) are motor proteins involved in actin cytoskeleton structure that are mainly responsible for mediating the traffic of proteins to their correct subcellular localization (Roland et al., 2011; Schneeberger et al., 2018; Vogel et al., 2015).

The formation of the microvilli (commonly referred to the brush border (BB)) at the apical membrane domain of enterocytes is a distinguishing feature of enterocyte cell polarity. Microvilli are extensions of the apical membrane providing the AE an elongated surface of contact for maximum absorption of nutrients as they pass through the lumen (**Figure 06A**) (Crawley et al., 2014; Noordstra et al., 2016; Tonucci et al., 2018). The core of each microvillus is maintained intercellularly by bundles of actin filaments emerging from a region beneath the apical membrane termed terminal web (TW). The actin bundles are attached at the TW by many proteins including Myosin (MYO1A, MYOII, MYOVI) (Hegan et al., 2012; Mazerik and Tyska, 2012), the major actin-modifying protein Villin as well as members of the ERM (ezrin-radixin-moesin) family (Solinet et al., 2013). Several *in vivo* studies have shown that genetic deletion

or depletion of structural components of the microvilli core structure such as *Ezrin*, *MyosinVI* and *Villin* result in dramatic defects in BB formation (Casaletto et al., 2011; Ferrary et al., 1999; Mazerik and Tyska, 2012; Saotome et al., 2004).

The microtubule (MT) network also plays an important role in enterocyte polarity. MTs are arranged in an apical-basal array with their minus end anchored at the terminal web and the plus ends oriented towards the basal membrane. The MT network plays crucial roles in the enterocyte cell shape, positioning of cytoplasmic organelles and contributes largely to internal cell trafficking (Crawley et al., 2014; Tonucci et al., 2018). It has been shown by two independent studies that the organization of the longitudinal apical-basal MT array is regulated by the calmodulin-regulated spectrin-associated protein 3 (CAMSAP3), which tethers MT minus-end to the apical membrane (Noordstra et al., 2016; Toya et al., 2016). In addition, another study has confirmed by inducible destruction of MT polymers in the mouse intestine through the expression of Spastin (microtubule-severing protein) that MT defects leads to several intracellular aberrations in organelle positioning and nutrient transport (Muroyama et al., 2018).

Generally, mislocalization of the actin cytoskeleton- and microtubule-associated proteins at the apical and basolateral membrane domains affects epithelial architecture, permeability and absorption of nutrients and, consequently, results in many intestinal diseases (Fatehullah et al., 2013; Overeem et al., 2016; Schneeberger et al., 2018).

Figure 06 - Nutrient transport in absorptive enterocytes. Mechanisms of nutrient absorption by enterocytes. **A:** Molecules are absorbed at the apical membrane (Brush Border) and transported to the submucosa capillaries via paracellular and transcellular transport. **B:** Paracellular transport (PT) refers to the passage of molecules through intercellular spaces between adjacent cells. It is a process regulated by junctional complexes that allow the passage of small and water-soluble molecules. **C:** Pinocytosis is an endocytic form of transportation in which the apical cell membrane forms invaginations to incorporate nutrients into vesicles in which they are digested and transported to the blood. **D:** Active Transport. In primary active transport, ATP is the source of the energy required to move a molecule against its concentration gradient. The Na^+/K^+ pump is a primary active transport system. In secondary active transport, the energy from ATP is not required. Instead, the electrochemical gradient of an ion, normally established through a primary active transport, is the force that moves molecules across the cell membrane. An example of secondary active transport is the cotransport of sodium and glucose molecules into the cell that is driven by the (Na^+) -dependent glucose cotransporter SGLT. Once the glucose concentration gradient inside the cell is high, it triggers the facilitated transport of glucose into the submucosa by GLUT. Facilitated transport (FT) is also a form of passive transport of molecules that uses the local gradient concentration.

1.1.7 Absorption of Nutrients

AEs are highly specialized cells whose primary function is to ensure absorption of nutrients and their transport into the submucosal layer where they are exported into capillaries and lymphatic vessels (Wang et al., 2009; Zimmer et al., 2016). Distinct compartments of the enterocytes such as the microvillus and the mucous layer covering it, as well as the junctional complexes connecting neighbouring cells, play an important part in the absorption process.

The mucus layer is a thick viscous matrix covering the tips of microvilli along the entire epithelium and is formed by several components, including transmembrane mucin glycoproteins with large extracellular domains, gel-forming mucins secreted from GCs and numerous enzymes (Johansson et al., 2011; Snoeck et al., 2005). This mucous coat prevents the passage of pathogen antigens and promotes the uptake and absorption of nutrient molecules (Maury et al., 1995). Because microvilli are negatively charged, they provide a diffusion barrier for charged molecules. In addition, the space separating microvilli is similar to the scale of some bacterial antigens, thus creating another line of mechanical defence. Morphological defects of microvilli alter the intestinal barrier function (Gonnella and Neutra, 1984). The junctional complexes, discussed later in this chapter, also contribute significantly to the intestinal uptake of molecules.

Once inside the cell, molecules are transported from the apical to the basolateral membranes. The mechanisms underlying the transport of nutrients across enterocytes into the submucosa depend upon molecule type and/or size (**Figure 6**) (Choudhuri and Chanderbhan, 2016). For example, sugars, peptides, lipids, ions and water are absorbed and transported in different ways.

Paracellular transport (PT) refers to the uptake of molecules crossing the epithelium through intercellular junctional complexes, which allows the passage of small and water-soluble molecules. (Lavin et al., 2007; Szefel et al., 2015). PT is a passive form of transport. Thus, it depends entirely on the concentration gradient of molecules in the luminal space (Edelblum and Turner, 2015; Roxas and Viswanathan, 2018). There are two described pathways of PT through junctional complexes: the pore and leak pathways (Roxas and Viswanathan, 2018; Snoeck et al., 2005). The pore pathway enables the passage of negatively charged molecules, size-selecting

small molecules ranging between 4 and 6 kDa (between 5 and 10 angstroms (Å)) (Pappenheimer, 2001; Pappenheimer et al., 1994). In contrast, the leak pathway is not selective for charge and enables the passage of larger molecules with a maximum size of 50 Å (Atisook and Madara, 1991; Buschmann et al., 2013). Paracellular transport function can be measured by monitoring the uptake of macromolecules across the epithelial junctions. The most frequent tracers used experimentally are ovalbumin, lactulose, inulin and or polymers of various sizes such as 4 kDa dextran (~4.9 Å) and 40 kDa dextran (~45 Å) (Al-Sadi et al., 2011; Richter et al., 2019; Volynets et al., 2016; Woting and Blaut, 2018).

Generally, absorption and transport of macromolecules occurs via transcellular mechanisms such as endocytosis as well as receptor-mediated endocytosis. The most frequently described mechanism of transcellular intake is pinocytosis, which is a fluid-phase endocytosis in which a molecule transits across the cell through invaginations of the apical membrane that form vesicles containing digestive enzymes (Kiss and Botos, 2009; Snoeck et al., 2005). In a recent study, Park et al. (2019), characterized a subclass of specialized enterocytes, referred to as lysosome-rich enterocytes (LREs), found to be morphologically and functionally conserved between zebrafish and mouse. These authors showed that LREs function essentially in the uptake of proteins by fluid-phase endocytosis and by receptor-mediated endocytosis modulated by a complex of transmembrane receptors comprising Cubilin (Cubn) and Amnionless (Amn), and the endocytic adaptor Dab2. Proteins are intercellularly digested by lysosomes before being exported across the basolateral membrane. Moreover, they demonstrated that conditional deletion of *Dab2* led to severe malnutrition and growth defects in suckling mice (Park et al., 2019).

In addition, other molecules are absorbed and transported across cells by protein transporters such as those involved in active transport (AT) and facilitated transport (FT). In primary AT, adenosine triphosphate (ATP) is the source of the energy required to transport molecules against their concentration gradient. For instance, the Na^+/K^+ pump is a form of primary active transport in the cell. In secondary AT, the energy from ATP is not required (Szefel et al., 2015). This transport system relies upon an ion electrochemical gradient, normally established through primary active transport, as the force to transport molecules across the cell membrane. An example of secondary active transport is the co-transport of sodium and glucose molecules into the cell, which is mediated by SGLT, a Na^+ -dependent glucose co-transporter. Once the glucose concentration gradient inside the cell is high, it triggers a form of facilitated transport in which glucose molecules cross the cell membrane into the blood via GLUT—a facilitative transporter (Choudhuri and Chanderbhan, 2016; Szefel et al., 2015).

Malfunction of components in the machinery that drives internal cell transport has long been implicated in malabsorption conditions in humans (Park et al., 2019; Tonucci et al., 2018, 2017). D-xylose, a small sugar that is readily absorbed via transcellular transport, has been frequently used as a molecule tracer to measure transcellular uptake in malabsorption conditions (Antunes et al., 2009; Craig and Atkinson, 1988; Hope et al., 2012).

1.1.8 Intestinal epithelium junctional complexes and permeability

AEs are bound together by structural complexes comprised of transmembrane proteins that join adjacent cells at their lateral membranes through direct interaction with the cytoskeleton. These complexes are responsible for sealing the epithelial lining and establishing a barrier that confers the epithelium its selective permeability competence. The assembly and maintenance of these protein complexes are regulated by a number of signaling pathways, involving the Rho GTPase (Rho), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways (Assimakopoulos et al., 2011; Camilleri et al., 2012; Ulluwishewa et al., 2011).

In epithelial cells, there are three main intercellular junctional complexes (**Figure 7**). First, the tight junction (TJ), the most apical cell-cell junctional complex, is formed by several components including occludins, claudins and tricellulin. These proteins are associated with the actin cytoskeleton via an intercellular plaque of zona-occludens (ZO-1,2 and 3) (Assimakopoulos et al., 2011; Camilleri et al., 2012; Ulluwishewa et al., 2011). The transmembrane receptor junctional adhesion molecule (JAM) is also a component of the TJ complex. The recruitment of JAM to the TJ complex has been shown to be regulated through its interaction with PDZ domain protein Afadin-6 (AF-6) and possibly ZO-1 (Ebnet et al 2000; Bazzoni et al 2000).

Second, Adherens junctions (AJs) are located right beneath the tight junctions. They are comprised of E-cadherin, which is a lateral transmembrane protein connected to actin filaments by cytoplasmic components such as α -catenin and β -catenin (Sumigray et al., 2014). Lastly, desmosomes are located beneath the AJs along the lateral membrane. They are comprised of two transmembrane proteins—Desmocollin and Desmoglein—which connect the keratin filaments of adjacent IECs

via an intracellular protein called Desmoplakin (DP). Interestingly, upon genetic deletion in the intestinal epithelium, DPs were found to be not essential for intercellular adhesion or cell integrity as the keratin filaments were not affected. However, lack of DPs in the intestine led to defects in microvilli morphology, a result that demonstrates a different function for these junctional components (Sumigray and Lechler, 2012). Desmosomes and AJs are junctional complexes that strongly bind IEC and provide mechanical stability to the epithelium barrier (Assimakopoulos et al., 2011; Meng et al., 2008; Sumigray and Lechler, 2015; Yu and Yang, 2009).

Figure 07 - Junctional complexes in epithelial cells. Junctional complexes regulating epithelial cell-cell interactions. **Tight junctions (TJ)** are the most apical protein complexes at the lateral membrane of epithelial cells. TJ components include Zona Occludens (ZO1,2 and 3), Occludin, Tricellulin, Claudin and junctional adhesion molecule (JAM). TJs seal the intercellular space and regulate the intestinal epithelial permeability. **Adherens junctions** and **desmosomes** are mainly responsible for providing mechanical strength to intercellular connections. All junctional components are connected to the actin cytoskeleton (*Adapted from Neunlist et al., 2012 and Luissint et al., 2016*).

The intestinal epithelium barrier function can be directly affected by dysregulation of cell-cell junctional complex components resulting in increased paracellular and transcellular permeability. Intestinal permeability defects have been described as key drivers of several inflammation-associated diseases of the GI tract. Changes in the expression and localization of several tight junction proteins, such as Claudin-2, have been reported in various cases of intestinal permeability diseases including IBD. In addition, altered transcriptional levels of inflammatory cytokines including IL-6 and IL-1 β , IFN- γ and IFN- α , have been observed in subsets of IBD (Cereijido et al., 2007; Gassler et al., 2001; Snoeck et al., 2005; Yu and Yang, 2009).

1.2 THE CENTROSOME

In the late 1800s, this organelle was first christened the centrosome of the cell owing it to its central intracellular localization. Structurally, the centrosome is a non-membrane bound organelle that occupies a small volume in the cell (between 1 to 2 μ m) normally around the nucleus radius (Doxsey, 2001). Now, it is well known that the subcellular localization of the centrosome is entirely dependent on the specific cell type. In some cells, its proximity to the nucleus is so intimate that the nuclear envelop forms an invagination harboring the centrosome (Hulspas et al., 1994). However, in ISC, for example, the centrosome is located in a region proximal to the apical membrane (Quyn et al., 2010).

Figure 08 - The centrosome. The centrosome is the microtubule organizing center (MTOC) of the cell. The centrosome structure is comprised of two barrel-shaped structures referred to as **centrioles**. The maternal centriole contains additional distal and subdistal appendages. Maternal and daughter centrioles are connected by fiber protein complexes. Centrioles are positioned perpendicularly and embedded into the pericentriolar material (**PCM**). PCM is an organized dense matrix containing several proteins involved in the nucleation and anchoring of the **microtubules** (in blue) (*Adapted from Doxsey, 2001*).

Structurally, the centrosome is comprised of two perpendicularly-arranged centrioles embedded into a dense matrix of pericentriolar material (PCM) that contains numerous proteins with distinct functional roles (**Figure 8**) (Doxsey, 2001; Feldman and Priess, 2012; Tang and Marshall, 2012). Centrioles are barrel-shaped structures with open ends. They are both the same size, with a range of 0.2 μm in diameter and 0.5 μm . One of the centrioles displays 2 sets of additional structures (distal and subdistal appendages) and therefore it is named 'mother centriole' (Badano et al., 2005). Apart from that, they are each formed of nine triplets of MTs arranged in manner that resembles a cylinder (Badano et al., 2005; Doxsey, 2001; Tang and Marshall, 2012). Of note, centrioles are not necessarily involved in the cell division process.

Their exact cellular function remains not well defined (Tang and Marshall, 2012). It is generally accepted that, in some cells, they play an important part in the assembly of the primary cilium (Lüders and Stearns, 2007), which propagates from the mother centriole (Badano et al., 2005; Lüders and Stearns, 2007). In cells that lack PCM, the centrioles are called basal bodies, which are mainly involved in the formation of motile cilia and flagella (Korinek et al., 1998; Tang and Marshall, 2012; Woodruff et al., 2014).

As mentioned above, the PCM is a dense matrix composed of fibers and protein complexes comprising a high amount of coiled-coil proteins (Doxsey, 2001; Pelletier et al., 2004; Woodruff et al., 2014). PCM components are mostly associated with nucleation and anchoring of MT polymers (Badano et al., 2005; Feldman and Priess, 2012). The organization of these proteins and their function in PCM assembly is yet to be explored. It is well-defined by sophisticated microscopy techniques that some of PCM components are located in close proximity to the centriole surface, but others are in the PCM periphery (Woodruff et al., 2014).

The centrosomal complex is a major player in numerous events in the cell. For instance, during cell cycle progression, the centrosome is duplicated and each newly assembled centrosomal complex migrates to opposite sides of the nucleus. Subsequently, MTs grow from each centrosome forming the mitotic spindle, responsible for correct chromosome segregation. Furthermore, the centrosome has also been implicated in the establishment of cell polarity by reorganizing MTs and the actin cytoskeleton and contributing to internal cell molecule trafficking (Korinek et al., 1998; Woodruff et al., 2014).

In most cells, MT polymers are organized such that their minus ends are proximal to the centrosome while their plus ends extend distally into the cytoplasm. To

achieve this level of organization, the centrosome must both anchor the MT minus ends to its core and mediate their dynamic growth. In fact, the centrosome is commonly referred to as the microtubule-organizing center (MTOC) of the cell due to its central role in MT nucleation (Ito and Bettencourt-Dias, 2018; Muroyama and Lechler, 2017; Sanchez and Feldman, 2017). Proteins such as CEP135, Dynactin, Ninein, and Centriolin anchor MTs at the centrosome whereas the release of MTs from the centrosome is mediated mainly by Katanin, a microtubule-severing protein (Doxsey, 2001; Muroyama and Lechler, 2017).

MTs are nucleated from their minus ends by specialized multiprotein complexes within the PCM that involve the protein γ -tubulin, an important player in MT organization, and other associated proteins. As confirmed by electron microscopy, these complexes resemble a ring-shaped structure and are so-called γ -tubulin ring complexes (γ -TuRC). γ -tubulin nucleates the polymerization of MTs by incorporating α - and β -tubulin subunits to the polymer (Badano et al., 2005; Mogensen et al., 1997). The γ TuRC complex is responsible for adding the GTP cap to the minus-ends of MTs, stabilizing the polymers and thereby preventing depolymerization. Other components of the PCM have also been strongly implicated in MT nucleation, including Aurora Kinases, Polo and Pericentrin. The molecular basis of MT nucleation and anchoring remains poorly understood (Badano et al., 2005; Muroyama and Lechler, 2017; Muroyama et al., 2016).

In epithelial tissues, during cell differentiation the centrosome becomes inactive. Fundamentally, the centrosome loses its classic MTOC function as its constituent proteins are reassigned to other sites in differentiated cells to establish a new MT anchoring/nucleating site. These new sites are termed non-centrosomal MTs

organizing centers (nc-MTOC) (Ito and Bettencourt-Dias, 2018; Muroyama and Lechler, 2017; Sanchez and Feldman, 2017).

In a recent study, Yang and Feldman (2015), detected a downregulation of the centrosomal protein CEP192 and the cell cycle modulator CDK in anticipation of the nc-MTOC assembly during differentiation of *C. elegans* intestinal cells. Another study in *C. elegans*, reported that the intestinal centrosomal PCM is eliminated once cells exit the mitotic cycle during larval stages (Lu and Roy, 2014).

Furthermore, cell differentiation is often accompanied by reorganization of MTs into cell-type specific arrays. In intestinal crypt cells, MTs are arranged radially along the apical–basal axis of the cell, with MT minus-ends anchored on the apically located centrosome—that is, the MTOC (Sallee et al., 2018; Sanchez and Feldman, 2017). In polarized differentiated IECs, however, MTs do not emanate from the centrosome, instead they adopt a more specific longitudinal apical-basal orientation that is organized by nc-MTOC located along the apical membrane (**Figure 9**) (Sallee et al., 2018; Sanchez and Feldman, 2017). The centrosomal anchoring protein Ninein is known to re-locate to apical sites of differentiated intestinal cells co-localizing with β -catenin at apical-lateral cell junctions (Goldspink et al., 2017; Mogensen et al., 2000). In addition, γ -tubulin localizes to the terminal web in differentiated enterocytes of the mouse intestine (Ameen et al., 2001). In the *C. elegans* intestine, γ -tubulin also localizes to apical sites of the cell. Moreover, MTs were shown to grow from the nc-MTOC of differentiating *C. elegans* embryonic intestinal cells following nocodazole (a drug that interferes with MT polymerization) washout, supporting the role of γ -tubulin in nucleating MTs (Brodu et al., 2010; Yang and Feldman, 2015). However, it is not clear if in mammalian intestinal differentiated cells, the nc-MTOC is the site of

nucleation. The molecular mechanism of such coordinated events involving redeployment of centrosomal proteins to nc-MTOC sites during epithelial differentiation remains equally poorly understood.

Figure 09 - Non-centrosomal MTOCs in the intestinal epithelium. Schematic representation of terminal differentiation in the intestinal epithelium. **A:** Transit amplifying cells migrate upwards along the crypt-villus axis while differentiating into polarized intestinal cells (e.g. enterocytes). **B:** Upon intestinal cell differentiation, microtubule (MT) anchoring proteins are redeployed to the apical membrane leading to centrosome inactivation and reorganization of MTs into a more characteristic longitudinal apical-basal array. The MT minus ends are anchored at the terminal web. **C:** Events underlying epithelial differentiation include both transcriptional downregulation of centrosomal components and a decrease in pro-proliferative cell-cycle signals in parallel with MT reorganization (*C is Adapted from Muroyama and Lechler, 2017*).

1.3 *MCC: MUTATED IN COLORECTAL CANCER*

1.3.1 Background and Clinical Significance

The gene *Mutated in Colorectal Cancer (MCC)* was identified more than 25 years ago through linkage studies and positional cloning as a culprit tumor suppressor gene for human hereditary colon cancer, as its name implies, familial adenomatous polyposis coli (FAP) (Ashton-Rickardt et al., 1991; Kinzler et al., 1991). Shortly after this initial finding, a now much more famous gene, *Adenomatous Polyposis Coli (APC)*, which is tightly linked to *MCC* on human chromosome 5q21 was established as the gene responsible for hereditary colon cancer (FAP) (Grodén et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991). FAP patients typically present hundreds to thousands of adenomas in the colon and rectum, but only a few of these lesions ever advance to colorectal cancer (CRC) (Rhodes and Bradburn, 1992). As APC grew in prominence, with its binding to β -catenin and link to canonical WNT signaling first established in 1993 (Rubinfeld et al., 1993; Su et al., 1993a), interest in *MCC* waned considerably.

Although *MCC* was mapped in both humans and mice in a region that is frequently mutated in cases of FAP (Kinzler et al., 1991; Luongo et al., 1993; Nishisho et al., 1991) the mechanistic roles that *MCC* might play in intestinal cancer development, if any, as well as its function in normal adult tissue homeostasis have not been explored.

A number of studies have nevertheless appeared intermittently over the last two decades that support a role for *MCC* as a bona fide tumor suppressor in some cancers. Loss-of-heterozygosity, deletions and mutations of the *MCC* gene are indeed frequently observed in CRC, but mutation of the remaining wild-type allele is extremely

rare (Curtis et al., 1994; Nakamura et al., 1992; Nishisho et al., 1991). These findings initially cast doubt on whether *MCC* fits the conventional definition of a tumor suppressor gene. However, it was subsequently reported that *MCC* is silenced through promoter hypermethylation in approximately 50% of primary sporadic CRCs and 80% of serrated polyps, suggesting that loss of *MCC* expression is an important event in early colon carcinogenesis via the “serrated polyp-neoplasia” pathway (Kohonen-Corish et al., 2007; Li et al., 2013). Interestingly, very recent evidence also indicates that *MCC* serves as a tumor suppressor in liver cancer. In 4 of 19 human hepatocellular cancer genomes analysed, germline retrotransposition of the LINE-1 element into the *MCC* locus was observed. This resulted in greatly decreased *MCC* mRNA expression and led to an accumulation of oncogenic nuclear β -catenin (Shukla et al., 2013). Furthermore, loss-of-function mutations, or decreased *MCC* expression are also detected in a number of other human cancers including lung, breast carcinoma, hepatocellular carcinoma, gastric carcinoma (Ashton-Rickardt et al., 1991; D’Amico et al., 1993; Fukuyama et al., 2008; Mukherjee et al., 2011, 2016; Shukla et al., 2013; Sud et al., 1996).

Additional evidence supporting the designation of *Mcc* as a bona fide tumor suppressor comes from experiments in mice. In a forward genetic screen aimed at identifying potential driver genes for CRC, Starr et al. (2009) employed the Sleeping Beauty transposon system to generate insertional mutations within the mouse gastrointestinal epithelium. Among the 77 common insertion sites identified by sequencing of tumor DNA, *Mcc* was recovered 7 times. In a second study, Lim et al., 2014 developed an inducible liver tumor model combining MYC and RAS activation and discovered that microRNA-494 (miR-494) is highly upregulated in tumor samples and specifically targets the 3’ UTR of *Mcc*. Overexpression of miR-494 and *Mcc*

knockdown increases, while miR-494 knockdown and *Mcc* overexpression decreases the proliferation of liver tumor-derived cell lines. Taken together, these findings in both human and mouse provide considerable evidence that loss of *Mcc* expression plays a rather causal role in cancer development.

Interest in *Mcc* in my home laboratory originated from a comparative microarray screen performed using human embryonic stem cells (hESCs) in which the goal was to identify novel markers of the definitive endoderm, the parental embryonic germ layer of organs such as the liver, lung, pancreas and intestines (Teo et al., 2012). *MCC* emerged among the leading candidates of endoderm-enriched genes. Surprisingly, despite its historical association with CRC and linkage to the *APC* tumor suppressor and strong evolutionary conservation, *Mcc* transcript distribution in the mouse had not been previously reported. The lab therefore performed a comprehensive analysis of *Mcc* expression and showed for the first time that *Mcc* is dynamically expressed during mouse embryonic development, labelling not only the gut endoderm, which validated the results of the initial *in vitro* screen using hESC, but also the organs it gives rise to (Young et al., 2011). In a follow up study, Young et al. (2014) evaluated the expression and function of the zebrafish *mcc* revealing that it plays essential roles during gastrulation and the establishment of the zebrafish body plan. In adult mouse tissues, *Mcc* has been reported to be expressed in the pancreas, kidney, liver and cerebellar cortex (Ashton-Rickardt et al., 1991; Matsumine et al., 1996; Senda et al., 1999; Young et al., 2011).

1.3.2 MCC Protein

The mouse *Mcc* and human *MCC* genes generate two transcripts that result from alternative promoter usage. In the mouse, the *Mcc* locus starts at 113022099 and ends at 113488830 bp on chromosome 18 (total of 382.3 Kb) and is comprised of 20 exons with the alternative ATG for isoform 2 in exon 4 (**Figure 10**). MCC is a highly evolutionarily conserved protein (from *Drosophila* to Human) that contains several regions predicted to form coiled-coils (Bourne, 1991; Nakamura et al., 1992). The two isoforms differ at their N-terminus and contain a highly conserved, extreme C-terminal type I (PSD-95/Dlg/ZO-1) PDZ binding motif (ETSL).

Figure 10 - *Mcc* locus and protein isoforms. Schematic representation of the mouse *Mcc* gene and the protein isoforms it encodes. The *Mcc* gene is comprised of 20 exons and generates 2 transcripts via alternative promoter usage. The ATG for isoform 2 is located in exon 4 (in green). **MCC protein:** Both isoform 1 (1003 amino acids (aa)) and isoform 2 (828 aa) contain a highly evolutionarily conserved, C-terminal type I (PSD-95/Dlg/ZO-1) PDZ binding motif (in red). They differ at the N-terminus with isoform 1 containing an EF-hand domain. The *Mcc* protein contains several regions (in blue) predicted to form coiled-coil structures (Adapted from Young et al., 2011).

A study published in 2011 by Young et al. (2011) has determined the relative expression of each *Mcc* transcript by semi-quantitative reverse transcriptase-

polymerase chain reaction (RT-PCR) of total RNA samples from mouse embryonic stem cells, mouse embryos as well as various adult tissues (**Figure 11A**).

Figure 11 - Mouse *Mcc* isoforms expression. Relative expression of the *Mcc* isoforms 1 and 2 measured by qPCR in assorted adult mouse tissues, mouse embryonic stem cells (mESC) and mouse embryos at different embryonic stages (embryonic day (E)) reveals that the isoform 2 transcript is most abundant in the majority of the analyzed samples. **B:** Western blot analysis of protein lysates from stage 10.5 frog embryos over-expressing either the mouse *Mcc* isoform 1 (iso 1: 112kDa) or 2 (iso 2: 93kDa) were used as control for isoform specificity. The same MCC antibody does not react with endogenous frog proteins (frog). Protein extracts from adult mouse tissues, mESC, and mouse embryo at different embryonic stages analyzed by western blot confirm that *Mcc* isoform 2 is the most abundant (Table 03) (Data published in Young et al., 2011).

In summary, this study revealed that *Mcc* isoform 2 expression is abundant in all tissues examined, but noticeable expression of the isoform 1 transcript was observed only in mouse embryonic stem cells, mouse embryos at embryonic days (E) 9.5 and 10.5 and lung. These findings were subsequently confirmed by western blot (WB) using a commercially available mouse monoclonal MCC antibody (**Table 03**) raised against a sequence of 141 amino acids that is common for both MCC isoform 1 and 2 of human origin. The isoform specificity of this antibody was examined by injecting mRNA encoding either the mouse *Mcc* isoform 1 or 2 into stage 10.5 frog embryos. WB analysis from frog embryos protein extracts detected both isoforms (MCC isoform 1 100 kDa and MCC isoform 2 93 kDa) (**Figure 11B**). However, only isoform 2 (93 kDa) was observed in WB analysis of different adult mouse tissues (Young et al.,

2011). Collectively, these findings provide compelling evidence that Mcc isoform 2 is the main protein encoded by the mouse *Mcc* locus.

Additionally, my home laboratory generated an *Mcc* mutant mouse line using mouse embryonic stem cells that harbor a classic splice acceptor β -galactosidase reporter gene trap in the *Mcc* locus (**Figure 12**). Molecular characterization of the gene trap allele confirmed that the gene trap insertion generates a complete loss-of-function (LOF) *Mcc* allele. Mice homozygous for this mutation were reported to be viable and fertile with no obvious phenotype (Young et al., 2011).

Figure 12 - *Mcc*^{lacZ} mutant allele. **A:** Schematic representation of the *Mcc-lacZ* gene trap allele into the *Mcc* locus. The gene trap insertion contains a splice acceptor (SA) sequence, an open reading frame encoding β -galactosidase (β -geo) and a polyadenylation sequence (PA), which generates a knock-out for *Mcc*. **B:** qPCR using primers targeting exons 17 and 18 downstream of the gene trap insertion shows the absence of *Mcc* expression in *Mcc*^{lacZ/lacZ} (*Mcc*^{-/-}) adult tissues, while expression in heterozygotes is diminished roughly by half. **C:** Western Blot analyses confirm that Mcc protein is absent in *Mcc*^{-/-} tissues (Data published in Young et al., 2011).

Numerous published studies revealed the presence of MCC protein in assorted widely used cell lines by WB analysis. However, the precise subcellular localization of MCC was controversial with various conflicting reports (Arnaud et al., 2009; Benthani et al., 2018; Fukuyama et al., 2008; Matsumine et al., 1996; Pagon et al., 2010, 2015; Senda et al., 1997). This is due both to the dearth of published studies investigating Mcc and their often-confusing results showing that Mcc, depending on the cell type, reagent and assay used, can be found in several organelles (mitochondria and endoplasmic reticulum) and cellular compartments (plasma membrane, cytoplasm and nucleus). For example, when overexpressed, MCC has been shown to bind β -catenin in the nucleus to negatively regulate canonical WNT signaling in cancer cell lines and to inhibit cell proliferation (Fukuyama et al., 2008; Pagon et al., 2015). MCC has also been implicated as a modulator of the Nuclear Factor Kappa B (NF κ B) signaling pathway through its interaction with the Inhibitor of Kappa B (I κ B), the cytoplasmic inhibitor of NF κ B signaling (Sigglekow et al., 2012).

The MCC protein contains a highly conserved, extreme C-terminal type I (PSD-95/Dlg/ZO-1) PDZ binding motif (ETSL) that can be phosphorylated at position -1 Ser828. Upon phosphorylation, MCC binds the PDZ-domain containing PCP protein SCRIBBLE at the active migratory edge of CRC cell lines (Pagon et al., 2012), and this interaction serves as a bridge to Myosin IIB and the regulation of cytoskeletal organization and cell migration (Caria et al., 2019). Lastly, a recent report argues that Mcc is membrane associated and interacts with β -catenin and E-cadherin to strengthen cell adhesion in CRC cell lines (Benthani et al., 2018).

The studies published on Mcc to date, using assorted conventional cell lines and biochemical studies, implicate Mcc in a wide range of cellular processes—cell migration, proliferation, adhesion and polarity. Fundamental, basic questions, however,

remain about *Mcc* and its function *in vivo*, such as the distribution of *Mcc* transcripts in adult tissues and the definitive subcellular localisation of Mcc protein, which has endured nearly three decades of controversy.

These questions as well as whether *Mcc* plays a role, if any, in tissue homeostasis and disease *in vivo* were the major motivation for the experiments and results outlined in the coming chapters, the answers to which offered a few surprises and opened new lines of exploration into Mcc function.

1.3.3 The Scope and Aims of this Study

Given the numerous studies emphasizing the contributions of MCC as a candidate tumor suppressor for intestinal cancers development, the scope of this project mainly focusses on understanding what are the contributions of *Mcc* to adult intestinal epithelium homeostasis.

In order to pursue these above-mentioned outstanding questions we have exploited proteomics, cell biology and a genetic model mouse system where *Mcc* is constitutively absent to (I) perform holistic assessment of *Mcc* transcript distribution in the murine intestinal epithelium and characterize the *Mcc*-expressing cell populations; (II) to ascertain MCC subcellular localization using not only cells lines but also *in vivo* systems; (III) to evaluate MCC network of interactions and characterize cellular processes that MCC is involved in by identifying its binding protein partners, and (IV) to investigate whether the genetic loss of *Mcc* has any impact on intestinal epithelium homeostasis.

2.1 - CLONING

2.1.1 - Overexpression Constructs

Full length cDNA constructs of MCC isoform 2, NDE1 and RASAL2 were PCR-cloned into pCMV2B (FLAG) and pCMV3B (Myc) expression constructs from Stratagene. Constructs for SCRIBBLE, NHERF1, CK1D, CK1E, GSK3e were generated by Gateway Cloning into pcDNA5 FRT-TO (3xFlag N-terminus or EGFP N-terminus) vectors (Invitrogen) according to the manufacturer's instructions. Point mutations were generated by site-directed mutagenesis using the QuickChange II Site-Directed mutagenesis kit (Agilent).

2.1.2 - mMcc2-emGFP Construct

The pCS2+_EmGFP-Mcc isoform 2 overexpression vector was previously described in Young et al., 2014. This plasmid robustly produces an N-terminal EmGFP-Mcc fusion protein in heterologous cells. (Plasmid inventory #525, Dunn lab).

2.2 - PCR - POLYMERASE CHAIN REACTION

PCR reactions were performed with a minimum of 200 ng DNA template. Primers were purchased from Integrated DNA Technologies (IDT, Singapore). Electrophoresis: A minimum of 10 µl amplicon from each reaction was run on a 1% - 2% agarose gel in TBE buffer and stained with ethidium bromide. The bands were visualized under UV illumination. A DNA ladder was included in each electrophoresis run (2-Log DNA Ladder 0.1-10.0 kb, N3200, New England Biolabs). DNA Sequencing:

Samples were diluted into sterile nuclease free water according to the manufacturer's recommendations and sequenced by AITBiotech, Singapore.

2.3 - CELL CULTURE

RPE-1, MDCK and HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM-High glucose, 11960-044, Gibco™) supplemented with 10% (v/v) FBS. HCT116 were cultured in McCoy's 5A Modified Medium (16600-082, Gibco™) supplemented with 10% (v/v) FBS. RPE-1. SW480 cells were cultured in RPMI medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine (25030-081, Gibco™), 1 mM sodium pyruvate (11360-070, Gibco™), and Penicillin-Streptomycin (15140-122, Gibco™).

2.4 - MOUSE WORK

2.4.1 - Mouse Husbandry

Mice used for this study were housed, intercrossed and euthanised for experiments according to the Institutional Animal Care and Use Committee (IACUC/Singapore) standard practices under the protocol #171200 and following the Biological Center Resources (BRC/A*STAR) institutional guidelines. All mouse experiments were conducted using adult animals with a minimum age of 120 days. No statistical method was used to pre-determine sample size. Experiments were not randomized, and there was no blinded allocation during experiments and outcome assessment.

2.4.2 - The *Mcc^{lacZ}* Mouse Model

Embryonic stem cells harboring the *Mcc^{Gt(D062B07)}* gene trap allele (often abbreviated as *Mcc^{lacZ}* or *Mcc-* on this study) were acquired from the German Gene Trap Consortium (GGTC) and used to generate mice maintained on a (129/Sv x C57BL/6) F1 mixed genetic background as previously described (Young et al., 2011). The *Mcc^{lacZ}* mouse line contains a splice acceptor (SA)-ROSA- β -geo+1 gene trap inserted 96.2 kb 3' of exon 4. The gene trap eliminates the expression of both *Mcc* isoforms and functions as a classic β -galactosidase reporter for endogenous *Mcc* expression.

2.4.3 - *Apc^{Min}* Mouse Model

Apc^{Min/+} mice (C57BL/6J-*Apc^{Min}/J*) (given to us by Dr. Dmitry Bulavin, previously at A*STAR IMCB) were mated to WT C57BL/6J animals to maintain a heterozygous colony. Next, *Apc^{Min/+}* mice were crossed to *Mcc^{-/-}* to generate double mutant animals.

2.4.4 Mouse Small Intestine Villus and Crypt Isolation

Villus and crypt compartments from mouse small intestine (duodenum) were dissociated as previously described (Jung et al., 2011). Duodenum portions from wild-type and *Mcc^{-/-}* age/sex-matched mice were dissected into 8-10 cm segments and further flushed with ice cold 1x PBS-0 (lacking Mg^{2+} and Ca^{2+}) to eliminate contaminants. Tissue pieces were cut open longitudinally. Villus fragments were obtained as described in Sato et al. (2009) then collected into conical tubes with ice-cold PBS-0. Remaining crypt-containing pieces of tissue were later incubated in a solution containing 2 mM-EDTA in ice-cold PBS-0 for 1 hour at 4°C on a rotating plate. After incubation, crypt-containing pieces of tissue were vigorously shaken 10 times

into a 50 mL conical tubes containing ice-cold PBS-0 to detach crypts from muscle layer. After shacking, pieces were transferred into a new 50 mL conical tube with ice-cold PBS-0 and the crypt-supernatant was kept on ice. This procedure was repeated 3 times resulting in 4 fractions of supernatant with detached crypts. The first tube was discarded and the last three crypt fractions were filtered through a 70-µm cell strainer (BD Biosciences) to remove remaining villi debris. Purified crypt and villus fractions were divided into two 1 mL samples for further processing either for RNA isolation, protein extraction or 3D organoid culture.

2.4.5 Mouse Intestinal Organoids Culture

About 50 crypts/well were processed and cultured in Mouse IntestiCult™ Organoid Growth Medium (STEMCELL Technologies, Cat#06005) organoid medium supplemented with 100 mg/ml of ampicillin and 100 mg/mL Primycin following standard protocols provided by the vendor. Only primary cells from mice were used for organoid culture. For inhibition of Casein Kinase 1 Delta and Epsilon (CK1D/E), a single dose of PF670462 (5 uM) was added to the organoid medium over the course of 72 hours (on days 3, 4 and 5 of culture). Treated organoids were harvested on day 6 for further analysis.

2.4.6 Mouse Intestinal Permeability Assays

In vivo intestinal permeability assays were performed using D-Xylose (Chondrex, Inc. - Cat#6601) to assess intracellular permeability and FITC-labelled Dextran-4kDa (Chondrex, Inc. - Cat#4013) to evaluate paracellular permeability. Briefly, water and food were removed from cages for 4 hours and then mice were administrated with a permeability tracer by oral gavage needles at concentrations of

50 mg/30 g of body weight for D-xylose and 60 mg/30 g body weight for FITC-labelled according to manufacturer's protocols. Mice remained in cages without food and water. After 4 hours mice were euthanized and blood was collected by cardiac puncture in EDTA tubes and later processed for serum extraction. D-xylose and FITC-dextran concentrations in serum were determined using standard curve with serial dilutions following manufacturer's instructions and measured by spectrophotometer (SpectraMax M5 – Molecular Probes).

2.5 - HISTOLOGICAL ANALYSIS

2.5.1 - Mouse Tissue Sections

Adult mice were gender- and age-matched to assure comparable outcomes. The proximal portion of the mouse small intestine (duodenum) and distal portion of the colon were used for intestinal epithelium analysis. Tissues were isolated as previously described (Williams et al., 2016). Briefly, duodenum and colon were carefully flushed with ice-cold PBS using a gavage needle and a 50 mL syringe. After removing lumen contaminants, intestinal tubes were cut open longitudinally, sectioned into roughly 3-cm pieces and later placed into biopsy cassettes and incubated overnight at 4°C with 4% Paraformaldehyde (PFA) (Formaldehyde 16% sol. Em Grade, #15710, EMS). Tissues were later dehydrated and embedded in paraffin. For swiss-roll sections, the entire small intestine was cut open longitudinally and pinned flat for fixation at 4°C with 4% PFA. After 24 hours, tissues were rolled up and placed into large cassettes. Following fixation, specimens were subsequently dehydrated in ethanol with increasing concentrations ranging from 70% to 100% followed by xylene, and later embedded in paraffin. For whole-mount tissue analysis, samples were fixed with 4%

PFA and later washed with PBS. Some analyses were performed in whole-mounts embedded in low melting point agarose and sectioned using microtomes.

2.5.2 Human Tissue Sections

Paraformaldehyde-Fixed Paraffin-Embedded (PFPE) human intestinal tissue sections were provided by Assoc. Prof. Christopher S. Williams, Department of Medicine and Cancer Biology at Vanderbilt University School of Medicine - Nashville, TN, USA.

2.5.3 - Histochemical Stains and *in situ* Hybridization

Staining for Beta-galactosidase activity was performed as described in Barker et al., (2007). Alkaline Phosphatase staining was performed using a commercially available kit (Vulcan Fast Red Chromogen kit FR805S, Biocare Medical, USA) following the manufacturer's recommendations. *in situ* hybridization (ISH) was performed using RNAscope® 2.5 HD Reagent Kit-Brown (322300) and 2.5 HD Duplex Reagent Kit (322430) from Advanced Cell Diagnostics according to the manufacturer's recommendations. The specificity of the *Mcc* probe set (**Table 05**) was confirmed by a lack of signal on sections of intestine from *Mcc^{lacZ}* homozygous adult mice (**Figure 15**).

2.5.4 Immunofluorescence – PFPE Tissues

Tissue sections (7 µm) were deparaffinized and rehydrated. Antigen retrieval was performed with 0.5x Tris/Borate/EDTA (TBE) buffer by heating slides using a pressure cooker. Sections were then blocked with 5% Bovine Serum Albumin (BSA) for 2 hours at room temperature and stained overnight with primary antibodies (**Table**

03). Tissues were washed with PBS before incubation with secondary antibodies (**Table 04**) for 1 hour at room temperature. Nuclei were stained with Hoechst 33342 (ThermoScientific, Cat#62249). Slides were mounted with water-based mounting medium (Hydromount, Electron Microscopy Sciences, Cat#17966,). Representative images of 3 repeats of immunostainings were included in this thesis.

2.5.5 Immunofluorescence - Cells

RPE-1, HCT116, HEK293 and SW480 cells were grown on glass coverslips and fixed in cold 100% methanol for 20 mins at -20°C. Cells were washed/permeabilized with 0.5% Triton-X100 in PBS and then blocked for 30 minutes with 5% BSA in 0.1% Triton-X100 in PBS. Immunostaining with primary antibodies was performed overnight at room temperature followed by PBS washes. Incubation with secondary antibody was performed for 2 hours at room temperature. Nuclei were stained using Hoechst 33342 (ThermoScientific, Cat#62249) at a dilution of 1:1000 in PBS followed by PBS wash. Slides were mounted with water-based mounting medium Hydromount (Electron Microscopy Sciences, Cat#17966,). For super resolution microscopy by Structured illumination microscopy (SIM) technique, cells were grown in 1.5H circular glass coverslip and mounted with VECTASHIELD® Mounting Medium (H-1000, Vector Laboratories, USA).

2.6 - MICROSCOPY

All images were acquired and processed using systems available at the A*STAR Microscopy Platform (AMP) in Singapore. Confocal microscopy images were generated either using an Olympus FV1000 upright confocal (IX81) or Olympus FV3000 confocal laser scanning microscope. Images were analysed using FlowView

software and further processed using Fiji (ImageJ). Conventional bright-field images were taken using Zeiss AxioImager Z1 upright EBL. Super resolution microscopy was performed using DeltaVision OMX microscope with 3D Structured Illumination Microscopy (3D-SIM). 3D images were analysed with IMARIS software. Samples for Electron Microscopy were processed and imaged at the Electron Microscopy (EM) division of the AMP using Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) techniques.

2.7 - RNA ANALYSIS

2.7.1 - RNA Isolation and qPCR.

RNA extraction from tissues was performed using Tryzol (Qiagen) and RNeasy Mini kit (Qiagen) following the manufacturer's protocol. High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) was used to reverse transcribe RNA into cDNA following the manufacturer's instructions. Quantification of gene expression by qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and the FastReal-Time PCR System (Applied Biosystems, USA). Each gene was analysed as technical triplicates for a minimum of three biological replicates. Relative gene expression was assessed using the $2^{-\Delta\Delta CT}$ method and data were normalized to the housekeeping gene *Beta-actin*. The qPCR primers are listed **Table 02**. Gene expression data were quantified and depicted as the mean $\pm$ SEM. Statistical analysis were performed using GraphPad Prism. Data were tested for significance by an unpaired two-tailed t-test. P-values of statistical significance are represented as *** $p < 0.001$, ** $p < 0.01$.

2.7.2 - RNA Sequencing

RNA was extracted from isolated crypts and villi fractions of WT and *Mcc*^{-/-} mouse small intestines. RT-PCR using specific markers for each fraction confirmed purity of the isolation. Quality control test showed RNA Integrity Number (RIN) above 8. RNA-Seq library preparation was performed using TruSeq RNA Sample Preparation Kit v2 kit (Illumina #RS-930-2001). The single-end reads were subsequently mapped to the mouse mm9 genome with RNA-seq aligner STAR 2.4.2a (Dobin et al., 2013) using default parameters. Mapped read counts against the ENSEMBL v65 mouse gene definitions were quantified using htseq-count within the HTSeq Python framework (Anders et al., 2015). Differential expression analysis of RNA-seq expression profiles between WT and *Mcc*^{-/-} was conducted using the R package edgeR (Bioconductor) (Anders et al., 2015).

2.8 - PROTEIN ANALYSIS

2.8.1 - Protein Extraction and Western Blot

Tissue/cell lysates were prepared using RIPA (R0278, SIGMA) lysis buffer supplemented with cOmplete ULTRA protease inhibitor (Roche, Switzerland). Tissue lysates were later sonicated for two cycles at 4°C for complete lysis. Protein concentration was determined for each sample using the Bradford Assay. Samples were boiled at 95°C for 5 min and loaded onto a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred onto PVDF membrane and incubated 1 hour at room temperature with 2% milk powder in PBS 0.2% TRITON 100 for blocking. Primary antibodies were applied overnight at 4°C and secondary antibodies for 1 hour at room temperature. Blots were revealed using SuperSignal™ West Femto Maximum

Sensitivity Substrate (34095, Thermo Scientific™). Beta-actin was routinely used as a loading control.

2.8.2 - Immunoprecipitation and Mass-spectrometry

The first round of immunoprecipitation (IP) and mass spectrometry (MS) performed to identify MCC-interacting proteins in this study was performed in collaboration with Dr Bernard Liu, formerly a member of the laboratory of Dr Tony Pawson (RIP 2013), Lunenfeld Tanenbaum Research Institute, ON, Canada. Briefly, for IP, N-terminal FLAG-tagged human MCC isoform 2 (pCMV2B-FLAG, Stratagene) was transfected generating stable HEK293 cell lines under drug selection. Stably empty pCMV2B vector were used as control. Cells lysates were prepared using HNTG lysis buffer (50 mM HEPES - pH 7.5; 150 mM NaCl; 1% Triton X-100; 10% glycerol; 1 mM EDTA) and immunoprecipitated using anti-Flag agarose beads overnight at 4°C and then washed with HNTG buffer.

Sample preparation for MS were carried out using two approaches. The first batch of samples was run on SDS-PAGE gel and bands were cut and digested with trypsin. A second batch of samples was eluted using 200mM glycine pH2.5 and then incubated on a strong-cation exchange SCX Column (ProPac™ SCX-20 HPLC Column - Thermo Scientific, #074643) followed by digestion with trypsin. Next, MCC interactions were captured using samples prepared by both approaches. Samples were run in an LTQ-Orbitrap-XL (PlanetOrbitrap – Thermo Scientific) mass spectrometer and data were analysed using both Mascot and ProHITS softwares (Liu et al., 2010) for protein identification and quantification.

2.8.3 Clustering - Alignment and Neighbour-Joining Tree of MCC.

Sequences from mouse and human MCC were used to BLAST the Human National Center for Biotechnology Information (NCBI) protein database using the various taxonomy codes for *Drosophila melanogaster* (Fruit Fly), *Danio rerio* (Zebrafish), *Branchiostoma floridae* (Lancet), *Bos taurus* (Cow), *Strongylocentrotus purpuratus* (Sea urchin), *Xenopus tropicalis* (Frog), and *Gallus gallus* (Chicken). Comparative genomics were confirmed using Ensembl Gene Tree analysis. Full length sequences were aligned using ClustalX software and a phylogenetic tree was generated using the neighbour-Joining algorithm and visualized using TreeView.

As described in the previous chapters, *MCC* was initially identified as a candidate tumor suppressor gene in a region of the human chromosome 5 that is frequently mutated in both sporadic and inherited colorectal tumors (Kinzler et al., 1991). Ever since *MCC*'s discovery in 1991, there have been numerous studies suggesting its contribution as a potential driver in the development of intestinal tumors. Particularly, two independent studies have demonstrated that genetic loss of *MCC* and *MCC* silencing through promoter hypermethylation are implicated in the development of cancer precursor lesions (polyps) and colorectal tumors via the serrated neoplastic pathway (Fukuyama et al., 2008; Kohonen-Corish et al., 2007; Li et al., 2013). Additionally, another study identified mutations in *Mcc* as drivers of colorectal cancers in mice through a transposon-based genetic screen (Starr et al., 2009).

Although studies have long reported that mutations in the *MCC* gene are associated with subsets of sporadic and inherited intestinal cancers, the fundamental role of *MCC* in intestinal homeostasis remains unclear. Furthermore, there is very little substantial evidence determining the expression of *Mcc* in the intestinal epithelium. Young et al. (2011) reported that *Mcc* is dynamically expressed during mouse embryonic development. In this study, they showed that *Mcc* expression not only labels the developing gut, but its expression persists in the adult intestine. However, the specific expression pattern of *Mcc* along the intestinal epithelium cells was poorly characterized. Given that stem cells located in the crypts have been identified as cell-of-origin of intestinal cancers, it prompted me to accurately investigate potential *Mcc*-expressing cell populations in the adult intestinal epithelium.

3.1 *Mcc* is Specifically Expressed in the Crypts of the Intestinal Epithelium.

I employed an *Mcc^{lacZ}* reporter allele generated by a loss-of-function gene trap insertional mutation (hereafter designated *Mcc⁻* or *Mcc^{lacZ}*) to document *Mcc* expression at the cellular level (**Figure 12**). The *Escherichia coli* (*E. coli*) *lacZ* gene is an extensively used reporter gene in transgenic mouse models. *E. coli lacZ* encodes the enzyme β -galactosidase (β -gal) whose activity can be chemically visualized with the hydrolysis of a substrate complex simply termed X-Gal (indoxyl glycoside 5-bromo-4-chloro-3-indolyl- β -galactopyranoside) (Burn, 2012). The reaction results in a stable blue precipitate labeling *lacZ* expressing cells.

To characterize *Mcc*-expressing cells in the adult intestinal epithelium, I harvested proximal small intestine (duodenum) and distal colon tissues from 120-day old *Mcc^{lacZ/lacZ}* mice and further processed the tissues for *Mcc^{lacZ}* expression analysis (β -gal activity) as previously described in Muncan et al. (2006) (**Figure 13**). Intestinal tissues from wild-type (WT) *Mcc^{+/+}* age- and gender-matched animals were used as negative control for *Mcc^{lacZ}* expression (**Figure 15A-B**). Histochemical analysis was performed under controlled pH conditions (pH8) to suppress potential endogenous intestinal β -gal activity (Merkwitz et al., 2016).

Microscopic analysis of paraformaldehyde-fixed and paraffin-embedded (PFPE) duodenum sections revealed that β -gal staining was absent in the villus compartment. Notably, *Mcc^{lacZ}* expression was found specifically in the crypt compartment of both small intestine (**Figure 13A**) and colon (**Figure 13B**). This unique *Mcc^{lacZ}* expression pattern in the crypt compartment was also observed in whole-mount small intestine vibratome sections (**Figure 13C**).

Figure 13 - *Mcc^{lacZ}* expression is restricted to the crypts in the intestine. β -galactosidase reporter mouse model (*Mcc^{lacZ}*) employed to analyze *Mcc* expression in the intestinal epithelium. **A - B:** Longitudinal sections of *Mcc^{lacZ/lacZ}* mouse small intestine (duodenum) and colon tissues, respectively, stained for β -galactosidase activity (β -gal). Black dashed-squares highlight regions selected for higher magnification. Images in A and B were counter-stained with Nuclear Fast Red. Scale bars, 50 μ m. **C:** Whole-mount β -gal staining of adult *Mcc^{lacZ/lacZ}* small intestine (duodenum) reveals that β -gal activity is restricted to the crypts (black arrows). Scale bar, 500 μ m. **D - F:** Staining for β -gal activity and alkaline phosphatase in whole-mount villus (white arrow) and/or crypts (black arrow) micro-isolated from the adult small intestine (duodenum). Scale bars, 50 μ m.

The crypt of the small intestine is a dynamic and hierarchic epithelial compartment harboring stem cells and rapid proliferating progenitors located at different positions along the crypt axis (**Figure 02**). To better characterize the *Mcc^{lacZ}* expression in the crypt compartment, small intestine tissues from WT and *Mcc^{lacZ/lacZ}* were harvested and processed to separate and purify crypt and villus compartments as previously described in Sato et al. (2009). Next, isolated villus and crypt structures were fixed and processed for detection of β -gal and alkaline phosphatase activities as whole-mounts. The enzyme alkaline phosphatase is uniquely secreted by differentiated cells in the villus (Sussman et al., 1989).

Whole-mount staining for β -gal activity in *Mcc^{lacZ/lacZ}* purified crypts and villi along with the differentiation marker alkaline phosphatase confirmed that β -gal-positive cells

are limited to the crypts (**Figure 13D-E**). Moreover, high magnification imaging revealed that β -gal is detected in cells throughout the entire crypt structure, indicating that *Mcc* is expressed from the base at the stem cell zone of intestinal crypts to the transit amplifying compartment (**Figure 13F**).

To more accurately resolve the *Mcc* expression in intestinal crypts, I performed high-resolution RNA section *in situ* hybridization (ISH) for endogenous *Mcc* transcript expression singly or together with *Lgr5*, which specifically labels CBC stem cells (**Figures 2 and 3**). In agreement with the *Mcc^{LacZ}* expression findings, I also detected robust endogenous *Mcc* expression specifically at the crypt compartment (**Figure 14A**).

Figure 14 - Endogenous *Mcc* expression is restricted to intestinal crypts. **A - B:** Section *in situ* hybridization (ISH) for *Mcc* and *Lgr5* expression on wild-type (WT) small intestine. **A** Brown dots represent endogenous *Mcc* expression in the crypt compartment. **B** Co-ISH for *Mcc* (blue) and *Lgr5* (red) shows that *Mcc* expression overlaps with *Lgr5* at the crypt base but extends further into the transit amplifying compartment. **C:** Section *in situ* hybridization for *Mcc* expression on WT colon. Brown dots represent endogenous *Mcc* expression. Black dashed squares in **A - C** highlight regions selected for higher magnification. Scale bar, 50 μ m. **D:** qPCR analysis for *Mcc* and *Lgr5* expression in purified small intestine (duodenum) crypts and villi (n = 3). Data were tested for significance by an unpaired two-tailed t-test. ***P < 0.001, **P < 0.01.

Co-in situ hybridization analysis showed that *Mcc* and *Lgr5* transcripts overlap in CBC cells at the crypt base. However, *Mcc* expression is broader, labelling not only the crypt base but also the transit-amplifying (TA) compartment (**Figure 14B**), further validating my *Mcc^{lacZ}* reporter allele expression data (**Figure 13A-F**). *Mcc* mRNA is similarly restricted to the crypts in the colon epithelium (**Figure 14C**). Additional *in situ* hybridization images showing controls and probe specificity in WT and *Mcc^{lacZ/lacZ}* can be found in **Figure 15C-D**. Next, I performed qPCR for endogenous *Mcc* and *Lgr5* expression in isolated crypts and villi samples from adult WT and *Mcc^{lacZ}* homozygous proximal small intestine (**Figure 14D**). As expected, based on my ISH analysis, expression of *Mcc* is only detected in WT crypt samples. *Lgr5* expression is detected in both WT and *Mcc^{lacZ/lacZ}* crypt samples.

In summary, my findings reveal that *Mcc* expression is restricted to proliferative cells along the entire crypt, from CBCs extending into the transit-amplifying compartment, but is not detected within the differentiated cells along the villus axis. From these comprehensive analyses, we conclude that *Mcc* is a novel marker for all proliferating cells within the crypt but its expression at the mRNA level is entirely absent in the differentiated cells within the villus.

Figure 15 - β -gal activity and *in situ* hybridization (RNAScope) controls. **A:** ~3cm pieces of wild-type (WT) *Mcc*^{+/+} and *Mcc*^{lacZ/lacZ} mouse small intestine duodenum stained for β -gal activity. WT intestine control does not react with the X-gal substrate. Scale bars not shown. **B:** Whole-mount isolated crypts from WT and *Mcc*^{lacZ/lacZ} small intestine stained for β -gal activity and Alkaline Phosphatase (Alk Phos). *Mcc-lacZ* expression is not detected by β -gal activity in WT crypts. Scale bars, 50 μ m. **C:** *in situ* hybridization (ISH) by RNAScope using a positive mouse control probe for *Ppib*, and probes for *Lgr5* and *Mcc* on WT and *Mcc*^{lacZ/lacZ} (*Mcc*^{-/-}) mouse small intestine tissues (cross-sections). Endogenous *Mcc* expression is not detected on *Mcc*^{-/-} small intestine. **D:** ISH by RNAScope for *Mcc* on WT and *Mcc*^{-/-} mouse small-intestine crypt transverse sections. *Mcc* expression is not detected on the *Mcc*^{-/-} crypts. Scale bars represent 20 μ m.

3.2 *MCC* Encodes a Centrosomal Protein.

MCC is a highly evolutionarily conserved protein that contains an extreme C-terminal type I (PSD-95/Dlg/ZO-1) PDZ binding motif (ETSL) (**Figures 10** and **16A**) (Caria et al., 2019; Pagon et al., 2012). The *MCC* protein also contains several regions predicted to form coiled coil structures (**Figure 16B-C**) (Bourne, 1991; Kinzler et al., 1991). In mouse and human, there are two isoforms of *MCC* (1 and 2) that are generated from alternative promoter usage and differ only at their N-termini (**Figure 10**). My laboratory has previously demonstrated that the shorter *Mcc* transcript encoding *Mcc* isoform 2 is the most abundant in the developing mouse embryo and in adult tissues, including the intestine (Young et al., 2011).

Despite this knowledge, the molecular function of *Mcc* within cells and tissues remains poorly understood. This is due both to the lack of published studies investigating *Mcc* and their often-confusing results showing that the *Mcc* protein, depending on the cell type, reagents and assays used, can be localized in different organelles (mitochondria and ER) and cellular compartments including plasma membrane, cytoplasm and nucleus (Arnaud et al., 2009; Benthani et al., 2018; Fukuyama et al., 2008; Li et al., 2013; Matsumine et al., 1996; Pagon et al., 2015).

Because of these rather controversial findings and the fact that we found *Mcc* specifically expressed in crypts of the adult mouse small intestine and colon, we were prompted to further investigate the subcellular localization of *MCC* in assorted cell lines and in the murine intestine, as well as to uncover the binding partners of *MCC* and establish its network of interactions.

We started our investigations by performing additional bioinformatic analysis on MCC sequence using online protein databases and first discovered that MCC protein shares multiple regions of sequence homology with the centrosomal protein NINEIN, a protein that also contains multiple coiled-coil regions (Bouckson-Castaing et al., 1996) (**Figure 16C**).

Figure 16 - Bioinformatic analysis of the MCC sequence. **A:** (Left) Neighbor-joining tree of protein sequences of various MCC orthologs and paralogs across different species using ClustalX sequence alignment and (Right) the conserved C-terminal PDZ binding motif sequences of MCC from the indicated species. **B:** Sequence analysis of MCC using the COILS prediction software (http://embnet.vital-it.ch/software/COILS_form.html) revealed multiple coiled-coil regions in MCC sequence. **C:** Schematic representation of MCC isoforms 1 and 2 and NINEIN. MCC isoforms share multiple regions of sequence homology with NINEIN. Graph: The X and Y axis correlation represents shared amino acid positions for MCC isoform 1 and NINEIN.

This guided us towards the next experimental approach in re-investigating MCC's subcellular localization. For this, an N-terminal EmGFP-mouse Mcc isoform 2 (EmGFP-mMcc2) fusion construct was transfected into HEK293 cells followed by immunofluorescence (IF) using a robust antibody (**Table 03**) against PERICENTRIN (PCNT), an integral component of the centrosome PCM. Interestingly, confocal microscopy revealed co-localization of the EmGFP-mMcc2 signal with endogenous PCNT at the centrosome (**Figure 17A**).

Figure 17 - EmGFP-Mcc subcellular localization and MCC interactome in HEK293 cells. A: HEK293 cells transfected with EmGFP-Mcc isoform 2. Transfection is not 100%, yielding a mosaic EmGFP signal, while all cells are PCNT-positive. Arrows indicate co-localization of EmGFP-Mcc with PCNT. Scale bar, 20 μ m. **B:** Immunoprecipitation of Myc-tagged human MCC isoform 2 in HEK293 cells followed by mass spectrometry identifies several classes of MCC binding partners, including proteins associated with the centrosome (yellow), GTPase regulators (orange), cell polarity proteins (red) and casein kinases 1 (CK1) (green). **C:** Immunoprecipitation (IP) in HEK293 cells followed by Western blotting for indicated interactions. WCL, whole cell lysate. Ø, pCMV empty vector control.

This preliminary and unexpected subcellular localization of EmGFP-mMcc2 and the fact that our understanding of MCC functional role in homeostasis or disease is largely unknown encouraged us to identify MCC-interacting proteins using a proteomics approach. To this end, we overexpressed FLAG-tagged human MCC isoform 2 in HEK293 cells and established the MCC interactome using conventional

immunoprecipitation with anti-FLAG agarose beads followed by mass spectrometry. Bioinformatic analysis revealed that MCC binds to a number of centrosomal proteins such as CEP131 (also known as AZI1), CEP170 and NDE1 (**Figure 17B**).

MCC was also further found to interact with the casein protein kinases CSNK1D and CSKN1E (CK1 δ and CK1 ϵ) and other polarity proteins containing PDZ domains such as SCRIBBLE, NHERF1/2 and SNX27. Several proteins that regulate small GTPases such as RASAL2, IQGAP1 and RAB11FIP5 (also known as RIP11) were also recovered (**Figure 17B**). Several of these interactions were further validated by additional immunoprecipitation of overexpressed Myc-tagged MCC and Western blotting for assorted endogenous proteins in HEK293 cells (**Figure 17C**).

We next aimed to reevaluate the subcellular localization of MCC in numerous cell lines including those known to contain endogenous MCC by Western blotting or in which MCC localization had been previously proposed (e.g., HEK293, SW480 and HCT116) (Benthani et al., 2018; Fukuyama et al., 2008). To this end, we surveyed numerous commercially available antibodies for MCC specificity and identified one that shows highly specific staining for endogenous MCC by IF and that works well in western blot analysis (**Table 03**).

Firstly, we independently confirmed some MCC interactions identified by mass spectrometry by performing IF co-localization with the integral centrosomal proteins NDE1, CEP131, CEP170; and proteins that also co-localize at the centrosome such as the polarity protein NHERF1 and the GTPase regulator RASAL2. Confocal microscopy demonstrates that MCC in fact co-localizes with all these proteins at the centrosome of RPE1 cells (**Figure 18A**). PCNT and NINEIN are well characterized centrosomal protein. Therefore, we also performed IF co-localization of MCC with

PCNT and NINEIN. My results also confirmed co-localization of MCC and PCNT at the centrosome in a variety of cell lines including SW480, HEK293, HCT116 and RPE1 (**Figure 18B**). MCC also co-localized with NINEIN in RPE-1 cell (**Figure 18B**).

To further elucidate the subcellular localization of MCC at the centrosome, we performed IF using both confocal and the super resolution technique Structured Illumination Microscopy (SIM) (Sonnen et al., 2012) to analyse the spatial proximity between MCC and either NINEIN or PCNT in SW480 cells (**Figure 18C**). These imaging analyses revealed that MCC is in close proximity to PNCT and NINEN in the centrosome, although the mass spectrometry did not reveal their direct physical interaction.

To substantiate our findings, we used correlation-based co-localization analysis to quantitatively measure the relationship of MCC versus NINEIN, CEP170, NDE1, CEP131, RASAL2 and NHERF1 in several independent IF co-localization experiments in individual RPE-1 cells. Our results (Pearson Correlation (r)) shows strong positive correlation between MCC signal position with the assorted proteins analysed (**Figure 18D**) (Aaron et al., 2018).

Collectively, these data provide strong evidence that recast MCC as a novel centrosomal protein. Importantly, we used several different approaches including Western blot and IF analyses to validate these findings in several cell lines from mouse and human and mass spectrometry to reveal several interacting partners of MCC.

Figure 18 - MCC co-localizes with well-characterized centrosomal proteins in assorted cell lines.

A: Co-immunofluorescence in RPE-1 cells shows co-localisation of MCC and NDE1, CEP131 and NHERF confirming interactions revealed by mass spectrometry. **B:** Co-localization of MCC and PERICENTRIN (PCNT) at the centrosome in SW480, HEK293, HCT116 and RPE-1 cells as well as MCC and NINEIN in RPE-1 cells. **C:** Co-immunofluorescence wide-field 3D and super resolution Structured Illumination Microscopy (SIM) demonstrate that MCC co-localizes with the centrosomal proteins PCNT and NINEIN in SW480 cells. Scale bars, 50 μ m. **D:** Pearson correlation coefficient (r) reveals strong positive linear correlation between the signal positions of MCC and several of its interacting partners at the centrosome in RPE-1 cells.

3.3 Mcc Re-Localizes from the MTOC in the Crypts to the Apical nc-MTOC in Intestinal Cells.

The unique *Mcc* transcript distribution in the murine intestinal epithelium revealed by ISH as well as the subcellular localization of MCC at the centrosome prompted me to examine to investigate the localization of the Mcc protein in the crypt cells of the intestinal epithelium. First, following the same experimental approach of the qPCR analysis in isolated crypt and villi (**Figure 14D**), we extracted protein from isolated crypt and villus samples to access the presence of Mcc protein by Western blot. Protein lysates from *Mcc^{lacZ/lacZ}* crypts and villi, which entirely lack Mcc protein, were used as negative control to verify the specificity of the anti-MCC antibody. As expected, we detected Mcc protein in WT crypt samples (**Figure 19A**). However, unexpectedly, we also detected Mcc protein in villi samples despite the absence of *Mcc* mRNA by ISH and qPCR (**Figure 14A/D** and **Figure 19A**).

Hence, we next sought to investigate the significance of this finding by looking at endogenous Mcc localization in the intestinal epithelium compartments. To this end, we performed IF in PFPE sections of the small intestine and colon. Confocal microscopy analysis confirmed that Mcc localizes to the centrosome in proliferating crypt cells of both the small intestine (**Figure 19B-D**) and colon (**Figure 20A**). Notably, however, we observed a highly specific staining for Mcc at the apical membrane of differentiated cells along the villus compartment in the murine small intestine (**Figure 19B/E-F**). The presence of Mcc protein in the villus compartment is consistent with my Western blotting results indicating that Mcc protein, in fact, localizes to the apical membrane of differentiated intestinal cells as no Mcc signal was detected by IF in *Mcc^{lacZ/lacZ}* intestinal epithelium tissue sections, confirming the specificity of the antibody (**Figure 21C-D**). Mcc is also localized at the apical membrane of differentiated cells on the surface of the colonic epithelium (**Figure 20A**).

Figure 19 - Mcc localizes to the centrosome in proliferating crypt cells and at the apical membrane in differentiated villus cells. **A:** Western Blot for Mcc protein in whole cell lysates from purified villi and crypts of the adult small intestine. **B:** Immunofluorescence (IF) for Mcc in the adult mouse small intestine (duodenum). The white dashed square indicates the region selected for higher magnification. Blue arrow indicates punctate Mcc staining at the centrosome in crypt cells and white arrow indicates Mcc localization at the apical membrane of differentiated cells. **C** and **D:** IF for Mcc in crypts of the adult small intestine. White dashed squares highlight regions selected for higher magnification (C, longitudinal and D, transverse sections). Punctate Mcc staining at the centrosome is observed throughout the entire crypt. Scale bar, 20 μ m. **E** and **F:** Mcc and β -catenin co-immunofluorescence in adult intestinal villi. Mcc (red) localizes to the apical and β -catenin to the lateral membrane of enterocytes (E, longitudinal and F, transverse sections). Scale bars, 20 μ m.

Furthermore, we wanted to investigate if another centrosomal protein would have the same localization pattern as Mcc in the intestinal epithelium. Therefore, we performed co-localization IF for Mcc and Pcnt and we detected their co-localization at the centrosome in intestinal crypts as well as at the apical membrane in villus differentiated cells (**Figure 20C-D**). Additional IF co-localization for Mcc and β -catenin in intestinal differentiated cells revealed that Mcc overlaps with β -catenin uniquely at apico-lateral junction (**Figure 19E-F** and **Figure 20B**).

Lastly, we sought to analyse the localization of MCC by IF in human small intestine sections. We confirmed that MCC also localizes to the centrosome in the crypt and re-localizes to the apical membrane of villus differentiated cells (**Figure 20E-F**).

Taken together, our results show that Mcc protein specifically localizes to the centrosome in proliferative cells of intestinal crypts from mouse and human. *Mcc* transcript is also detected in this compartment. However, upon intestinal differentiation *Mcc* is no longer being expressed, while the Mcc protein is re-located to the apical membrane of differentiated cells in the villus compartment.

Figure 20 - Mcc protein localization in the small intestine and colon epithelium. **A:** Immunofluorescence (IF) for Mcc localization on mouse colonic epithelium reveals Mcc at the centrosome in colonic crypt cells and at the apical membrane of differentiated cell at the surface. **B:** IF colocalization for Mcc and β -catenin on transverse section of WT small intestine villus shows overlap at the apico-lateral cell junction. **C:** IF for Mcc and Pcnt on section of the small intestine shows colocalization at the centrosome in the crypts and apical membrane of differentiated villus cells. **D:** Cross section of a villus showing co-localization of Mcc and Pcnt by IF. **E** and **F:** IF for MCC on human small intestine cross-sections showing Mcc staining at the centrosome in crypt cells and (F) at the apical membrane of differentiated cells in the villi. White dashed-square indicates selected region for higher magnification. Scale bars represent 20 μ m.

Figure 21 - Anti-MCC antibody specificity control for immunofluorescence in the mouse intestine. **A - B** : Immunofluorescence (IF) for *Mcc* showing protein localization on WT transverse sections of mouse small intestine crypts and Villus. White dashed-square indicates selected region for higher magnification. **A**: *Mcc* localizes to the centrosome (punctuate staining) in the crypts and **B**, at the apical membrane of differentiated cells in the villus compartment. **C** and **D**: Immunofluorescence (IF) for *Mcc* in *Mcc* $-/-$ small intestine crypts and villus confirms the specificity of the anti-MCC antibody for IF as no signal is detected, despite unspecific background. Scale bars represent 20µm.

3.4 MCC is Phosphorylated by CK1 δ/ϵ

The centrosome is the major microtubule-organizing center (MTOC) in most eukaryotic cells. MTs are nucleated and anchored at the MTOC by the γ -TuRC and several components of the pericentriolar material (PCM) in the centrosomal complex (Muroyama and Lechler, 2017; Woodruff et al., 2014). In specific types of differentiated epithelial cells, such as in the intestinal epithelium, the MTs undergo reorganization during terminal differentiation and are anchored at the apical membrane by the nc-MTOC (non-centrosome microtubule-organizing center), exactly where I identified Mcc to be localized in the villus cells as shown in my previous result (**Figure 19 B/E-F**) (Muroyama and Lechler, 2017; Sanchez and Feldman, 2017). MTs anchoring components are known to re-localize to the nc-MTOC in the intestinal epithelium, however the molecular mechanism is not well understood (Goldspink et al., 2017; Noordstra et al., 2016; Toya and Takeichi, 2016).

Interestingly, among the major MCC-interacting proteins uncovered by our mass spectrometry analysis are the casein kinases CK1 δ/ϵ , which are known to play both positive and negative roles in the regulation of the WNT/ β -Catenin signaling pathway and to play regulatory functions at the centrosome (Brockman et al., 1992; Cruciat, 2014; Greer et al., 2014; Schitteck and Sinnberg, 2014; Stöter et al., 2005).

Numerous reports using proteomic studies have revealed that MCC is a phosphoprotein (Arnaud et al., 2009; Caria et al., 2019; Pangon et al., 2012). In addition, we identified multiple phosphorylation sites on MCC by CK1 ϵ in our mass-spectrometry and bioinformatic data (**Figure 22**). We therefore sought to investigate whether MCC is a direct target of CK1 δ/ϵ phosphorylation.

Figure 22 - Mass-spectrometry identifies multiple phosphorylation sites on MCC by CK1ε. Stably expressing Flag-MCC HEK293 cells transfected with or without CK1ε revealed numerous phosphorylation sites across the MCC sequence. **A:** MCC peptide sequence showing coverage of phosphorylation sites identified by the software Mascot (Matrix Science). **B:** Phosphorylation on Ser(828) within the PDZ motif (last 4 amino acids E-T-S-L of MCC). The resolving MS-MS spectrum map of a phospho-SRPHT-NETpSL peptide is shown. Mascot score 52.9. Evidence for S828 phosphorylation is presented by b-series (highlighted in red).

Our first confirmation that MCC is a direct target of CK1δ/ε comes from western blot analysis for endogenous MCC in the colon cancer cell line SW480. Specifically, we used a commercially available MCC antibody and detected 2 bands in SW480 cells, one running at about 93 kDa, which refers to the wild-type, and another one with a higher molecular weight band that is most likely due to phosphorylation. Upon treatment with a highly-specific small molecule inhibitor of CK1δ/ε (PF670462), the upper MCC band is no longer detectable indicating that MCC is indeed a target of CK1δ/ε phosphorylation (**Figure 23A**).

Next, we performed *in vitro* co-expression of Myc-MCC and FLAG-CK1 ϵ or FLAG-CK1 ϵ _{D1280} (a truncated non-active form of CK1 ϵ) in HEK293 cells. We observed that CK1 ϵ prompts an upward mobility shift of MCC, which is not observed in the presence of the catalytically inactive form CK1 ϵ _{D128A} (**Figure 23B**). Furthermore, we confirmed that overexpression of Myc-MCC and FLAG-CK1 ϵ in the presence of PF670462 also abolishes the higher molecular weight version of MCC, revealing that phosphorylation of MCC is triggered by CK1 ϵ (**Figure 23B**).

To investigate whether phosphorylation of MCC regulates its interactome, we overexpressed Myc-MCC singly or in combination with FLAG-CK1 ϵ in HEK293 cells and selected RASAL2 (RAS GTPase regulator) and NDE1 for immunoblotting. Both proteins are actin/MT regulators. NDE1 is a well-known integral component of the centrosome (Hirohashi et al., 2006). We observed that in cells where MCC is phosphorylated by CK1 ϵ (+ Myc-MCC and +FLAG-CK1 ϵ) the interaction with RASAL2 is stabilized while binding to NDE1 appears to be diminished indicating a regulation of interactions between MCC and other centrosomal components upon phosphorylation (**Figure 23D**).

Several proteins including SCRIBBLE, NHERF1 and NHERF2 have been previously identified to interact with MCC through its PDZ binding motif (the last 4 amino acids, E-T-S-L) (Arnaud et al., 2009; Pangon et al., 2012). SCRIBBLE and NHERF1 both were identified as MCC binding partners by our mass spectrometry analysis (**Figure 17B**). Interestingly, NHERF1 (Na/H exchanger regulator factor 1) also known as EBP50 (ezrin-radixin-moesin binding phosphoprotein 50) is a PDZ scaffold protein that plays important roles in organization of epithelial cell apical domains through assembly of protein complexes at the membrane or by linking

proteins to apical actin filaments (Garbett et al., 2010; Georgescu et al., 2016; Kreimann et al., 2007; Treat et al., 2016; Vaquero et al., 2017).

To investigate if MCC binding to NHERF1 occurs via PDZ motif/domain interaction, we overexpressed an MCC mutant protein, in which the last 4 amino acids, (E-T-S-L) are truncated (MCC- Δ ETSL) in HEK293 cells, and immunoblotted for NHERF1, NDE1 and RASAL2. Indeed, we observed that interaction between MCC and NHERF1 was dependent on the MCC PDZ-binding motif, while RASAL2 and NDE1 interactions were not affected (**Figure 23E**).

Figure 23 - Phosphorylation of MCC by Casein Kinases 1 (CK1) shifts its affinity for interacting protein partners. **A:** In the human colorectal cancer cell line SW480, endogenous MCC is phosphorylated (higher mobility species in the DMSO lane). Phosphorylation is inhibited by treatment with the CK1 δ/ϵ inhibitor (PF670462) with increasing concentrations (5 μ M and 10 μ M). SCRIBBLE, β -CATENIN and β -TUBULIN were used as controls. **B:** Co-expression of Myc-MCC and FLAG-CK1 ϵ in HEK293 cells results in the phosphorylation of Myc-MCC (lane 2). Phosphorylation of MCC is not observed with a catalytically dead mutant of CK1 ϵ (CK1 ϵ -D128A). ($\emptyset$ = Empty). **C:** Phosphorylated MCC (upper band, asterisk) is more abundant in HEK293 cells co-transfected with Myc-MCC and FLAG-CK1 ϵ expression constructs (as in B). Treatment with the PF670462 eliminates the phosphorylated form of MCC. **D:** Simultaneous expression of Myc-MCC and FLAG-CK1 ϵ in HEK293 cells followed by immunoblotting (IB) analysis reveal that interaction of MCC with RASAL2 is stabilized upon MCC phosphorylation while interaction with NDE1 is weakened. **E:** Truncation of the PDZ binding motif (-ETSL) of MCC disrupts binding to NHERF1 and SCRIB, but not to RASAL2 or NDE1. WCL, whole cell lysate.

Collectively, these results indicate that MCC is a direct target of CK1 ϵ phosphorylation and that phosphorylation of MCC decreases its affinity to centrosomal core proteins such as NDE1. In addition, we demonstrated that MCC interacts with the PDZ domain of NHERF1 through its C-terminal PDZ binding motif. Hence, our hypothesis is that phosphorylation of MCC by CK1 ϵ triggers MCC re-localization to the apical membrane in differentiated intestinal cells where MCC is anchored through its PDZ binding motif with NHERF1 (**Figure 24**).

Figure 24 - Working model for Mcc redeployment from the centrosome in proliferative crypt cells to the apical membrane of differentiated cells. **A:** Schematic diagram of small intestinal cell differentiation. **B:** Phosphorylation of Mcc by CK1 δ/ϵ releases Mcc from the centrosome by decreasing its affinity to proteins such as NDE1 (left panel). Mcc moves to the apical membrane to form the nc-MTOC where it interacts with NHERF1 and β -catenin at the apico-lateral junction (right panel). (**A** is adapted from Barker, 2014).

To explore this hypothesis, we generated small intestinal *ex vivo* organoids as previously described by Sato et al. (2009) (**Figure 25A-B**). Firstly, we observed that *Mcc*^{-/-} mutant homozygous organoids present no obvious phenotypes over a period of 5 days in culture. Next, I treated wild-type (WT) *Mcc*^{+/+} organoids with single daily doses of 5 μ M of the CK1 δ/ϵ PF670462 inhibitor at day 3, 4 and 5 post seeding. I then isolated and examined Mcc localization by IF in PFPE sections of PF670462 treated

and non-treated control (NTC) WT organoids. Mcc signal was detected along the apical membrane of NTC WT organoids. However, Mcc fails to localize at the apical membrane upon inhibition of CK1 δ/ϵ (**Figure 25C**).

Figure 25 - Treatment of *Mcc* $+/+$ small intestinal organoids with the CK1 δ/ϵ inhibitor (PF670462) displaces Mcc from the nc-MTOC. A: Schematic diagram of small intestinal organoids showing crypt-like and villus-like domains. **B:** Small intestinal organoids derived from wild-type (WT) *Mcc* $+/+$ and *Mcc* $-/-$ mice. Whole-mount bright filed images reveal no ostensible phenotypes in *Mcc* $-/-$ organoids at day 7 of culture post-seeding. Scale bars 500 μ m **C:** Immunofluorescence (IF) of WT organoid sections. IF for Mcc in WT non-treated control (NTC) organoids showing Mcc localization along the apical membrane (left panels). IF for Mcc in WT organoids treated with 5 μ M of PF670462 (CK1 δ/ϵ inhibitor) reveals that Mcc localization at the apical membrane is abolished upon inhibition of CK1 δ/ϵ . Scale bars 50 μ m.

These results demonstrate that MCC localizes to the centrosome (MTOC) in proliferative cells in the crypt and upon terminal differentiation *Mcc* re-localization to the apical membrane is triggered CK1 δ/ϵ phosphorylation.

3.5 Analysis of the *Mcc* Loss-of-function Phenotype.

Homozygous *Mcc^{lacZ}* mice (referred to as *Mcc^{-/-}*) (**Figure 12**) were initially reported to present no ostensible phenotypes. Throughout the course of my experiments, however, we noticed at the time of harvesting that the intestinal tissue of *Mcc^{-/-}* mice presents villi with a compromised morphology (**Figure 26A**). Furthermore, various histological analyses reveal shrinkage with frequent indentations along the villus axis (**Figure 26**). Notably, the mutant villi are generally much shorter than WT (**Figure 26B**). In addition, *Mcc^{-/-}* animals weighed considerably less than their WT littermates by three months of age (**Figure 26C**). Because of this obvious weight loss in *Mcc^{-/-}* animals, we were driven to examine the microvilli/ brush border (BB) and check if loss of *Mcc* would affect the assembly of the BB and thus result in absorption defects. To this end, we prepared small intestine tissue samples for analysis by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (**Figure 27**). In agreement with my histological findings the SEM analysis confirmed a compromised morphology and shrinkage with frequent indentations/invaginations of *Mcc^{-/-}* villi. The microvilli in *Mcc^{-/-}* enterocytes, however, do not display any obvious aberrations (**Figure 27**).

Interestingly, as previously mentioned, *Mcc^{-/-}* mutant homozygous organoids did not present obvious morphological differences as compared to WT intestinal organoids over a period of 5 days. After 8 days in culture, however, particularly *Mcc^{-/-}* organoids disintegrated and presented a high number of disconnected cells, indicating that *Mcc* expression is not essential for organoid outgrowth but potentially important

for maintaining the epithelial integrity of intestinal organotypic cultures over time (Figure 28).

Figure 26 - *Mcc* loss-of-function phenotypes. **A:** Various histological analyses reveal shrinkage and architectural aberrations in the villi morphology of *Mcc*^{-/-} mouse small intestine. Whole-mount bright field images of freshly harvested wild-type (WT) *Mcc*^{+/+} and *Mcc*^{-/-} duodenum revealed indentations along the villus structure in *Mcc*^{-/-} (Scale bars not shown). Vibratome sections stained with F-actin and PFPE sections stained with β -catenin confirm morphological defects in the intestinal epithelium of *Mcc*^{-/-} mice. Scale bars 50 μ m. **B:** Comparisons of villus length between WT and *Mcc*^{-/-} mice confirms that *Mcc*^{-/-} villi are significantly shorter than the WT. **C:** *Mcc*^{-/-} mice show reduced body weight as compared with age-matched WT littermates. Data were tested for significance by an unpaired two-tailed t-test *** $p < 0.001$.

Figure 27 - *Mcc* loss-of-function intestinal phenotype. Panoramic view of wild-type (WT) *Mcc* $+/+$ and *Mcc* $-/-$ small intestinal villi by scanning electron microscopy (SEM) exposing the compromised architecture of villi in *Mcc* $-/-$ mouse small intestine. Scale bars 500 μ m. No ostensible morphological defects are observed in the microvilli (brush border) of *Mcc* $-/-$ by SEM or Transmission electron microscopy (TEM) as compared to the WT microvilli. Scale bars 1 μ m.

Figure 28 - *Mcc* loss-of-function phenotype in intestinal organoids. Wide field microscopy of wild-type (WT) *Mcc* $+/+$ and *Mcc* $-/-$ mutant small intestinal organoids at indicated time points post seeding. Scale bars 200 μ m.

Next, we investigated whether differentiated cells in the villus compartment displayed defects in the MT network. Therefore, we performed IF for α -tubulin and found that differentiated epithelial cells in the villus of *Mcc*^{-/-} intestinal epithelium maintained the characteristic apical-basal array of the MTs with no obvious phenotype, indicating that *Mcc* is not essential for microtubule reorganization during intestinal epithelium turnover (**Figure 29A**). However, we observed changes in the morphology of the *Mcc*^{-/-} differentiated epithelial cells such as in cell length, width and aspect ratio nuclei (**Figure 29B**), suggesting that the polarity of the cell might be affected.

Figure 29 - Loss of *Mcc* affects the morphology of intestinal differentiated cells. **A:** Small intestine sections of villus differentiated cells from wild-type (WT) *Mcc* ^{+/+} and *Mcc* ^{-/-} tissue stained with α -tubulin shows no gross aberrations of the microtubules array in *Mcc* ^{-/-}. Scale bars 10 μ m. **B:** Comparison measurement analyses of intestinal differentiated cell length, width and aspect ratio nuclei reveal morphological defects in *Mcc* ^{-/-} villus cells. Data were tested for significance by an unpaired two-tailed t-test ***p < 0.001.

Recently, in an attempt to further understand the molecular origins of the *Mcc* loss-of-function intestinal phenotype, we performed a comparison of RNA-seq gene expression analysis in isolated villus and crypt samples from WT and *Mcc*^{-/-} mice (see Materials and Methods for details regarding the complete experimental set up). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for biological processes and pathways revealed that most upregulated genes in *Mcc*^{-/-} crypts samples were involved in immune response and cell adhesion processes (**Figure 30A**) (gene list is available in **Table 06**).

This preliminary comparative transcriptome analysis prompted us to evaluate the intestinal permeability function in *Mcc*^{-/-} mice. Inflammation-associated diseases of the intestinal epithelium are frequently linked to abnormalities in the intestinal barrier function due to defects in the tight junctions and intercellular adherent connections. We hypothesized that loss of *Mcc* affects intestinal barrier function through displacement of components of the intestinal epithelium intercellular junctional complexes or/and apical domain complexes.

To assess intestinal barrier function, we performed 2 initial in vivo tests using molecule tracers to evaluate both paracellular permeability using FITC-labelled Dextran-4 kDa and D-xylose, which is an easily absorbed monosaccharide commonly used to evaluate intracellular uptake function. Mice (n = 4/genotype) were administrated with one molecular tracer by oral gavage, and the concentrations of Dextran and D-xylose were subsequently analysed in the serum 4 hrs later (see Materials and Methods for details regarding the complete experimental set up). We found that levels of Dextran were higher in the *Mcc*^{-/-} mice as compared to the WT, suggesting that the barrier function is affected (**Figure 30B**). The concentration of FITC-Dextran (4 kDa) in the blood is a measure of paracellular permeability. Moreover,

we observed that the levels of D-xylose were notably lower in the mutants, suggesting that the uptake of molecules via intracellular transport is additionally compromised (Figure 30C).

Figure 30 - *Mcc* loss-of-function intestinal phenotype. **A:** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of upregulated genes in *Mcc*^{-/-} small intestine isolated crypts as compared to wild-type (WT) *Mcc*^{+/+}. Enrichment scores were calculated as the negative logarithm of a p value (-Log p). **B** and **C:** *in vivo* Intestinal permeability assays. **B:** Levels of 4 kDa FITC-Dextran in the plasma of WT and *Mcc*^{-/-} measured 4 hours after oral gavage. Photospectrometry reveals higher levels of FITC-Dextran in *Mcc*^{-/-} mice serum indicating that loss of *Mcc* affects intestinal paracellular permeability. **C:** Levels of D-Xylose in the plasma of WT and *Mcc*^{-/-} measured 4 hours after oral gavage. Photospectrometry reveals lower levels of D-Xylose in *Mcc*^{-/-} mice serum suggesting that loss of *Mcc* compromises the intracellular uptake of molecules. n=4/genotype. P value of statistical significance represented as ***p < 0.001.

Next, we extended our analysis of the intestinal epithelial barrier by performing IF for proteins associated with intercellular junctional complexes (**Figure 31**). Confocal images of differentiated intestinal cells showed that the localization of β -catenin, Epithelial cell adhesion molecule (Ep-CAM), Epithelial Cadherin (E-Cad) and Zona occludens 1 (ZO1) is not affected, indicating that, perhaps, other integral components of junctional complexes or apical domain are affected upon loss of *Mcc*. The investigation of the intestinal epithelial integrity is ongoing.

Figure 31- Epithelial intercellular junctional complex proteins localization in villus cells. Immunofluorescence (IF) of epithelial junctional complex markers including β -Catenin, Ep-CAM, E-Cadherin and ZO-1 in transverse sections of WT *Mcc* $+/+$ and *Mcc* $-/-$ small intestine villus. No defects are observed in the localization of these markers in the *Mcc* $-/-$ villus cells. Hoechst is shown in grey. Scale bars 50 μ m.

Our recent results from histological and scanning electron microscopy analysis have revealed a prominent *Mcc* loss-of-function intestinal morphology phenotype that could results in potential defects in intestinal barrier and promote an inflammation response, resulting in significant weight loss in homozygous mutant mice. However, the origin of this intestinal phenotype, and its genesis, either embryonically or

postnatally, are at present still unknown and are the focus of on-going experiments described in the Future Direction section on this thesis.

3.6 *Mutated in Colorectal Cancer (MCC)* and Intestinal Cancers.

During the course of my thesis, I obtained some tantalizing, though preliminary data supporting a role for *Mcc* in the pathogenesis of intestinal cancers. As canvassed in the Introduction, there is abundant evidence implicating *Mcc* as a tumor suppressor gene both in mice and man. For instance, loss-of-heterozygosity, deletions and mutations of the *MCC* gene have been described as common occurrences in human CRC, although mutation of the remaining wild-type allele is rare (Curtis et al., 1994; Nakamura et al., 1992; Nishisho et al., 1991). Further supporting evidence comes from a study aimed at identifying potential driver genes for CRC using the Sleeping Beauty transposon system to generate insertional mutations in the mouse intestinal epithelium. In a total of 77 insertion sites identified by sequencing of tumor DNA, *Mcc* was recovered 7 times (Starr et al., 2009). Moreover, *MCC* silencing through promoter hypermethylation was described in approximately 50% of primary sporadic CRCs and 80% of serrated polyps, suggesting that loss of *MCC* expression is involved in early colon carcinogenesis via the serrated-polyp-neoplasia pathway (Kohonen-Corish et al., 2007; Li et al., 2013). However, adenoma formation was never observed along the length of the intestine in *Mcc* null mutant mice on a mixed (129S2 x C57BL/6J) genetic background. Thus, we conclude that *Mcc* loss-of-function does not fit the conventional definition of a driver mutation for tumor initiation.

To date, many pathways have been identified that govern epithelial morphogenesis and are also frequently implicated in cancer formation such as the

Wnt- β -catenin pathway. Loss of *APC*, an essential intracellular component of the Wnt- β -catenin pathway, represents the most common initiating mutation in the outgrowth of benign adenomas as a consequence of hyper-activation of the WNT signaling pathway due to accumulation of cytoplasmic β -catenin and its subsequent translocation to the nucleus (**Figure 04**) (Su et al., 1992, 1993a).

Given the fact the *MCC* and *APC* are tightly linked in both human and mouse, we therefore chose to further explore whether *Mcc* genetically plays a role in its namesake disease. To this end, we intercrossed *Mcc^{lacZ}* (**Figure 12**) homozygous mice and *Apc^{Min}* heterozygous mice to interrogate what is the contribution, if any, of *Mcc* to the development of CRC. The consequences of this genetic interaction for tumorigenesis have not been previously described.

Mcc^{lacZ} homozygous mutant mice on a mixed (129S2 x C57BL/6) genetic background were crossed to C57BL/6J *Apc^{Min/J}* mice (mice were a gift from Dr. Dmitry Bulavin, previously at A*STAR IMCB). After extensive intercrosses, recombination occurred such that the *Apc^{Min}* and *Mcc^{lacZ}* (*Mcc*-) were linked on the same chromosome, and thus *Apc^{Min/+};Mcc^{+/-}* and *Apc^{Min/+};Mcc^{-/-}* were obtained. Subsequent analysis of the tumor burden (distribution) along the entire length of the small intestine of 90-day-old mice was performed. *Mcc* deficiency in the C57BL/6J *Apc^{Min/+}* background resulted in significantly increased tumor burden and tumor size as well as significantly shortened survival (**Figure 32** and survival curve data not shown). No significant tumor formation (>3 adenomas) was observed in the colonic epithelium of the indicated genotypes. Specifically, we observed that *Apc^{Min/+}* mice developed on average 30 adenomas in the small intestine, similar to previous studies (Haigis et al., 2004; Kwong and Dove, 2009; Kwong et al., 2007; Philipp-Staheli et al., 2002).

Figure 32 - Loss of *Mcc* in *Apc^{Min}* mice increases intestinal tumor burden. *Apc^{Min/+}* and *Mcc^{lacZ/+}* (designated *Mcc^{+/-}*) were intercrossed, giving rise to the genotypes displayed on the graphs. At 90 days of age, mouse intestines were harvested, fixed and stained with Alcian Blue for tumour count and size measurement using a stereo microscope. **A:** Total intestinal tumor count. Y axis shows average number of tumors per mouse. Each symbol represents one animal. For *Apc^{Min/+}; Mcc^{+/+}* (N=14), *Apc^{+/+}; Mcc^{+/-}* (N=14), *Apc^{Min/+}; Mcc^{+/+}* (N=15), *Apc^{Min/+}; Mcc^{+/-}* (N=19) and *Apc^{Min/+}; Mcc^{-/-}* (N=18). **B:** Y axis shows the average number of tumors per genotype. Tumors were measured and categorized into small (>2 mm), medium (2-4 mm) and large (>4 mm). Data were tested for significance by an unpaired two-tailed t-test. ***P < 0.0001.

Interestingly, the presence of one *Mcc* mutant allele in an *Apc^{Min/+}* genetic background caused an increase in the tumor burden (**Figure 32A**). There were no significant differences in tumor load between *Apc^{Min/+}; Mcc^{+/-}* and *Apc^{Min/+}; Mcc^{-/-}*. This observation indicates that one mutant *Mcc* allele is sufficient to augment tumor formation in the *Apc^{Min}* genetic background.

Quantitative analysis of tumor size along the length of the small intestine was performed. All adenomas were counted and manually measured using calipers and a stereo microscope. Tumors were subsequently classified in three categories according to their size: small (>2 mm), medium (2-4 mm) and large (>4 mm). No tumors larger than 6 mm were found (**Figure 32B**). Large tumors (>4 mm) were observed only in the double mutants (*Apc^{Min/+}; Mcc^{+/-}* and *Apc^{Min/+}; Mcc^{-/-}*). No

significant differences were observed in tumor density or size between different sexes among the indicated genotypes.

Next, histological sections (swiss-rolls) of the entire small intestine were prepared to investigate phenotypic differences in tumor density and size at the cellular level. Swiss-rolls of the *Apc^{Min/+};Mcc^{-/-}* intestinal epithelium revealed not only a significantly higher tumor density but also larger (>4 mm) adenomas when compared to *Apc^{Min/+};Mcc^{+/-}* or *Apc^{Min/+}* alone (**Figure 33A**). These animals also succumbed more rapidly most likely due to the severely compromised intestinal epithelium (data not shown). High magnification analysis of adenomas stained with the proliferation marker Ki67 shows not only typical proliferation in the crypts but also confirms abnormal proliferation of cells in different compartments of the intestinal adenomas from all genotypes (**Figure 33B**). Additionally, nuclear accumulation of β -catenin is also observed in high magnification images of adenomas of all indicated genotypes (**Figure 33C**).

As summarized in the Introduction, data accumulated over the last 30 years implicate human *MCC* mutations as a ‘second hit’ in the pathogenesis of both inherited and sporadic human CRC (Li et al., 2013; Kohonen-Corish et al., 2007; Curtis et al., 1994; Nakamura et al., 1992; Nishisho et al., 1991). Excitingly, my observations detailed in this section provide the first genetic evidence that this ‘second hit’ can be modeled in a well-established and well-characterized mouse model of CRC—that is, accompanying loss of *Mcc* in the context of *Apc^{Min}* significantly increases the tumor burden and accelerates the demise of double mutant animals.

Figure 33 - *Mcc* deficiency in *ApcMin/+* background leads to significant increase of tumor burden and tumor size. **A:** Swiss-roll sections of mouse small intestine from *ApcMin* and *Mcc* null mutants (*ApcMin/+;Mcc +/+* , *Apcmin/+;Mcc +/-* and *Apcmin/+;Mcc -/-*). Sections are stained with the proliferation marker ki67 and counter stained with haematoxylin. Arrowheads indicate some tumors. **B:** High magnification images of tumors stained with Ki67. Dashed lines indicate some crypt structures, which are normally labelled by Ki67. **C:** High magnification images of tumors stained with β -catenin. Arrowheads indicate membrane staining of β -catenin and yellow asterisks indicate regions where nuclear accumulation/staining of β -catenin can be observed. Scale bars not shown.

What awaits beyond the scope of this thesis is a rigorous dissection of the molecular mechanisms that underlie this robust genetic interaction for tumor initiation. Tantalizing cues, discussed later, emerge from similar observations in both *Apc^{Min};Nherf1* and *Apc^{Min};Cftr* double mutant mice.

Mutated in Colorectal Cancer (MCC), identified nearly thirty years ago, has long been overshadowed by its chromosomal neighbor, *Adenomatous Polyposis Coli (APC)*, whose prominent role in the pathogenesis of colorectal cancer has been firmly established. Consequently, the number of reports investigating MCC, whether at the level of mRNA or protein distribution or potential functions in homeostasis and disease, have been intermittent and infrequent, and in many cases present conflicting and confusing data. For example, Mcc protein, depending on the cell type, reagent and assay used, was reported to localize to several organelles (mitochondria and endoplasmic reticulum) and cellular compartments (plasma membrane, cytoplasm and nucleus) (Arnaud et al., 2009; Benthani et al., 2018; Fukuyama et al., 2008; Matsumine et al., 1996; Pangon et al., 2010, 2015; Senda et al., 1997). Thus, one of the major motivations behind the work presented in this thesis was to definitively establish the subcellular localization of MCC.

As Henry Bourne (1991) predicted in an accompanying “News and Views” article in the same issue of *Science* that first characterized the human *MCC* gene, our modern bioinformatic analysis confirms that the MCC protein contains multiple regions predicted to form coiled-coils as well as a C-terminal type I (PSD-95/Dlg/ZO-1) PDZ binding motif (PBM) that is evolutionarily conserved from *Drosophila* to humans (**Figure 16A**). Further bioinformatic analysis reveals that MCC shares multiple regions of sequence homology with the centrosomal coiled-coil MT-anchoring component NINEIN (Bouckson-Castaing et al., 1996) (**Figure 16C**). Interestingly, the centrosome/PCM complex comprises a large number of coiled-coil proteins

(Bouckson-Castaing et al., 1996; Doxsey, 2001; Kuhn et al., 2014; Woodruff et al., 2014).

These bioinformatic analyses presaged the outcome of our proteomic analysis that were performed in close collaboration with Dr. Bernard Liu, a former post doc in the laboratory of the late Dr. Tony Pawson. The MCC interactome was established by overexpression of an epitope-tagged version of MCC in HEK293 cells followed by conventional immunoprecipitation and mass spectrometry. Among the MCC binding partners, a number of centrosomal proteins including CEP131 (also termed AZL1), CEP170 and NDE1 were recovered, and these interactions were confirmed by independent transfection experiments and IP-Western (**Figure 17B-C**).

4.1 MCC is a Novel Centrosomal Protein.

Our first attempt to investigate MCC subcellular localization relied on an overexpression construct encoding an N-terminal EmGFP-Mcc isoform 2 (mouse) fusion protein in heterologous cell lines. Consistent with both the structural similarities between MCC and NINEIN and the results of MCC mass spectrometry, we observed highly specific localization of EmGFP-Mcc at the centrosome. This result was surprising as another independent study reported that an N-terminal EGFP-MCC fusion protein was broadly distributed in the cytoplasm and at the cell membrane in T47D breast cancer epithelial cells (Arnaud et al., 2009). Notably, however, the EGFP-MCC signal is clearly enriched in a punctum close to the nucleus of these cells, reminiscent of centrosomal localization, especially when the last three amino acids within the PBM are deleted (see Figure 3A in Arnaud et al. (2009)). Anecdotally, in the course of our experiments, we observed that overexpression of MCC using strong viral

promoters (*e.g.*, in pCAGGS) can lead to mislocalization of MCC, resulting in the formation of large cytoplasmic aggregates (data not shown). The possibility remains that the differences in MCC localization between Arnaud et al. (2009) as well as other studies and those results reported here are cell line dependent. This is however unlikely given the diverse cell lines we analysed by IF using a specific anti-MCC antibody. These include HCT116, SW480, RPE1 and HEK293. Significantly, we further show by IF that MCC co-localizes with PCNT, NINEIN, CEP131, CEP170 and NDE1. Super-resolution microscopy techniques emphasize the close proximity of MCC to both NINEIN and PCNT at the centrosome in HEK293 cells.

Given the confusion within the literature surrounding MCC subcellular localization, we carefully considered whether our fixation techniques impacted our interpretation of our MCC IF results. Hua and Ferland (2017) thoroughly investigated the effect of buffers and fixatives and their impact on the localization of centrosomal and ciliary proteins by immunolabeling after conflicting reports emerged about the localization of Centrosome and Spindle Pole Associated Protein 1 (CSPP1). These authors reported that methanol (MeOH) fixation produced less cytoplasmic background while enhancing centrosome and MT staining when compared to paraformaldehyde (PFA) fixation in assorted cell lines. In an independent study (Goldspink et al., 2017), it was reported that PFA fixation commonly results in the loss of antigenicity of centrosomal proteins. These findings were carefully considered when developing the experimental IF protocols described in the Materials and Methods and when interpreting our results. Most significantly, and discussed later, the absence of Mcc staining at the centrosome in intestinal tissue harvested from *Mcc* homozygous null mice confirms the specificity of our IF techniques and allows us to christen Mcc for the first time as a novel centrosomal protein.

The centrosome is the major MTOC of the cell that anchors and nucleates MTs. PCNT is a well-characterized component of the MTOC, which is mainly responsible for nucleation of MTs through interaction and recruitment of γ TuRCs (Dictenberg et al., 1998). NINEIN, on the other hand, is a major MT-anchoring protein (Goldspink et al., 2017; Mogensen et al., 2000). Functionally, MT-anchoring and MT-nucleation are two different processes regulated by different components of the centrosome (Mogensen et al., 1997). This distinct concept comes from studies in epithelial cells where a unique MT-anchoring site is assembled for organization of MTs. At these sites, MTs are anchored through their minus-ends by centrosomal MT-anchoring proteins and, seemingly, proteins involved in nucleating MTs such as PCNT and γ -Tubulin are not part of it (Doxsey, 2001; Mogensen et al., 1997; Sanchez and Feldman, 2017). NINEIN has been previously reported to re-localize from the centrosome/MTOC to non-centrosomal (nc-) MTOC sites in the epithelial cell for reorganization of MTs (Goldspink et al., 2017; Mogensen et al., 2000).

Our findings that MCC localizes to the centrosome in proliferating intestinal crypt cells (**Figures 20C/D**) and is redeployed to the apical membrane of polarized cells (where the nc-MTOC is situated) (**Figure 19E/F**) underpins the central hypothesis of the work presented in this thesis—that is, MCC plays a functional role at the MTOC either anchoring or nucleating MTs.

4.2 *Mcc* mRNA Distribution in the Mouse Intestine.

The murine *Mcc* locus generates two transcripts as a result of alternative promoter usage (Young et al., 2011). Both *Mcc* transcripts are detected throughout mouse embryonic development by qPCR and whole-mount *in situ* hybridization. Combining expression studies with two independent *Mcc* gene trap reporter lines and

other biochemical experiments, (Young et al., 2011) firmly established that the shorter mouse *Mcc* transcript encoding a protein of 828 amino acids is most abundant both embryonically and postnatally. These authors additionally reported that *Mcc* expression, monitored by β -gal staining in *Mcc^{lacZ}* mutant mice, is present in the developing gut and persists into adulthood. However, the expression in the adult intestinal epithelium was poorly characterized, which for reasons described earlier (*e.g.*, relationship to *Lgr5* expression and studies implicating *Mcc* in intestinal cancer), served as a departure point for the experiments described in this thesis.

Employing the *Mcc^{lacZ}* reporter mouse, we exhaustively characterized the expression of *Mcc* iso 2 in the adult small intestine and colonic epithelium by staining for β -gal activity in both conventional PFPE sections and whole-mount tissues. β -gal-positive cells are specifically detected in crypt proliferative cells of the small intestine and are also observed clustered in the crypts of the colonic epithelium. Whole-mount analysis of β -gal activity confirms that *Mcc^{lacZ}* expression is conspicuously restricted to the crypt compartment along the length of the small intestine (**Figure 13**). In an effort to better resolve the expression of *Mcc* expression within the crypt compartment, we examined β -gal activity in whole-mount isolated crypts. High-magnification imaging revealed that *Mcc^{lacZ}*-expressing cells are detected throughout the entire crypt structure, including the CBC stem cells located at the bottom of the crypt as well as rapid proliferating cells within the TA compartment (**Figure 13F**). The activity of β -gal in the intestine was meticulously controlled using methods described by Merkwitz et al. (2016) and no β -gal activity was observed in wild-type tissue section controls.

These observations with the *Mcc^{lacZ}* reporter mouse were confirmed using RNAScope, a commercially available high-resolution section *in situ* hybridization technique for the detection of endogenous mRNAs. Importantly, the specificity of the

bespoke *Mcc* probe set was verified using tissue harvested from *Mcc* mutant mice (*Mcc^{lacZ/lacZ}*). Co-*in-situ* hybridization for *Mcc* and *Lgr5*, which specifically labels CBC stem cells (Barker et al., 2007), conclusively revealed for the first time that *Mcc* transcript expression overlaps with *Lgr5* in CBC cells at the crypt base. However, as anticipated from our *Mcc^{lacZ}* reporter mouse studies, *Mcc* is more broadly expressed, labeling not only the crypt base, but also the TA compartment. RT-PCR for relative expression of *Mcc* in isolated crypt and villus samples confirmed lack of *Mcc* expression in the villi.

4.3 *Mcc* Protein Localization in the Intestinal Epithelium.

In contrast to *Mcc* mRNA distribution in the mouse small intestinal epithelium, *Mcc* protein was surprisingly, and at first confusingly, detected in crypt as well as villus cell lysates by Western blot (**Figure 14** and **Figure 19A**). Fortunately, we were able to optimize IF protocols such that the anti-*Mcc* antibody we used for our *in vitro* IF studies performed robustly on tissue sections, expectedly revealing *Mcc* localization at the centrosome in proliferative cells of intestinal crypts in both mouse and human. Intriguingly, we also observed highly specific staining for *Mcc* along the apical membrane of differentiated cells in the villus compartment of the small intestine by immunofluorescence—a result consistent with our Western studies (**Figure 19**). *Mcc* protein was similarly distributed in the colonic epithelium (**Figure 20A**). The absence of *Mcc* staining in *Mcc^{lacZ}* homozygous mice provides incontrovertible evidence in support of the specificity of the commercial anti-*Mcc* antibody used throughout this thesis (**Figure 21**).

In an effort to gain insight into the potential function of Mcc, it is necessary to consider Mcc in the context of other proteins whose subcellular trajectory and function begins at the centrosome and terminates at the non-centrosomal MTOCs in the apical domain of differentiated cells. In most epithelial polarized cells, the cytoskeleton consists of actin filaments, microtubule (MT) filaments, and cytokeratin intermediate filaments (CIF) that are organized and interconnected with numerous associated proteins (Fuchs and Cleveland, 1998; Ku et al., 1998). In intestinal epithelium cells, the actin filaments extend into the intracellular apical/lateral domains and form compact bundles within the microvilli (Hirokawa et al., 1982). CIFs are also predominantly concentrated at the apical domain of intestinal cells, which express cytokeratins type I (CK18/19/20) and type II (CK7/8) (Fuchs and Cleveland, 1998; Rodríguez et al., 1994; Zhou et al., 2003). The MT filaments display a specific parallel organization throughout the cytoplasm having their minus-ends anchored at the intracellular apical membrane (Buendia et al., 1990; Mogensen et al., 1989). In proliferating intestinal crypt cells, MTs are organized radially along the apical–basal axis of the cell, with MT minus-ends anchored to the apically located centrosome—that is, the MTOC (Sallee et al., 2018; Sanchez and Feldman, 2017), where Mcc localization is detected (**Figure 19C**). The Centrosomal MTOC functions not only in anchoring, but also in nucleating MTs (Dammermann et al., 2003).

In order to terminally differentiate, epithelial cells must undergo several intracellular modifications in their cytoskeleton to adopt their characteristic shape and polarity. Differentiation in the epidermis, for instance, is associated with rearrangement of centrosomal MT filaments into cortical arrays (Lechler and Fuchs, 2007; Sumigray and Lechler, 2011; Sumigray et al., 2012). In differentiated intestinal cells, the parallel apico-basal MT array is achieved once their minus-end is anchored at non-

centrosomal MTOC sites situated along the apical membrane (Achler et al., 1989; Sanchez and Feldman, 2017). A number of centrosomal proteins have been described to be reallocated to the apical membrane for non-centrosomal MTOCs assembly through mechanisms that are poorly understood (Cabral et al., 2019; Ito and Bettencourt-Dias, 2018; Orlofsky, 2019; Sanchez and Feldman, 2017; Toya and Takeichi, 2016).

As previously described in the Introduction, γ -TuRC is essential for both MT nucleation at the centrosome and MT stabilization as it acts as a minus-end-capping protein complex (Anders and Sawin, 2011; Wiese and Zheng, 2000; Zheng et al., 1995). In differentiated intestinal cells, however, the major γ -TuRC-component γ -tubulin is redeployed to the apical nc-MTOC (Ameen et al., 2001; Muroyama et al., 2016, 2018; Salas, 1999). Ameen et al. (2001) showed that in the intestine of *Ck8*-deficient mice, differentiated cells lacked CIFs and the localization of γ -tubulin was found to be dispersed within the cytoplasm of enterocytes, in contrast to its normal apical membrane localization. Furthermore, the MT filaments in these cells were disorganized validating an essential role of CIFs in MTOC formation (Ameen et al., 2001).

Additionally, Nedd1 (a γ -TuRC associated protein) is also described to be redeployed to the apical membrane of enterocytes (Muroyama et al., 2018). The re-localization of MT-nucleating γ -tubulin upon differentiation indicates that the MTOC activity is reassigned from the centrosome to the apical domain of intestinal cells where microtubule minus-ends are thenceforth anchored. In a previous study from the same laboratory, the authors showed that molecularly distinct γ -tubulin complexes (CDK5RAP2- and Nedd1- γ -TuRC) play specific functions at the MTOC during epidermal differentiation to regulate MT filaments or coordinate the loss of

centrosomal MTOC activity. CDK5RAP2- γ -TuRCs present a robust MT-nucleating function that is maintained at the centrosomal MTOC until the first stages of differentiation whereas Nedd1- γ -TuRCs are strictly required for MT-anchoring activity and therefore rapidly redeployed from centrosomes to different sites (nc-MTOC) of the cytoplasm after cell cycle exit (Muroyama et al., 2016).

In the *C. elegans* intestine, γ -tubulin has also been reported to localize at apical sites in differentiated cells accompanied by a downregulation of the centrosomal protein CEP192 and the cell cycle modulator CDK in anticipation of nc-MTOC formation during differentiation (Brodu et al., 2010; Yang and Feldman, 2015). Additionally, Feldman and Priess (2012) reported that *C. elegans* intestinal cell centrosomes lose their PCM and other several MT-associated components as these proteins re-localize toward the future apical surface nc-MTOC. Immunostaining confirmed, for instance, enrichment of γ -tubulin and MT-associated proteins (MAP), including XMAP215 family members of MT polymerases, at the nc-MTOC sites (Feldman and Priess, 2012).

In agreement, another independent study in *C. elegans* reported that the intestinal centrosomal PCM is eliminated once cells exit the mitotic cycle during larval stages (Lu and Roy, 2014). Moreover, MTs were shown to re-grow (or repolymerize) from the nc-MTOC of differentiating *C. elegans* embryonic intestinal cells following nocodazole (a drug that interferes with MT polymerization) washout, supporting the role of γ -tubulin in nucleating MTs at nc-MTOC sites. However, it is not clear if in mammalian intestinal differentiated cells, the nc-MTOC is the exact site of MT nucleation (Yang and Feldman, 2015). In the Future Directions section, experiments aimed at addressing this outstanding question are described in detail.

Most recent reports have associated loss of the centrosomal MTOC with redeployment of centrosomal proteins to other sites within the cell. For instance, NINEIN, a minus-end MT anchoring protein, has been reported to re-localize from the centrosome to nc-MTOCs in cochlear supporting epithelial cells during differentiation. The re-localization of NINEIN was reported to be dependent upon MT organization. However, the molecular mechanism underlying this process remains unknown (Mogensen et al., 2000). In a more recent study, Goldspink et al. (2017a) showed that re-localization of Ninein from the centrosome to the apical nc-MTOC in differentiated intestinal cells was found to be essential for MT apico-basal organization and for establishing the columnar shape of differentiated intestinal cells. In addition, by using genetic deletion or functional inhibition-based experiments, NINEIN re-localization was proposed to occur via a mechanism involving the capture of CLIP-170 by the IQGAP1 receptor which activates Rac1. Activated Rac1 releases the minus-end of MTs from the centrosome resulting in the recruitment of NINEIN to the apical membrane. The loss of NINEIN or CLIP-170, however, does not impair formation of the apical nc-MTOCs in differentiated intestinal cells, invoking compensatory mechanisms (Goldspink et al., 2017a).

Interestingly, during the course of my studies, I have consistently observed that the MT-nucleating protein Pcnt co-localizes with Mcc not only at the punctate centrosomal MTOC in intestinal crypt cells but also at the nc-MTOC along the apical membrane in differentiated villus cells (**Figure 20C**). In contrast, a study by Muroyama et al. (2018) showed that Pcnt does not localize to the apical membrane in differentiated cells of the mouse small intestine. However, a number of other reports have previously described the redeployment of Pcnt to nc-MTOC sites during cell differentiation. Mogensen et al. (1997), for instance, has reported that Pcnt is

redeployed to apical domains in differentiated mouse epithelial cells including inner pillar cells. In addition, during skeletal muscle differentiation, centrosomal proteins including Ninein and Pcnt have been described to re-localize from centrosomes to other sites in differentiated myotube cells for MTs nucleation (Bugnard et al., 2004; Srsen et al., 2009).

There is accumulating evidence showing that the centrosomal MTOC function is reassigned from the centrosome to the cytoplasmic apical domain during epithelial cell differentiation. This event is concomitantly associated with loss of the PCM and reposition of several components of the centrosomal complex to an nc-MTOC site. The molecular mechanism of such coordinated events during epithelial differentiation remains poorly understood. The redistribution of Mcc protein from the centrosome to the apical membrane (from the MTOC to the nc-MTOC) is a novel finding, raising the simple hypothesis that Mcc functions to organize and re-organize the cytoskeleton/MTs as proliferating cells undergo terminal differentiation. As discussed later in this chapter, *Mcc*^{lacZ} homozygous null mice are viable and fertile, though they present highly penetrant defects in the intestinal epithelium. Mcc is therefore necessary, but not absolutely required for the development and maintenance of the intestinal epithelium.

4.4 MCC Phosphorylation Triggers its Redeployment from MTOC to nc-MTOC.

Casein Kinases 1 δ and ϵ (CK1 δ/ϵ) are two MCC-interacting proteins uncovered by our mass spectrometry studies that quickly drew our attention. An independent study using a mass spectrometry-based approach in a large-scale protein–protein interaction screen in human cells also identified MCC as an interacting

partner of CK1 δ/ϵ . CK1 proteins are known to play both positive and negative roles in the regulation of the WNT/ β -Catenin signaling pathway and to play regulatory functions at the centrosome (Brockman et al., 1992; Cruciat, 2014; Greer et al., 2014; Schitteck and Sinnberg, 2014; Stöter et al., 2005).

Indeed, this work and prior studies confirm that MCC is a phosphoprotein (Caria et al., 2019; Pangon et al., 2012). For example, in SW480 cells two versions of MCC protein are detected by western blot—phospho-MCC running with an approximate molecular weight of 110 kDa and wild-type MCC (calculated molecular weight of 93kDa). Our data demonstrate that the phosphorylated version of MCC is eliminated upon treatment with a highly specific, small molecule CK1 δ/ϵ -inhibitor (PF670462) (Badura et al., 2007), indicating that MCC is a specific target of CK1 δ/ϵ phosphorylation (**Figure 23A**). Additional support for this interaction comes from a series of *in vitro* co-expression experiments with Myc-tagged MCC and FLAG-tagged CK1 ϵ or with a catalytically dead form of CK1 ϵ (FLAG-tagged CK1 ϵ^{D1280}) in HEK293 cells. When co-expressed, wild-type CK1 ϵ prompts an upward mobility shift of MCC, which is not observed in the presence of CK1 ϵ^{D128A} (**Figure 23B**). Furthermore, overexpression of Myc-MCC and FLAG-CK1 ϵ in the presence of PF670462 also abolishes the higher molecular weight version of MCC (**Figure 23C**).

In addition, we identified multiple amino acid residues in the MCC protein that are phosphorylated by CK1 ϵ overexpression in our comparative mass spectrometry data, including a previously described phosphorylation site at position -1 (Ser 828 in human) within the C-terminal MCC PDZ binding motif (PBM) (**Figure 22**) (Caria et al., 2019; Pangon et al., 2012a). Moreover, phosphorylation of MCC at residues 115, 118 and 120 (mouse protein nomenclature) has been previously described (Pangon et al., 2010, 2012b).

In a previous work published by my group using the Zebrafish system, they showed that the residue most critical for Mcc function during convergence and extension movements that narrow and elongate the body axis was position -1 within the C-terminal PDZ interaction motif (Young et al., 2014). The injection of mouse mRNA encoding alanine mutations in serine positions 115, 118 or 120, either singly or in combination, was able to fully rescue the phenotype of *mcc* morpholino-injected Zebrafish embryos, which display a foreshortened body axis and anterior patterning defects, at a percentage nearly equivalent to wild-type mouse *Mcc* mRNA. In contrast, mRNA encoding an alanine mutation at position -1 (position 827 in the mouse protein) was unable to rescue the prominent *mcc* morphant phenotype (Young et al., 2014). We therefore believe that residue -1, supported by evidence in this thesis, is critical, perhaps most central, to MCC function.

Taken together, these results provide compelling evidence that MCC is a direct target of CK1 δ/ϵ phosphorylation. Although CK1 δ/ϵ emerged in the MCC interactome, we have not formally tested whether CK1 δ and CK1 ϵ phosphorylate MCC equally. We nevertheless believe that CK1 δ and CK1 ϵ function nearly identically in phosphorylating MCC given that their kinase domains are 98% identical (Fish et al., 1995). Moreover, they both show conspicuous redundant functional roles in the intestinal epithelium (Morgenstern et al., 2017; Fish et al., 1995).

There is abundant evidence in the literature connecting CK1 δ/ϵ to the centrosome. For example, neurite extension from the neuronal cell body has been reported to be a process associated with centrosomal localization of CK1 δ . Both delocalization of CK1 δ from the centrosome and inhibition of CK1 δ/ϵ appear to impair neurite extension (Greer and Rubin, 2011). Another study demonstrated that CK1 δ/ϵ co-localize with γ -tubulin at the meiotic spindle during mouse oocyte maturation,

although depletion of CK1 δ/ϵ by RNAi failed to disrupt spindle formation and progression of meiosis (Qi et al., 2015). Greer et al. (2017) has described that CK1 δ is important during mitotic checkpoints for repair of DNA damage by activating and stabilizing checkpoint kinase 1 (Chk1). Interestingly, a study by Zyss et al. (2011) in T lymphocytes showed that phosphorylation of MT-associated protein EB1 by CKI δ resulted in re-localization of centrosomal components to a cell-cell junctional complex termed immunological synapse (IS). These authors also showed that inhibition of CKI activity reduced microtubule growth in RPE-1 epithelial cells (Zyss et al., 2011).

Our *in vitro* experiments reveal that phosphorylation of MCC stabilizes its binding to RASAL2 while decreasing its interaction with the integral centrosomal component NDE1 (**Figure 23D**). Significantly, we show that Mcc fails to localize at the apical membrane of intestinal organoids upon treatment with the CK1 δ/ϵ inhibitor (**Figure 25C**). These observations indicate that Mcc redeployment to the apical membrane during intestinal differentiation is a mechanism triggered by CK1 δ/ϵ phosphorylation. In the Future Directions chapter, experimental plans to further investigate the molecular basis of MCC phosphorylation by CK1 δ/ϵ are described. For example, site-directed mutagenesis to determine the precise MCC amino acid residues phosphorylated by CK1 δ/ϵ and the analysis of Mcc redeployment by live cell imaging using intestinal organoids. The intestinal organoid platform additionally affords the opportunity to incorporate stable dyes such as Sir-Tubulin to evaluate the effects of CK1 δ/ϵ inhibition on MTs arrangement during differentiation. Lastly, we have already initiated additional comparative proteomic experiments in HEK293 cells expressing our original epitope-tagged MCC construct with or without the potent CK1 δ/ϵ inhibitor. These data will also reveal valuable information about which residues in MCC are specifically phosphorylated by CK1 δ/ϵ .

Our interactome data also reveals that MCC partners with various polarity proteins containing PDZ domains such as SCRIBBLE (SCRIB), NHERF1/2 and SNX27. Moreover, several proteins that regulate small GTPases such as RASAL2, IQGAP1 and RAB11 (also known as RIP11) were recovered. In agreement with our findings, previous studies confirm the interaction of MCC to PDZ-domain containing proteins including SCRIB, SNX27, NHERF1/2 and EZRIN through a series of immunoprecipitation experiments followed by mass spectrometry (Arnaud et al., 2009). In T47D mammary gland epithelial cells, MCC co-localizes with SCRIB at the cell membrane and siRNA-mediated knockdown of *MCC* in these cells compromises cell migration (Arnaud et al., 2009). In addition, the interactions between MCC and SCRIB are dependent upon the C-terminal E-T-S-L motif (PDZ Binding Motif (PBM)) of MCC (Caria et al., 2019; Pagon et al., 2012a). Here, we provide additional evidence that deletion of the C-terminal (E-T-S-L) of MCC PBM eliminates its interaction to NHERF1 and in agreement with prior work, we also confirmed that the MCC PBM is essential for its interaction with SCRIB (**Figure 23E**).

In summary, we identified several proteins that interact with MCC including the Casein Kinases 1 CK1 δ and CK1 ϵ , which we demonstrate biochemically directly phosphorylate MCC and alters its affinity for binding partners. We propose that such phosphorylation events govern the subcellular localization of Mcc in the small intestinal epithelium. For example, phosphorylation decreases MCC affinity to centrosomal protein NDE1. Furthermore, inhibition of CK1 impairs Mcc re-localization to the apical membrane in intestinal organoids. We also reported that MCC interacts to the PDZ scaffold proteins SCRIB and NHERF1 via its PDZ binding motif. Given that, in the intestinal epithelium, NHERF1 localizes to the apical membrane of enterocytes (Georgescu et al., 2008; Hayashi et al., 2010) and SCRIB co-localizes with cell-cell

junctional complex components such as occludins and ZO-1 (Ivanov et al., 2009), we hypothesize that MCC is interacting with NHERF1 at the apical membrane and with SCRIB at the cell-cell junctions of differentiated intestinal epithelial cells (**Figure 24**). To validate this hypothesis, I anticipate pursuing additional co-localization experiments described in Chapter V (Conclusion and Future Directions).

4.5 *Mcc* loss-of-function Intestinal Phenotype.

Homozygous *Mcc*^{lacZ} mice (**Figure 12**) were originally reported to present no overt phenotypes (Young et al., 2011). However, over the course of the experiments described in this thesis, distinct defects in the intestinal epithelium of *Mcc*^{lacZ} null homozygous mutant mice were uncovered, which were most extensively characterized at 3 months of age. Histologically, these animals reproducibly present a phenotype characterized by villi atrophy and/or shortening, with prominent indentations along the lumen-facing, apical surface, that we hypothesize are inextricably linked to significant weight loss (**Figure 26**).

Normally, defects in microvilli architecture are directly implicated in nutrient malabsorption and chronic diarrhea (Crawley et al., 2014a, 2014b). Consequently, the dramatic weight loss observed in *Mcc* null mutant mice along with the fact that *Mcc* protein is found at the apical membrane of differentiated intestinal cells suggested a role for *Mcc* in microvilli formation and/or maintenance. Enterocyte microvilli in *Mcc* null mutant mice are however unaffected, displaying an ultrastructural morphology that is indistinguishable from the wild type (**Figure 27**). Interestingly, simultaneous genetic deletion of three genes *Epsin*, *Villin* and *Plastin-1*, which are associated with actin filament bundles in the brush border, results in normal microvilli formation, although

the actin filaments are compromised (Revenu et al., 2011). Moreover, Sumigay and Lechler (2012) showed that a desmosome-associated protein, Desmoplakin, is required for proper microvilli morphology and length. Several other studies have demonstrated that microvilli assembly involves a number of protein complexes, therefore invoking the possibility of highly efficient compensatory regulation (Crawley et al., 2014b; Revenu et al., 2011; Saotome et al., 2004; Tonucci et al., 2018).

A number of reports in the literature describe intestinal phenotypes similar to that of *Mcc* mutants, specifically in mouse models in which genes encoding *Mcc*-interacting proteins are mutated. For example, the loss of *CK1δ/ε* in the mouse intestine was shown to cause shrinkage of both crypt and villus structures due to rapid death of CBC cells (Morgenstern et al., 2017). Despite phenotypic similarities in the intestine, *Mcc*-deficient mice do not seem to present any defects in intestinal cell proliferation or differentiation. For instance, ISH for *Lgr5* expression (**Figure 14B-15C**), which marks crypt stem cells, as well as intestinal Alkaline Phosphatase activity (**Figure 13A-F**), which is detectable only in differentiated cells in the intestine, conclusively demonstrate that the composition of the crypt and villus compartments is not markedly different from the wild-type intestinal epithelium.

Furthermore, Morales et al. (2004) showed that loss of *Ezrin* leads to defects in villus formation and neonatal death. *Nherf1* interacts with the ezrin-radixin-moesin (ERM) apical membrane complex (McClatchey and Fehon, 2009; Reczek et al., 1997). The ERM complex is responsible for linking actin filaments to the apical domain of epithelial cells (Saotome et al., 2004). Moreover, when *Ezrin* is conditionally deleted in the adult mouse intestinal epithelium with *Villin-CreERT2*, dramatic defects are observed in villus morphology including discontinuous F-actin staining along the apical membrane, morphological aberrations in cell geometry and defects in the extrusion of

enterocytes into the intestinal lumen (Casaletto et al., 2011).

Interestingly, we observe that *Mcc*^{-/-} intestinal organoids on day 8 of *in vitro* culture shed increased numbers of cells into the Matrigel matrix when compared to wild-type controls (**Figure 28**). Although not reported in this thesis and unpublished, our collaborator Dr. Anne-Claude Gingras (University of Toronto) has interestingly shown that Mcc interacts with Ezrin using the BioID proximity-labeling technique (Samavarchi-Tehrani et al., 2018 and data not shown). It remains to be determined whether this interaction is direct or indirect, especially given that Ezrin was not recovered in the mass spectrometry data described in this thesis. Biological triplicate proteomic experiments are underway to re-interrogate the Mcc interactome in HEK293 cells that will undoubtedly shed additional light on the interactions between Ezrin and Mcc.

Similar to *Ezrin* loss, the loss of *Mcc* results in defects in differentiated epithelial cell morphology as compared to the wild type, including cell length, width and nuclear aspect ratio (**Figure 29**), suggesting that cell polarity is affected. Maintenance of polarity in differentiated epithelial cells is a process fundamentally regulated by molecular mechanisms that drive the establishment of the cell cytoskeleton — that is MT, actin and keratin intermediate filaments organization — as well as intracellular and membrane trafficking of molecules (Rodríguez et al., 1994; Rodriguez-Boulan and Nelson, 1989; Salas et al., 1988, 1997; Simons and Fuller, 1985). This enables epithelial cells to achieve their distinct apical-basolateral domains required to perform their dedicated functions in organs such as the intestine.

MTs have been shown to play important roles for proper intestinal epithelial cell morphology as well as for nucleus and organelle positioning. For example, genetic

depletion of *Camsap3*, which encodes a minus-end associated protein, disrupts MT organization, thereby compromising the cell architecture (Noordstra et al., 2016). In this context, the misplacement of a number of organelles was also observed (Toya et al., 2016; Wang et al., 2016). Additionally, conditional expression of *Spastin*, which encodes a MT-severing protein, in the mouse intestine resulted in misplacement of organelles (Muroyama et al., 2018). These studies indicate that MT filament organization are essential for the establishment of intestinal cell shape and organelle distribution. However, MT array organization does not appear to be vital for intestinal function as these mutant mice display no major defects in intestinal homeostasis.

A recent study by Sallee et al. (2018) reported that MT arrays at the nc-MTOC remained robustly organized even upon depletion of numerous well characterized MT regulators and MT-associated proteins including Patronin (PTRN), XMAP215/ch-TOG, and Ninein in *C. elegans* embryonic intestinal cells. Additionally, co-depletion of Aurora A kinase AIR-1 and γ -TuRC did not impair MT organization (Sallee et al., 2018).

In this thesis, we reported morphological discrepancies in the aspect-ratio of nuclei of *Mcc*-deficient enterocytes compared to wild-type (**Figure 29B**) and defects in nutrient absorption (**Figure 30C**). Heterozygous mice for the loss of *Mcc* do not display any phenotype in intestinal cells. Moreover, our investigation of the MT filament network by α -tubulin immunostaining revealed no obvious (or consistent) differences between wild-type and *Mcc*^{-/-} intestinal epithelial cells as their parallel apical-basal organization seemed to be unaffected by conventional confocal microscopy analysis (**Figure 29A**). A deeper, more granulated analysis of the MT network in *Mcc*^{-/-} intestinal cells is clearly warranted, perhaps using more sophisticated microscopy techniques such as super resolution (Heintzmann and Huser, 2017).

The CIF network has long been described as a mechanical component that underlies the apical membrane (or terminal web) of epithelial cells, providing a multifaceted scaffold that connects a number of proteins to the apical domain of these cells (Franke et al., 1979; Rodríguez et al., 1994). Bundles of CIFs also extend along the apical-lateral domain of intestinal cells and are connected to cell-cell desmosomes (Kottke et al., 2006; Kowalczyk et al., 1999; Oriolo et al., 2007).

Importantly, accumulating evidence indicates essential roles of CIFs in attaching MTOCs at the apical domain of epithelial cells during differentiation and polarization. For instance, Salas et al. (1997) showed that downregulation of CK19 in CACO-2 epithelial cells resulted in a distinctive polarity phenotype characterized by disorganization of MT arrays with impairment of apical F-actin localization. Cell-cell junctional complexes appeared unaffected as ZO-1 localization was intact (Salas et al., 1997). This potential role of CIFs in the organization of epithelial polarity was further investigated by analysing the consequences of Type I cytokeratin (CK18 and CK19) knock-downs in CACO-2 cells. The results confirmed that CIFs are the sites where γ -tubulin structures attach to the terminal web (Salas, 1999). Moreover, in *Ck8*-deficient mice, terminally differentiated intestinal cells were shown to lack CIF structures and γ -tubulin immunostaining was found to be dispersed in the cytoplasm of enterocytes. As a result, the MT filaments organization was disturbed. In addition, enterocyte apical membrane markers including Alkaline phosphatase and Cystic fibrosis transmembrane conductance regulator (Cftr) were lost in some villus cells (Ameen et al., 2001).

Collectively, these studies indicate CIFs are important for the apical distribution of MTOCs proteins and the organization of MT filaments in epithelial cells. A complete mechanistic role for these functions is still missing. Considering the level of

organization achieved by the cytoskeleton in epithelial cells, it is tempting to hypothesize that complex interactions between other centrosomal proteins with CIFs are involved in the regulation MT filaments organization in epithelial cells. I will expand the investigations of MT filaments in *Mcc*-deficient mice with thorough analysis of the CIF components (CKs) and other apical membrane proteins in intestinal cells.

Finally, RNAseq gene expression data reveal that the most differentially expressed genes in *Mcc*^{-/-} crypts are associated with biological processes and pathways (KEGG profile) that are mainly involved in immune responses and cell adhesion processes (**Figure 30A** and full gene list in **Table 06**). However, IF and confocal microscopy of various cell-cell adhesion proteins such as β -catenin, Ep-CAM, E-Cad and ZO1 in *Mcc*^{-/-} villus cells reveal their ostensibly normal cellular localization. Nevertheless, evaluation of both paracellular permeability and intracellular uptake functions with FITC-Dextran and D-xylose, respectively, suggests that the loss of *Mcc* affects intestinal barrier permeability and the absorption or transport of molecules via transcellular mechanisms (**Figure 30B-C**). These findings are supported by numerous studies that have used D-xylose, a small sugar that is readily absorbed via transcellular transport, to measure transcellular uptake in malabsorption conditions (Antunes et al., 2009; Craig and Atkinson, 1988; Hope et al., 2012). In addition, dysfunction of protein complexes involved in internal cell transport mechanisms have long been implicated in malabsorption conditions in humans (Park et al., 2019; Tonucci et al., 2018, 2017).

Furthermore, intestinal permeability defects have been described as key drivers of several inflammation-associated diseases of the GI tract. Changes in the expression and localization of several tight junction proteins, such as Claudins, have

been reported in various cases of chronic inflammatory diseases including IBD (Cereijido et al., 2007; Gassler et al., 2001; Snoeck et al., 2005; Yu and Yang, 2009). It is tempting to speculate that the expression and/or localization of Claudins and other components of the intercellular junctional complexes may be linked to or regulated by *Mcc*. We will expand these findings by selecting and further investigating those genes whose expression is associated with intestinal permeability and inflammation-associated GI diseases. We are confident that these future experiments (Chapter V) will provide a better understanding of the origins of the *Mcc*-deficient intestinal phenotype.

4.6 *Mutated in Colorectal Cancer (MCC)* and Intestinal Cancers.

In this section of my thesis, I return to *Mutated in Colorectal Cancer (MCC)* and the disease for which it is named—the third most commonly diagnosed malignancy and the second leading cause of cancer death (Keum and Giovannucci, 2019). As shown in **Figure 32**, homozygous loss of *Mcc* in mice does not lead to tumor formation. *Mcc* null mutant mice are in fact viable and fertile and can live a long life (> 1.5 years) despite the gross intestinal defects described at length in **Figures 26-27** and **29-30**. However, when introduced into the *Apc^{Min/+}* mouse model of intestinal cancer, loss of even one *Mcc* allele surprisingly yields an increase in tumor burden and accelerated death (**Figure 32** and survival curve not shown). This strong genetic interaction firmly establishes a role for the *Mcc* gene in the pathogenesis of its namesake disease.

The *Apc^{Min}* mutant mouse arose during a forward genetic screen with N-ethyl-N-nitrosourea (ENU) that resulted in Multiple Intestinal Neoplasias (Min) (Moser et al.,

1990). *Min* mice harbor a nonsense mutation at codon 850 of the *Apc* tumor suppressor gene (hence termed *Apc^{Min}*), whose human homolog (*APC*) is commonly perturbed in FAP and sporadic CRC patients. The *Apc^{Min}* allele produces a truncated *Apc* protein and is embryonically lethal when homozygous (Su et al., 1992, 1993). Phenotypically, *Apc^{Min}* heterozygous mice present a pathology frequently observed in FAP patients, developing on average 30-60 adenomas over a period of 3 months on the inbred C57BL/6J genetic background (Kwong and Dove, 2009; Kwong et al., 2007). However, in contrast to FAP patients, *Apc^{Min/+}* mice develop adenomas primarily in the small intestine (Moser et al., 1990, 1992). Adenoma formation in these animals as well as in FAP patients is caused by the loss of the remaining wild-type *Apc* allele—the ‘second hit in the ‘two-hit’ hypothesis first postulated by Knudson in 1971 (Knudson, 1971; Luongo et al., 1994; Su et al., 1992). This can occur via a variety of mechanisms, including partial or full gene deletion, recombination, regulatory mutations and epigenetic silencing (Grodin et al., 1991; Nishisho et al., 1991; Su et al., 1992).

The loss of *APC* represents the most common initiating mutation in the outgrowth of benign adenomas via hyper-activation of the WNT signaling pathway (Su et al., 1992, 1993a). As described in the Introduction (**Figure 04**), *APC* is a key component of the β -catenin ‘destruction complex’, playing a critical role in the regulation of β -catenin cytoplasmic levels. The accumulation of β -catenin in intestinal cells, which is frequently observed in adenomas, is a consequence of the inability of the remaining truncated *APC* protein to negatively regulate the levels of cytoplasmic β -catenin, leading to an up-regulation of the WNT pathway and tumor initiation (Munemitsu et al., 1995; Rubinfeld et al., 1993).

The cell of origin of intestinal tumors in the *Apc^{Min}* mouse model is well established. In a seminal paper, Barker et al. (2009) showed that the loss of *Apc* in

CBC (*Lgr5*⁺) cells leads to their rapid transformation into dysplastic cells that seed several microadenomas. Within 3 to 5 weeks, these microadenomas progressively grow upwards along the crypt-villus axis generating larger adenomas. This process is also driven by upregulation of WNT signaling in the *Lgr5*⁺ cells bearing loss of *Apc*. In crypt TA cells, however, conditional deletion of *Apc* leads to only a few microadenomas that rarely progress into adenomas. In such cases, tumor progression is most likely fueled by additional hits (Barker et al., 2009).

Earlier in Chapter III, we showed that *Mcc* is expressed throughout the crypt compartment of the small intestine and colon, labeling *Lgr5*⁺ cells well as TA cells (**Figure 13-15**). Additionally, human *MCC* is located in a region of chromosome 5q21 that is frequently mutated in both sporadic and inherited CRCs, residing 800 kb from the *APC* gene in a head-to-tail configuration (Ashton-Rickardt et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991). In the mouse, *Mcc* and *Apc* are similarly situated head-to-tail 10.6 Mb apart on chromosome 18 (about 5 centimorgans or, in other words, with a recombination efficiency of 5%) (Luongo et al., 1993). The proximity of *Mcc* and *Apc* as well as its long-postulated and thinly explored link to intestinal cancer formation prompted us to further investigate the contributions of *Mcc* loss to adenoma growth in the *Apc*^{Min/+} mouse model.

By intercrossing *Mcc* homozygous null and *Apc*^{Min} heterozygous mice, we first found that loss of one *Mcc* allele is sufficient to significantly increase tumor number in *Apc*^{Min/+} mice on a mixed (129/Sv x C57BL/6J) genetic background (**Figure 32**). After repeated intercrossings, recombination was achieved, resulting in homozygous loss of *Mcc* in the context of *Apc*^{Min/+}, and these *Apc*^{Min/+};*Mcc*^{-/-} animals presented larger adenomas (>4 mm), which consequently led to a more severely compromised intestinal epithelium (**Figure 32 - 33**). Additionally, we observed that such double

mutants more rapidly succumbed to their tumor burden when compared to their *Apc^{Min/+};Mcc^{+/+}* wild-type counterparts (data not shown in this thesis). *Apc^{Min/+}* animals as well as CRC patients suffer from chronic intestinal hemorrhage because adenoma outgrowth compromises the intestinal epithelium resulting in severe anemia (Su et al., 1992). We therefore believe that the shorter survival of *Apc^{Min/+};Mcc^{-/-}* animals is most likely due to the presence of more abundant and larger adenomas that harshly compromise the function of the intestinal epithelium—a hypothesis that will be tested in the future.

Further studies are required to elucidate what specific protein complexes and molecular mechanisms (signaling pathways) are impacted by the loss of *Mcc* that underlie the increased tumor burden in the *Apc^{Min}* background. It is noteworthy that *Mcc* co-localizes with β -catenin at the apical-lateral membrane junction of intestinal epithelial cells (**Figures 19E-F and 20B**) (Benthani et al., 2018) and specifically interacts with the Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1) through its C-terminal PDZ binding motif (**Figure 23E**). NHERF1 also interacts with β -catenin at the apical/lateral membrane of intestinal epithelial cells (Georgescu et al., 2008; Hayashi et al., 2010). In CRC lesions, NHERF1 fails to localize at the apical membrane (Hayashi et al., 2010).

Interestingly, a study by Georgescu et al. (2016) demonstrated that homozygous loss of *Nherf1* in combination with *Apc^{Min/+}* also resulted in a significant increase in tumor burden, with a concomitant increase in the expression levels of β -catenin, YAP and the WNT target gene *CyclinD1*. These authors showed that *Nherf1* depletion results in release of E-cadherin and β -catenin from cell membrane junctions. This consequently leads to an accumulation of cytoplasmic and nuclear β -catenin that in the context of *Apc^{Min}* results in upregulation and oncogenic activation of WNT/ β -

catenin signaling pathway (Georgescu et al., 2008). Like *Mcc*, homozygous loss of *Nherf1*, however, does not trigger adenoma formation along the length of the intestine (Georgescu et al., 2016; Morales et al., 2004).

NHERF1 functions in providing a scaffold for the interaction between ERM (EZRIN, RADIXIN, and MOESIN) proteins and the cytoskeleton in epithelial tissues (Reczek and Bretscher, 1998; Reczek et al., 1997). ERM proteins are involved in assembling apical membrane protein complexes, linking them to the actin cytoskeleton and participating in cell-cell junction formation (Bretscher et al., 2002; Reczek et al., 1997). In *Nherf1* null mice, ERM proteins are significantly decreased in the apical domains of small intestine epithelial cells (Morales et al., 2004). Ezrin is the only ERM expressed in the mouse intestinal epithelium (Berryman et al., 1993; Reczek et al., 1997). Loss of Ezrin leads to apical defects affecting cell-cell junctions and cell polarity (Casaletto et al., 2011; Saotome et al., 2004). Moreover, both *Nherf1*- and *Ezrin*-deficient mice display phenotypes in enterocyte morphology and microvilli (Casaletto et al., 2011; Garbett et al., 2010; Georgescu et al., 2008; Saotome et al., 2004) that are intriguingly very similar to those observed in *Mcc* null mutant mice, including villus cell disorganization and defects in intestinal cell morphology (**Figures 26-27, 29 and 33**).

NHERF1 has been reported to bind EZRIN with high affinity through its C-terminal ERM-binding (EB) region (Finnerty et al., 2004; Reczek and Bretscher, 1998; Reczek et al., 1997). For this reason, NHERF1 was also independently named ERM-binding phosphoprotein-50 (EBP50) (Reczek 1997). In addition, NHERF1/EBP50 contains two N-terminal PDZ domains (PDZ-1 and PDZ-2) (Reczek et al., 1997). Similar to the other ERM proteins, EZRIN contains a long coiled-coil region, a short carboxy C-terminal domain and an N-terminal FERM (Four-point one, Ezrin, Radixin,

Moesin) domain whose amino acid sequence is closely related to a domain found in the erythrocyte cytoskeletal component Protein/Band 4.1 (Conboy et al., 1986; Gould et al., 1989). Through its C-terminal EB region, NHERF1/EBP50 binds to the FERM domain of ERM proteins, while other NHERF1/EBP50-interacting proteins bind to the PDZ-1 domain with exception of only a few proteins including β -catenin and YAP that specifically interact with the PDZ-2 domain (Finnerty et al., 2004; Reczek and Bretscher, 1998; Shibata et al., 2003; Weinman et al., 2003).

Morales et al. (2007) previously published that NHERF1/EBP50 adopts an intramolecular conformation in which the C-terminal EB region binds to its own N-terminal PDZ-2 domain, resulting in a head-to-tail configuration that prevents the association of other proteins to NHERF1/EBP50 PDZ domains. This intramolecular configuration is disrupted by the interaction between EZRIN to the EB region of NHERF1/EBP50 (Georgescu et al., 2008; Morales et al., 2007). Therefore, the binding of EZRIN to NHERF1/EBP50 is important for the assembly of apical macromolecular protein complexes via PDZ motifs, their interaction with the cytoskeleton and ultimately for the establishment of cell polarity.

NHERF1 was additionally shown to regulate the activity of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in the intestine (Broere et al., 2007). The CFTR protein localizes to the apical membrane of enterocytes where it functions as a transporter of chloride ions across the epithelial membrane, maintaining bicarbonate secretion and extracellular alkalization (Jakab et al., 2010; Strong et al., 1994). Significantly, *Cftr*-deficient mice also show similar intestinal defects to *Nherf1* null mutant mice and, on an *Apc*^{Min/+} background, *Cftr* deficiency leads to an increase in tumor burden (Than et al., 2016).

In this study, we have demonstrated that MCC physically interacts with NHERF1 through its C-terminal PDZ binding motif (**Figures 17B** and **23E**). Whether this interaction occurs via PDZ-1 or -2 domain of NHERF1 is yet to be determined. Additionally, in agreement with our mass spectrometry results, the interaction between MCC with NHERF1 (**Figure 17B**) was independently reported via a series of immunoprecipitation experiments using MCC peptides as bait followed by mass spectrometry (Arnaud et al., 2009). EZRIN was also recovered by Arnaud et al. (2009), but EZRIN was not identified in our initial mass spec in HEK293 cells, perhaps due to technical limitations. Although not reported in this thesis, and yet unpublished, our collaborator Dr. Anne-Claude Gingras (University of Toronto, Canada) has however shown using a robust lentiviral-based BioID proximity-labeling technique that MCC “interacts” with EZRIN (data not shown), perhaps via NHERF1/2 as an intermediate (Arnaud et al., 2009; Samavarchi-Tehrani et al., 2018). At present, biological triplicate proteomic experiments are underway to re-interrogate the MCC interactome in HEK293 cells. We are additionally developing tools and reagents to determine the Mcc interactome *in vivo* in purified intestinal villi and crypts, such as gene-edited *Mcc* alleles that introduce common epitopes (*e.g.*, FLAG) for efficient immunoprecipitation followed by mass spec as well as even more robust anti-Mcc antibodies. These analyses will undoubtedly shed additional light on the interactions between MCC and EZRIN and other apical proteins.

Based on the experimental findings presented in this thesis: *a)* that Mcc protein is localized at the apical membrane of mouse and human intestinal epithelium cells; *b)* that MCC interacts and/or co-localizes with NHERF1, EZRIN, β -catenin and E-cadherin; *c)* that loss of *Mcc* causes morphological aberrations in the villi that are strikingly similar phenotypically to the loss of *Nherf1*, *Ezrin* or *Cftr* in mutant mice, we

speculate that Mcc plays a mechanistic role in the NHERF1-ERM complex at the apical domain of intestinal epithelial cells. In the context of tumorigenesis, genetic loss of *Mcc* in the *Apc^{Min}* background may therefore exacerbate the oncogenic accumulation of β -catenin due to a potential disruption of apical protein complexes involving NHERF1, ERM and other cell junction proteins.

Although these results are preliminary, they indisputably implicate Mcc in the pathogenesis of CRC and provide a valuable platform to further investigate how the loss of a centrosomal protein that re-localizes to the apical membrane in enterocytes contributes to super-activation of the WNT signaling pathway and increases adenoma formation. Indeed, the *Apc^{Min};Mcc^{-/-}* model will be a useful tool for a detailed mechanistic and biochemical interrogation of the functional participation of Mcc in intestinal tumorigenesis and for deeper analysis of individual aspects of intestinal homeostasis and disease. In ongoing experiments, we will leverage on the published studies describing *Nherf1*-, *Ezrin*- and *Ctfr*-deficient mice as guideposts for the design of experiments aimed at not only understanding the etiology of the intestinal phenotype in *Mcc*-deficient mice but also interrogating the unexpected increase in tumor burden in the context of *Apc^{Min}*. It is anticipated that the findings presented thus far in conjunction with data that will emerge from the Future Directions (final chapter) will pave the way for a reinvestigation of the relationship between an undisputed “driver” mutation (*APC*) and a poorly described, somewhat neglected, “passenger” mutation (*MCC*) in a devastating human disease (Luongo et al., 1994; Zeineldin and Neufeld, 2013).

The gene *Mutated in Colorectal Cancer (MCC)* was discovered through linkage studies and positional cloning nearly 30 years ago and christened, as its name implies, a tumor suppressor for inherited and sporadic human colorectal cancers. To date, despite its historical association with CRC, linkage to the cardinal *APC* tumor suppressor gene and strong evolutionary conservation, the expression of *MCC*, both at the mRNA and protein level, as well as its role in tissue homeostasis and disease remain poorly studied, even neglected. Moreover, those studies that did emerge over the years yielded contradictory findings.

Prior work by former members of my lab investigated the requirement of *Mcc* for normal murine embryonic development using a novel *Mcc^{lacZ}* reporter mouse model (**Figure 12**) (Young et al., 2011). Molecular characterization of this allele established that it fully inactivates the *Mcc* gene, generating a bona fide loss-of-function mutation, and the resulting homozygous mice were viable and fertile with no ostensible phenotypes. This result was unexpected because it was in stark contrast to findings in the literature that mutations in *MCC* contribute to the development of a variety of human cancers, including CRC (Benthani et al., 2018; Currey et al., 2019; Edwards et al., 2014; Mukherjee et al., 2011, 2016; Shukla et al., 2013). This mystery around *Mcc* and its role in the intestine both genetically and biochemically motivated the experimental work chronicled in the preceding chapters.

Firstly, we focused our attention on the distribution of *Mcc* transcripts using both our *Mcc^{lacZ}* reporter mouse model and a high-resolution section *in situ* mRNA localization technique called RNA Scope. In parallel, we definitively determined at the

subcellular level in which compartment the Mcc protein resides, which was accomplished with the identification of a high-affinity, commercially available Mcc antibody (**Table 03**). My results reveal that *Mcc* is expressed throughout the crypts of the intestine and clarify decades of confusion in the literature by indisputably establishing that Mcc is not found in the nucleus, but conspicuously localizes to the centrosome in crypt proliferating cells and is redeployed to the apical membrane in terminally differentiated intestinal epithelial cells. To elaborate Mcc function, we embarked on rigorous proteomic studies and the results of these investigations laid the groundwork for a number of future experiments, described in detail below, aimed at fully unveiling the role of Mcc in tissue homeostasis and disease.

Mcc Function at the nc-MTOC in Enterocytes.

The fact that MCC is a centrosomal protein that re-localizes to the apical membrane of differentiated intestinal cells, where the nc-MTOC is situated, supports our hypothesis first stated in Chapter IV, that MCC likely functions at the nc-MTOC by either anchoring or nucleating MTs. Evidence supporting this hypothesis comes from our observations of the intestinal phenotype in *Mcc* null mutant mice that present gross morphological aberrations in the villi as well as defects in the architecture of differentiated cells resident within the villi.

The nc-MTOC is a protein complex largely involved in MT regulation, organelle distribution and establishment of cell polarity (Mogensen et al., 1997; Keating and Borisy, 1999; Toya and Takeichi, 2016; Goldspink et al., 2017; Muroyama and Lechler, 2017; Sanchez and Feldman, 2017). In the intestinal epithelium, other centrosomal proteins, including Ninein, have been reported to be redeployed to the nc-MTOC to

anchor MTs. Like Mcc, Ninein also co-localizes with β -catenin at apical-lateral cell junctions and is essential for the establishment of apico-basal MT arrays in intestinal cells. (Goldspink et al., 2017; Mogensen et al., 2000). Another example is Camsap3, which also anchors the minus-ends of MTs to the nc-MTOC in the mouse intestine. In *Camsap3*-deficient mouse intestine, the MTs present a tangled appearance with loss of apico-basal directionality (Toya and Takeichi, 2016).

Despite the extensive experimental efforts outlined in this thesis, the precise molecular mechanism underlying the prominent intestinal phenotype in *Mcc*-deficient mice remains frustratingly unexplained. Leveraging on the published literature as a guide to decode this phenotype, it has been difficult to ascribe an overall “general” mechanism. This is principally due to the fact that components of the centrosome that are recruited to the nc-MTOC are involved in a diverse array of MT regulatory functions, including, but not exclusive to, nucleating, anchoring and/or stabilizing MT filaments or simply transporting other components to the nc-MTOC (Doxsey, 2001; Sallee et al., 2018; Sanchez and Feldman, 2017; Tang and Marshall, 2012). Thus, there is a clear need for a deeper mechanistic examination of the role of Mcc, not only at the centrosome but also at the nc-MTOC.

To this end, we are rigorously reinvestigating the apico-basal MT arrays with fluorescent stains for α/β -tubulin in sections of wild-type and *Mcc*^{-/-} intestine using more sophisticated imaging techniques such as super resolution microscopy, e.g. stochastic optical reconstruction microscopy (STORM) or structured illumination microscopy (SIM), which will deliver an improved image analysis of the organization of MT filaments in the *Mcc*-deficient intestinal cells (Heintzmann and Huser, 2017; Komis et al., 2014; Rust et al., 2006; Sonnen et al., 2012).

One powerful platform for interrogating Mcc function in the nc-MTOC is the use

of *ex vivo* 3D intestinal organoid culture (Sato et al., 2009). First, we aim to capture the rearrangement of MT arrays by live cell imaging microscopy using MT-fluorescent dyes such as SiR-tubulin (Lukinavičius et al., 2018) during differentiation in wild-type compared to *Mcc*^{-/-} intestinal organoids. Second, nocodazole washout assays will also be employed in intestinal organoids to observe MT repolymerization. If MT regrowth is impaired after nocodazole treatment in *Mcc*-deficient organoids, this result would suggest that Mcc is important in the process of MTs nucleation in intestinal cells. In this light, it has been demonstrated, for instance, that MTs regrow from the nc-MTOC sites in differentiating *C. elegans* embryonic intestinal cells after nocodazole washout (Yang and Feldman, 2015).

The Molecular Mechanism of Mcc Redeployment.

Although various centrosomal components are known to be recruited to nc-MTOC sites at the point of epithelial differentiation, the molecular mechanisms of such coordinated events remain largely unknown (Keating and Borisy, 1999; Sanchez and Feldman, 2017; Tang and Marshall, 2012; Toya and Takeichi, 2016). In this study, we identified several proteins that interact with MCC and are likely involved in MCC re-localization, including the Casein Kinases 1 (CK1δ and CK1ε), NHERF1 and NDE1 (**Figure 17B**). We provided experimental evidence that phosphorylation of MCC by CK1ε governs its redeployment to the apical membrane (**Figure 25C**). Phosphorylation of MCC by CK1ε, for example, decreases its affinity to core centrosomal proteins such as NDE1 (**Figure 23D**). At the apical membrane, we hypothesize that MCC becomes anchored to NHERF1 through its PDZ binding motif

(**Figure 23E**). We anticipate pursuing immunoprecipitation assays to uncover which PDZ domain of NHERFF1 MCC binds to using mutant versions of NHERF1 where either the PDZ-1 or -2 domain is truncated.

Furthermore, by inhibiting phosphorylation using a highly specific CK1 δ/ϵ inhibitor, we observed that Mcc apical localization was disrupted in intestinal organoids (**Figure 25C**). Additional IF experiments are ongoing to investigate *a*) the localization of numerous enterocyte apical proteins including Nherf1, Ezrin, and Cfr; *b*) the localization of Scribble; *c*) intestinal differentiation markers (e.g. villus-specific marker Alkaline Phosphatase and the crypt-specific Paneth cell marker Lysozyme), in both wild-type and *Mcc*-deficient intestinal tissues and in organoids in cultured in the presence and absence of the CK1 δ/ϵ inhibitor. Additionally, these results will be further analysed by quantitative fluorescence microscopy (Esposito et al., 2009). We anticipate that the examination of the localization of MCC-interacting partners upon inhibition of phosphorylation and/or in the absence of Mcc (in the *Mcc*^{-/-} mice) will greatly expand our understanding of the dynamics involved in Mcc redeployment to apical membrane of intestinal differentiated cells.

To reinforce the hypothesis that phosphorylation is the trigger of Mcc re-localization, we have employed a high-fidelity CRISPR/Cas9 system (Havlicek et al., 2017) to precisely fuse an eGFP to the Mcc N-terminus in intestinal organoids (data not shown). With this system, we will track by live cell imaging (time-lapse) the Mcc re-localization under normal conditions and with CK1 δ/ϵ inhibition. MTs will also be stained with SiR-Tubulin in this approach. To date, the redeployment of centrosomal proteins to nc-MTOC by live cell imaging has not been reported. We believe that this system once established will provide a powerful platform to study the molecular

mechanisms and dynamics that govern the redeployment of centrosomal components and nc-MTOC assembly.

Lastly, we have already initiated additional comparative proteomic experiments in HEK293 cells expressing our original epitope tagged MCC construct (**Figure 17B**) with or without CK1δ/ε inhibition. These data will establish the interactome for MCC and its interacting partners in a phosphorylation-dependent manner. We predict that this will shed additional light into our understanding of the Mcc mechanism of re-localization. Moreover, we are optimistic that this experiment, performed with more sophisticated proteomic tools will provide an enhanced identification of MCC binding proteins as well as precise information about which residues in MCC are specifically phosphorylated by CK1δ/ε.

Intestinal Permeability in the *Mcc*^{-/-} Intestinal Epithelium.

The morphological defects observed at both tissue and cellular levels appear to disturb intestinal homeostasis in *Mcc*-deficient homozygous mice, as the weight in these animals is considerably less when compared to their wild-type littermates (**Figure 26C**). Exome sequencing of *Mcc*^{-/-} intestinal samples analysed by KEGG profiling showed upregulation of many pathways that are mostly associated with inflammatory responses (**Figure 30A**), including upregulation of cell adhesion molecules (CAM). Apart from their regulatory functions in the immune response, the CAM family also comprises members associated with cell-cell junctional complexes, such as Cadherins (Harjunpää et al., 2019).

The exome sequencing results will be further explored in a series of experimental approaches. For example, genes whose expression is associated with intestinal permeability and inflammation-associated intestinal diseases will be

rigorously investigated by quantitative expression analysis (RT-PCR) in wild-type and *Mcc* mutant intestine (Graham and Xavier, 2013; Harjunpää et al., 2019; Khor et al., 2011). In addition, we intend to further investigate the inflammation process in *Mcc*-deficient intestine using histopathological and immunofluorescence techniques to analyse markers of inflammatory cell infiltrates as well as to fully characterize the morphological changes in the *Mcc* mutant intestinal epithelium. My experimental approach investigating the severity of the inflammatory response is closely guided by work performed by Erben et al. (2014).

The KEGG profile results prompted investigation of the intestinal barrier as intestinal permeability can be compromised by defects in the cell-cell junctions, apical protein complexes and in the cytoskeleton (Al-Sadi et al., 2011; Assimakopoulos et al., 2011; González-González et al., 2018). Indeed, our intestinal permeability studies using D-Xylose and Dextran assays, as shown in **Figure 30B-C**, revealed that both intestinal paracellular and intracellular permeability are compromised in *Mcc*-deficient mice. We will expand and validate these findings by selecting tracers of different molecular weights to re-evaluate paracellular and intracellular permeability defects in *Mcc*^{-/-} (Woting and Blaut, 2018). In addition to the D-xylose assay which revealed defects in the intracellular absorption function of the *Mcc*^{-/-} intestinal cells (**Figure 30C**), other tracers will be utilized to assess the apical-membrane uptake in enterocytes (Howe et al., 2014).

Moreover, the Lucifer yellow (LY) tracer, which was recently applied in 3D intestinal organoids to evaluate epithelial integrity (Bardenbacher et al., 2019), will be used to strengthen our recent findings. We will also analyse by RT-qPCR the expression levels of several genes encoding for proteins involved in transport and endocytosis processes in enterocytes including the members of the large SLC family,

CORO1A, HFE, and SNX (Gassler et al., 2005). Additionally, several components of the cell-cell intercellular junctional complex will be further investigated using IF techniques followed by densitometric fluorescence intensity analysis in wild-type and *Mcc*^{-/-} enterocytes using both *in vivo* and *in vitro* approaches (Cowen and Thrassivoulou, 1992; McFarland et al., 2014).

Lastly, it has been shown that Paneth cells contribute largely to both the response and the pathogenesis of numerous inflammation-associated diseases along the length of the intestine (Clevers and Bevins, 2013; Schmitt et al., 2018). In inflammatory bowel disease (IBD), for instance, metaplastic Paneth cells can be found in the colonic epithelium (Cunliffe et al., 2001). In the small intestine, Paneth cells have been described to lose their secretory expression signature and acquire stem cell like properties (Schmitt et al., 2018). The morphological and molecular characteristics of Paneth cells in the context of inflammatory response will be investigated using immunohistochemical analysis in the intestine of the *Mcc*^{-/-} mice.

Loss of *Mcc* and Tumor Development.

Significant evidence has accumulated to date that loss of cell polarity and disruptions of cell-cell adhesion complexes are correlated events commonly observed in early cancer formation and that also contribute to tumor invasion (Bilder, 2004; Perl et al., 1998; Wodarz and Näthke, 2007). To explore the tumor suppressor function of *Mcc*, we examined the consequences of the loss of *Mcc* in the *Apc*^{Min} CRC mouse model. As reported in previous chapters of this thesis, one mutant allele of *Mcc* is sufficient to promote an increase in tumor burden frequency and tumor size in the *Apc*^{Min} background (**Figure 32**). We showed by IHC that Ki67 and β -catenin are both

increased in tumor tissues compared to adjacent normal mucosa in intestinal samples from the indicated genotypes (**Figure 33**).

A more holistic investigation is necessary to better understand these preliminary findings and conclusively characterize how the loss of *Mcc* is correlated with intestinal adenoma development in *Apc^{Min}* mice. One prospect that deserves thorough consideration as a probable cause for the tumor progression in *Apc^{Min};Mcc^{-/-}* is the fact that the loss of *Mcc* leads to alterations in intestinal cell polarity. The first obvious line of investigation that we are currently pursuing is the analysis of *Mcc* expression levels by RT-qPCR in tumors from *Apc^{Min}* intestine and the distribution of *Mcc* mRNA by RNAScope in *Apc^{Min/+};Mcc^{+/+}* tumor sections. Additionally, we are also examining the localization of *Mcc* in these tumor sections.

In Chapter IV, we described that the loss of *Mcc* in *Apc^{Min/+}* mice yields similar phenotypic outcomes as the loss of *Nherf1*, *Ezrin* and *Cftr* in the *Apc^{Min/+}* background. We are therefore further investigating the localization of *Nherf1*, *Ezrin*, *Cftr*, *Mcc*-interacting partners including β -catenin and other candidates such as Scribble, E-Cadherin as well as several components of the cell-cell junctions by immunofluorescence in tumor sections of *Apc^{Min/+};Mcc^{-/-}* compared to *Apc^{Min/+};Mcc^{+/+}*. We anticipate that these studies will further expand our understanding of the role of *Mcc* in the establishment of intestinal polarity in normal tissue and tumors.

Additionally, the expression levels of Wnt signaling components such as β -catenin, *Axin* and CK1 and Wnt-target genes including *Cyclin-D1*, *Axin2*, *CD44*, *c-MYC* will be quantitatively compared in dissected tumor samples from *Apc^{Min/+};Mcc^{+/+}* and double mutants by RT-qPCR to explore whether the loss of *Mcc* contributes to the oncogenic hyper activation of Wnt/ β -catenin pathway *in vivo*.

Another experimental approach to study the relationship of *Mcc* loss and tumor initiation is to employ the well-established *ex vivo* intestinal organoid system as it provides a homogenous population of intestinal epithelial cell lineages (Sato et al., 2009). Therefore, we plan to use it as an optimal platform to study changes at transcriptional levels as well as changes in tissue integrity and cell polarity of wild-type, *Mcc*-deficient, *Apc*^{Min/+} and double mutant mouse intestinal organoids (Sato et al., 2011; Xue and Shah, 2013). The CRC-derived Caco-2 cell line is an alternative *in vitro* system that can be used to study the intestinal epithelium. These cells harbor a truncated APC that should impair the degradation of cytoplasmic β -catenin and, importantly, they are capable to differentiated into intestinal polarized cells under appropriate conditions (Gayet et al., 2001; Simon-Assmann et al., 2006). Moreover, we anticipate analysing *MCC* expression and protein localization in human intestinal adenomas and carcinomas from surgically removed tumors. We have teamed up with colleagues at Lee Kong Chian School of Medicine in NTU for exciting collaborations that will grant us access to human CRC samples.

Moreover, a few years ago, we had engaged the services of the RIKEN institute (Japan) to generate a new *Mcc* mutant mouse model in which the insertional mutation was designed to introduce into *Mcc* exon 1(iso 2) the coding sequence for Enhanced Green Fluorescent Protein (eGFP) followed by the self-cleaving 2A peptide (Wang et al., 2015), the open reading frame (ORF) encoding CreERT2, which is a fusion version between Cre recombinase and a mutant form of the ligand-binding domain of the mutated estrogen receptor (Feil et al., 1997), and strong polyadenylation (pA) transcriptional termination sequence (Neve et al., 2017). The 2A-CreERT2-pA vector backbone was provided by Dr. Nick Barker (A*STAR IMB) and its utility was recently validated in (Leushacke et al., 2017). Although not mentioned thus far and nor used

in this study, this recently arrived *Mcc^{eGFP-CreERT2}* mouse model (in a pure C57BL/6 genetic background) will be a valuable tool for a variety of purposes. The CreERT2, for example, offers a range of possible strategies that can be applied to study tumor formation by activating mutations in conditional alleles such as *Apc^{flox}* (Barker et al., 2007, 2009). Additionally, *Mcc^{eGFP-CreERT2}* mouse model can be used to validate our observations regarding the *Mcc* loss-of-function phenotype.

Indeed, prospective experiments to investigate whether the onset of the intestinal phenotype in *Mcc^{-/-}* is developmental or adaptative are ongoing. Thus far, we only analysed 120-day old mice, as specified in the Materials and Methods. Indeed, the mammalian intestinal epithelium undergoes extensive morphogenesis to ensure the final compartmentalization of crypt and villus structures (Sumigray et al., 2018) and we have not established *Mcc* expression during this critical period. We will therefore examine the expression of *Mcc* and the morphology of the *Mcc^{-/-}* intestinal epithelium at different embryonic and postnatal days of development using whole-mount tissue analysis and different histological techniques, including ISH and IHC.

The data described throughout this thesis provide strong evidence that resolve an almost 30-yearlong controversy and finally recast *Mcc* as a novel centrosomal protein. Importantly, the data reveal clinically relevant insights into the involvement of a centrosomal component in intestinal tissue homeostasis and disease

TABLES

Table 01 - Mouse Models Genotyping Primers.

Alleles		5' > 3' Sequence	TM
ApcMin #01	FWD	TTCTGAGAAAGACAGAAGTCA	52.2
ApcMin #02	REV	TTCCACTTTGGCATAAGGC	53.9
ApcMin #03	REV	GCCATCCCTTCACGTTAG	55.9
Mcc-lacZ #01	FWD	TGCTATTGCTAACAACCCCC	55.8
Mcc-lacZ #02	FWD	AGGCGGGAAGTGTTTTAGGT	56.3
Mcc-lacZ #03	REV	GTGTGACCCCATTACCATCC	58.4
Mcc-eGFP #01	FWD	AGACTTCAGGTAGCCCCTCT	58.4
Mcc-eGFP #02	REV	AAGATGGTGCGCTCCTGGAC	60.4
Mcc-eGFP #03	REV	AGACTGAACAAAAGGGCCTGTC	58.6

Table 02 - RT-PCR Primers.

Gene - Target		5' > 3' Sequence	TM
<i>Mcc</i> Exon 17	FWD	ACTTGCCGAAGTAAGGACGA	52.2
<i>Mcc</i> Exon 18	REV	CTGGTGTCTGATTTCACTGC	53.9
<i>Lgr5</i>	REV	CCACAGCAACAACATCAGGT	55.9
<i>Lgr5</i>	FWD	AACAAATTGGATGGGGTTGT	55.8
<i>β-Actin</i>	FWD	CTTCTTTGCAGCTCCTTCGTTG	56.3
<i>β-Actin</i>	REV	CGCAGCGATATCGTCATCCA	58.4

Table 03 - Primary Antibodies.

Antibody	Species	Catalog #	Brand	Application
Alpha-Tubulin	Mouse IgG1	T9026	Sigma	WB, IF
Beta-Catenin	Rabbit	AB32572	Abcam	WB, IF
Casein Kinase 1E	Mouse	(A6) SC-374069	Santa Cruz	WB, IP
CD10	Mouse	AB951	Abcam	WB, IF, IHC
CEP170	Rabbit	AB72505	Abcam	WB, IF, IHC
CEP131	Rabbit	AB84864	Abcam	IF
Ep-CAM	Rabbit	AB71916	Abcam	IF
E-Cadherin	Rabbit	AB15148	Abcam	IF
K167	Rabbit	AB833	Abcam	IF
MCC	Mouse	SC-135982	Santa Cruz	WB, IF, IP
NDE1	Rabbit	AB127032	Abcam	WB
Ninein	Mouse IgG2a	637327	Merck	IF
Ninein	Rabbit	AB4447	Abcam	IF
NHERF1	Rabbit	SC-271552	Santa Cruz	WB, IF, IP
Pericentrin	Rabbit	AB4448	Abcam	IF
RasaL2	Rabbit	A302-109A	Bethyl Lab	WB, IF
Scribble (H300)	Rabbit	SC-28737	Santa Cruz	WB, IF, IHC
ZO1	Mouse	339100	Molecular Prob.	

Table 04 - Secondary Antibodies and Fluorescent Dyes.

Antibody	Species	Catalog #	Brand	Application
Alexa Fluor 488 Anti-Mouse	Donkey	A21202	Invitrogen	IF
Alexa Fluor 488 Anti-Rabbit	Donkey	A21206	Invitrogen	IF
Alexa Fluor 594 Anti-Mouse	Donkey	A21203	Invitrogen	IF
Alexa Fluor 594 Anti-Rabbit	Donkey	A21207	Invitrogen	IF
Alexa Fluor 488 Anti-Mouse	Goat	A28175	Invitrogen	IF
F-Actin (Phalloidin - 488)	---	A12379	Life Tech.	IF
Hoechst (33342)	---	62249	Thermo Scient.	IF
Dapi	---	D1306	Thermo Scient.	IF

Table 05 - ISH - RNA Scope Probes

Probe	Species	Catalog #	Brand	Application
Mm-Mcc	Mouse	411961	ACD Bio	ISH - RNA Scope
Mm-Mcc-C2	Mouse	411961-C2	ACD Bio	ISH - RNA Scope
Mm-Lgr5	Mouse	312171	ACD Bio	ISH - RNA Scope
Mm-Lgr5-C2	Mouse	312171-C2	ACD Bio	ISH - RNA Scope
Mm-Ppib	Mouse	313911	ACD Bio	ISH - RNA Scope
Mm-MKi67	Mouse	416671	ACD Bio	ISH - RNA Scope

Table 06 - KEGG Pathway Gene List

KEGG Entry	Pathways	Genes
mmu04660	T cell receptor signaling pathway	Cd8b1/Lat/Cd4/Itk/Cd3d/Zap70/Grap2/Cd3e/Pik3cd/Lck/Cd8a/Cd3g/Ppp3cc/Ptpn6/Vav1
mmu04650	Natural killer cell mediated cytotoxicity	Lat/Itgal/FasI/H2K1/Zap70/Itgb2/Hcst/Rac2/Pik3cd/Lck/Fcer1g/Ppp3cc/Ptpn6/Vav1/Plcg2
mmu04659	Th17 cell differentiation	Lat/Cd4/Il27ra/Cd3d/Zap70/Cd3e/Il21r/Il2rg/Lck/Il2rb/Cd3g/Runx1/Ppp3cc
mmu04514	Cell adhesion molecules (CAMs)	Ptpm/H2Q7/Cd6/Cd226/Cd8b1/Tigit/Itgal/Cd4/Fam120c/H2-K1/Itgb7/Selplg/Itgb2/Cd8a/Itga9/Pvr
mmu05340	Primary immunodeficiency	Cd8b1/Cd4/Cd3d/Zap70/Cd3e/Il2rg/Lck/Cd8a
mmu04658	Th1 and Th2 cell differentiation	Lat/Cd4/Cd3d/Runx3/Zap70/Cd3e/Il2rg/Lck/Il2rb/Cd3g/Ppp3cc
mmu04664	Fc epsilon RI signaling pathway	Lat/Derl2/Rac2/Pik3cd/Inpp5d/Fcer1g/Alox5ap/Vav1/Plcg2
mmu04666	Fc gamma R-mediated phagocytosis	Amph/Lat/Bin1/Was/Rac2/Pik3cd/Inpp5d/Hck/Vav1/Plcg2
mmu04640	Hematopoietic cell lineage	Cd5/Cd8b1/Il3ra/Cd4/Cd3d/Cd3e/Csf2ra/Cd7/Cd8a/Cd3g
mmu04062	Chemokine signaling pathway	Ccr3/Ccl27a/Cxcr3/Itk/Was/Adcy2/Rac2/Pik3cd/Xcl1/Plcb2/Prex1/Hck/Vav1

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