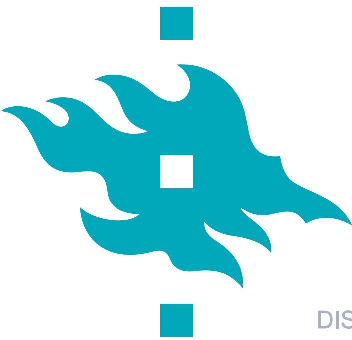
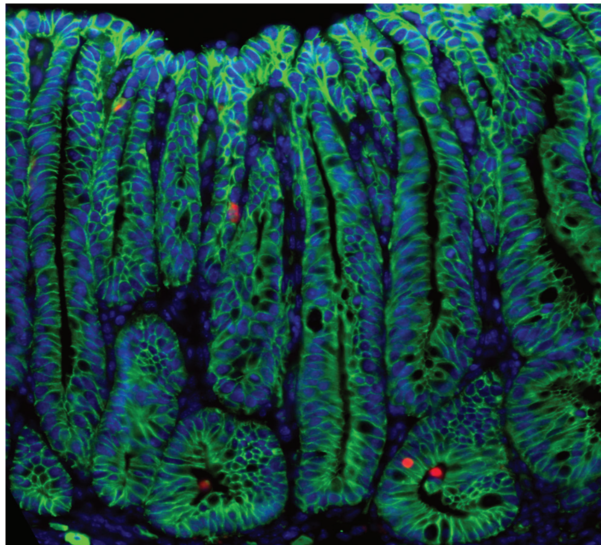


DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

MARIANNE LÄHDE

DISCOVERY OF A NOVEL FUNCTION FOR R-SPONDIN1 AS A SUPPRESSOR OF INTESTINAL ADENOMAS



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TRANSLATIONAL CANCER MEDICINE PROGRAM
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN BIOMEDICINE
UNIVERSITY OF HELSINKI

Faculty of Medicine
University of Helsinki

DISCOVERY OF A NOVEL FUNCTION FOR R-SPONDIN1 AS A SUPPRESSOR OF INTESTINAL ADENOMAS

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"Obstacles are those frightful things you see when you take your eyes off your goal."

- Henry Ford

To my family and friends

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

Lähde, M., Heino, S., Högström, J., Kaijalainen, S., Anisimov, A., Flanagan, D., Kallio, P., Leppänen, V-M., Ristimäki, A., Ritvos, O., Wu, K., Tammela, T., Hodder, M., Sansom, O.J., & Alitalo, K. (2021). Expression of R-Spondin 1 in *ApcMin/*+ Mice Suppresses Growth of Intestinal Adenomas by Altering Wnt and Transforming Growth Factor Beta Signaling. *Gastroenterology*, *160*(1), 245-259.

The thesis is further based on unpublished data.

ABBREVIATIONS

4-OH-Tam	4-hydroxytamoxifen
β-cat	β-catenin
AAV	Adeno-associated viral vector
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
BIM	Bcl2-like protein 11
BMP	Bone morphogenic protein
BRAF	v-Raf murine sarcoma viral oncogene homolog B
CAF	Cancer-associated fibroblasts
CBC	crypt base columnar cell
CIMP	CpG Island Methylator Phenotype
CIN	chromosoma instability
CRC	colorectal cancer
CTNNB1	β-catenin
DKK	Dickkopf
DLL	Delta-like ligand
EdU	5-ethynyl-2´-deoxyuridine
EGF	Epidermal Growth Factor
EMT	epithelial to mesenchymal transition
FAP	Familial adenomatous polyposis
FZD	Frizzled
GI	gastrointestinal
GPCR	G-protein-coupled receptor
hCRC	human colorectal cancer
ISC	intestinal stem cell
KRAS	Kirstin rat sarcoma virus
LEF	Lymphoid enhancer-binding factor
LGR	Leucine-rich repeat-containing G-protein-coupled receptor
LCR	label-retaining cell
LRP	LDL-receptor-related protein
MAPK	Mitogen-activated protein kinase
Min	Multiple intestinal neoplasia
MLH	mutL homologue
MMR	Mismatch repair
mRNA	Messenger RNA

MSH	mutS homologue
MSI	microsatellite instability
MSX1	Msh homeobox 1
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellucar domain
PI3K	phosphatidylinositide 3-kinase
PROX1	Prospero-related homeobox 1
p21	protein 21/ cyclin-dependent kinase inhibitor 1
RAS	rat sarcoma virus
RNF43	Ring finger protein 43
RSPO	R-spondin
RT-qPCR	real-time quantitive polymerase chain reaction
scRNA-seq	single-cell RNA-sequencing
SMAD	Small / Mothers Against Decapentaplegic
TA	transient-amplifying
TCF	T-cell factor
TCGA	The cancer genome atlas
TGFβ	Transforming growth factor beta
TGFβR1	Type 1 TGFβ receptor
TGFβR2	Type 2 TGFβ receptor
TGFβRi	Transforming growth factor beta receptor kinase inhibitor
TNF-α	Tumor necrosis factor alpha
TP53	Tumor protein 53
UMAP	uniform manifold apporximation and projection
vp	virus particle
Wnt	Wingless-related integration site
WT	wild type
ZNRF3	Zink and ring finger protein 3

ABSTRACT

Colorectal cancer (CRC) is the third leading diagnosed malignancy and the fourth most common cause of cancer-related mortality globally. CRC burden has been expected to increase by 60% within the next decade. Up to every third of the patients die from the disease despite the improvements in the CRC therapy. However, knowledge gained in understanding the molecular subtypes of the CRC has improved the ability to target CRC treatment strategies and to predict the treatment outcomes. Aberrant activation of the Wnt//β-catenin pathway drives intestinal tumorigenesis, often as a result of loss-of-function mutation of the tumor suppressor adenomatous polyposis coli (*APC*) gene. APC inactivation leads to disrupted proteasomal degradation of cytoplasmic β-catenin, which accumulates in the cytoplasm. This leads to dislocation of β-catenin into the nucleus, where it binds to transcription factors of the TCF/LEF family and initiates the transcription of target genes related to augmented cell cycle and tumorigenesis, such as Prospero homeobox protein 1 (*PROX1*), which has been shown to induce dysplasia and an invasive phenotype in CRC.

R-spondins (RSPO1-3) are ligands that bind to leucine-rich G-protein-coupled receptors (LGR4-6) that promote Wnt signaling. Most of the RSPOs enhance the growth of intestinal stem cells (ISCs) in healthy mice. Furthermore, RSPO gene fusions were recently identified in human (h)CRC. Thus, inhibition of RSPOs was considered to suppress intestinal tumorigenesis. However, a recent report suggested that RSPO2 expression level negatively correlates with intestinal tumor cell differentiation, size, and metastasis. RSPO2 expression was also downregulated via promoter hypermethylation in hCRC, suggesting that RSPO2 would have a tumor-suppressive role in hCRC. One study suggested that LGR5 could enhance transforming growth factor β (TGFβ)/SMAD signaling pathway upon RSPO1 stimulation in two tested hCRC cell lines. In general, the role of TGFβ/SMAD signaling in CRC is exceptionally complex, as it can turn from tumor suppressive to pro-metastatic throughout the progression of adenomas to carcinomas. Thus, the role of RSPO in intestinal tumorigenesis remains controversial.

I was interested in analyzing if exogenous Wnt-signals, especially RSPO1, could affect PROX1 expression induced by β-catenin activation in the early phase of intestinal tumorigenesis. For this analysis, we generated adeno-associated virus vectors (AAV) to induce systemic RSPO1 expression in mice. I then used *Apc*-deficient $Apc^{Min/+}$ mice to investigate the effect of exogenous RSPO1 on intestinal

adenomas, and surprisingly found that instead of enhancing intestinal tumorigenesis, RSPO1 functions as a suppressor of Wnt/ β-catenin signaling in *Apc* mutant adenoma cells and concomitantly activates the TGFβ/SMAD signaling pathway, leading to apoptosis and growth inhibition of the adenoma cells. Thus, RSPO1 treatment leads to regression of intestinal adenomas in the $Apc^{Min/+}$ mice, resulting in significantly increased survival of the mice.

My thesis provides a novel insight into the regulation of intestinal stem cells in intestinal adenomas. Our data reveal a dual role of RSPO1 in modulating Wnt signaling, proliferation, and apoptosis in the intestinal stem cells. Furthermore, RSPO1 regulates the competition equilibrium between healthy intestinal stem cells and *Apc*-mutated tumor stem cells. These findings revise and expand our knowledge on the signaling pathways that regulate tumor progression and they could provide possibilities for the development of novel cancer treatment strategies.

INTRODUCTION

The human intestinal epithelium self-renews every 4-5 days, thus being the fastest self-renewing tissue in the body (Van Der Flier and Clevers, 2008). Hence, it has become an exquisite model for adult stem cells studies (Clevers, 2013). The intestinal epithelium comprises stem cell-containing proliferative crypts and villi, consisting of several differentiated cell types (Van Der Flier and Clevers, 2008).

Physiological Wnt signaling has been shown to play a critical role in maintaining the self-renewal capacity of the intestinal epithelium and of the crypt stem cell niche, consisting of Paneth cells that provide the intestinal stem cells essential paracrine Wnt, Notch, and EGF signals (Sato *et al.*, 2011). The transmembrane protein Leucine-rich repeat-containing G-protein-coupled receptor (Lgr5) is a cell surface component that has been shown to mark actively cycling stem cells at the bottom of the intestinal crypt (Barker *et al.*, 2007). Furthermore, in intestinal adenomas, the Lgr5+ stem cells act as adenoma stem cells, fueling the growth of the tumor (Schepers *et al*., 2012). Importantly, Lgr5 binds R-spondins that promote Wnt-signaling (de Lau *et al*., 2011).

CRC is one of the leading causes of cancer-related mortality and morbidity in the Western world. Environmental and genetic risk factors promote CRC initiation, but only 5-10 % of CRCs have a robust inherited component (Peters *et al*., 2015). Pathogenesis of both sporadic and inherited forms of CRC depend on mutations in well-known oncogenes or tumor suppressor genes, such as *APC*, *TGFβ*, Kirsten rat sarcoma viral oncogene (*KRAS*), and tumor protein 53 (*TP53*) (Fearon, 2011). Traditionally, the initial steps of the CRC have been considered to occur via an adenoma-carcinoma sequence, also known as the Voglogram, in which specific, stepwise mutations in the genes controlling cell renewal, growth, and apoptosis, lead to progression of the tumor (Fearon and Vogelstein, 1990). However, around a decade ago, due to a thorough genetic and epigenetic analysis, hCRC was divided into three major molecular subtypes with distinct features contributing to their treatment responses (Shen *et al.*, 2007; Jass, 2007).

This study focused on mouse intestinal adenomas that commonly have *Apc* mutations and enhanced Wnt signaling compared to the normal intestinal epithelium. We wanted to further analyze Wnt signaling regulation via the Lgr5/R-spondin signaling module and its effect on Wnt target gene expression. We sought to investigate the impact of exogenous RSPO1 on the expression of the Wnt target PROX1 in intestinal adenoma cells. Surprisingly, we discovered

a novel role for RSPO1 as a suppressor of intestinal adenomas, a finding that provides valuable insight into the mechanisms of the initiation of intestinal tumors. When considering the clinical relevance of these remarkable results, one must keep in mind the anterior dislocation of the intestinal tumors in mice compared to hCRC. However, I present a novel concept of modifying the competitive positions between intestinal epithelial cells and the adenoma cells by enhancing renewal of healthy tissue, while suppressing the growth of the adenoma cells. This concept provides a potential strategy for future cancer treatments.

The signaling patterns in the tumor development are highly complex, resulting from a network of different pathways with different roles in several phases of the tumorigenesis (**Figure 1**). This study focuses mainly on the Wnt signaling pathway, which must be considered when evaluating the data in different contexts. Nevertheless, the crosstalk between Wnt and other major pathways has been assessed, and even novel data regarding these interactions have been represented.

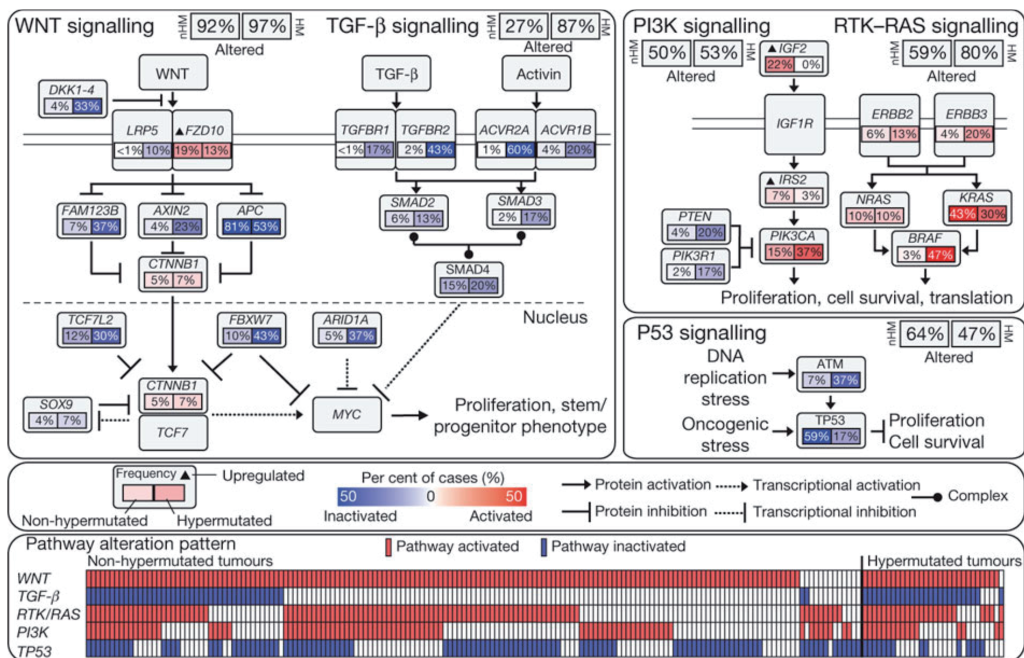

Figure 1. Frequency and variety of mutations leading to aberrant signaling activity in hCRC. A schematic describing the alterations in members of different signaling pathways involved in colorectal tumorigenesis based on a broad, comprehensive analysis of patient samples by The Cancer Genome Atlas Network (2012). 165 non-hypermutated (NhM) and 30 hypermutated (HM) tumors were analyzed. Alterations included somatic mutations, focal amplifications, homozygous deletions, and significant changes in the gene expression levels (for example, *IFG2*, *FZD10*, and *SMAD4*), and their frequencies are given as a percentage of all samples. Red color indicates upregulation and blue downregulation of the genes. TCGA, 2012.

REVIEW OF THE LITERATURE

1. STRUCTURE OF THE INTESTINAL EPITHELIUM

During embryonic gastrulation, the gastrointestinal (GI) tract is derived from the embryonic endoderm, which forms the gut tube further divided into foregut, midgut, and hindgut. These regions develop further to the different anatomical segments of the digestive tract. The small intestine and colon are derived from the midgut, whereas the hindgut gives rise to the distal colon. The small intestine is composed of three anatomical segments: duodenum, jejunum, and ileum. The specific timing and pattern of growth factor and transcription factor expression in the different regions of the endoderm determines the anterior-posterior-patterning of the epithelium and the entire GI tract. The BMP, Hedgehog, PDGF, TGFβ, and Wnt signaling pathways play essential roles in regulating the development and differentiation of the intestinal epithelium. (Noah *et al.*, 2011)

The intestinal wall consists of three layers: mucosa, submucosa, muscularis externa and serosa. The outermost layer is called the serosa. It contains a serous membrane (mesothelium), connective tissue, extensive blood and lymphatic vessels, and nerve trunks. Muscularis externa has two smooth muscle layers: circularly oriented cells and longitudinally oriented cells. The submucosa contains a dense, irregular connective tissue layer, including blood and lymphatic vessels, nerves, and glandular structures. The innermost layer, mucosa, comprises a thin epithelial layer, lamina propria, and muscularis mucosae. The mucosa is responsible for the main functions of the small intestine, namely the intestinal barrier and its absorptive and secretion functions. The absorptive area of the intestinal epithelium is significantly increased by surface projections of the mucosa and submucosa. These protrusions are called plicae circulares, villi, and microvilli. (Balcerzak *et al.*, 1970; Treuting *et al.*, 2003; Ross and Pawlina 2011) Submucosal invaginations called Lieberkühns crypts are present around the villi, and the intestinal stem cell compartment resides at the bottom of these crypts (Sancho *et al.*, 2003). The colonic epithelium lacks villi, but also contains crypts (Gehart and Clevers, 2019).

1.1. CELL LINEAGES

The human intestinal epithelium renews itself every 4-5 days; thus, it is the fastest renewing tissue in adults (Van Der Flier and Clevers, 2009). As indicated in

Figure 2, the intestinal stem cells, marked by expression of the transmembrane protein Lgr5, are located at the bottom of the intestinal crypts of Lieberkühn (Snippert *et al*., 2010). Approximately 15 crypt base columnar (CBC) cells function as intestinal stem cells at the bottom of each crypt. They proliferate approximately once in 24 hours and create a continuous flow of new, differentiating cells that migrate towards the tip of the villus (Gehart and Clevers, 2019). The CBC cells reside between Paneth cells that provide the stem cells with several essential growth factors (Gehart and Clevers 2019). A transient-amplifying (TA) zone is located above the stem cell zone. It contains lineage-specific progenitor cells derived from the intestinal stem cells. The TA-cells continuously divide to maintain the rapid intestinal epithelial cell turnover (Snippert *et al*. 2010; Gehart and Clevers 2019). At the crypt-villus junction, the TA cells are further differentiated into enterocytes, mucus-secreting goblet cells, enteroendocrine cells, Paneth cells, immune system-related tuft cells, or absorptive microfold cells (M cells) (van der Flier *et al*., 2009; Sato *et al*., 2011; de Lau *et al*., 2012). After reaching the tip of the villus, the cells undergo apoptosis and are replaced by advancing cells in a process called anoikis (in Greek: without house) (Gehart and Clevers 2019). In contrast to other mature intestinal epithelial cell types, upon their differentiation, the Paneth cells migrate to the bottom of the intestinal crypts, whereas the immune system-related M cells become translocated to the epithelium overlaying the Peyer's patches (immune cell clusters resembling lymph node stroma) (de Lau *et al*., 2012).

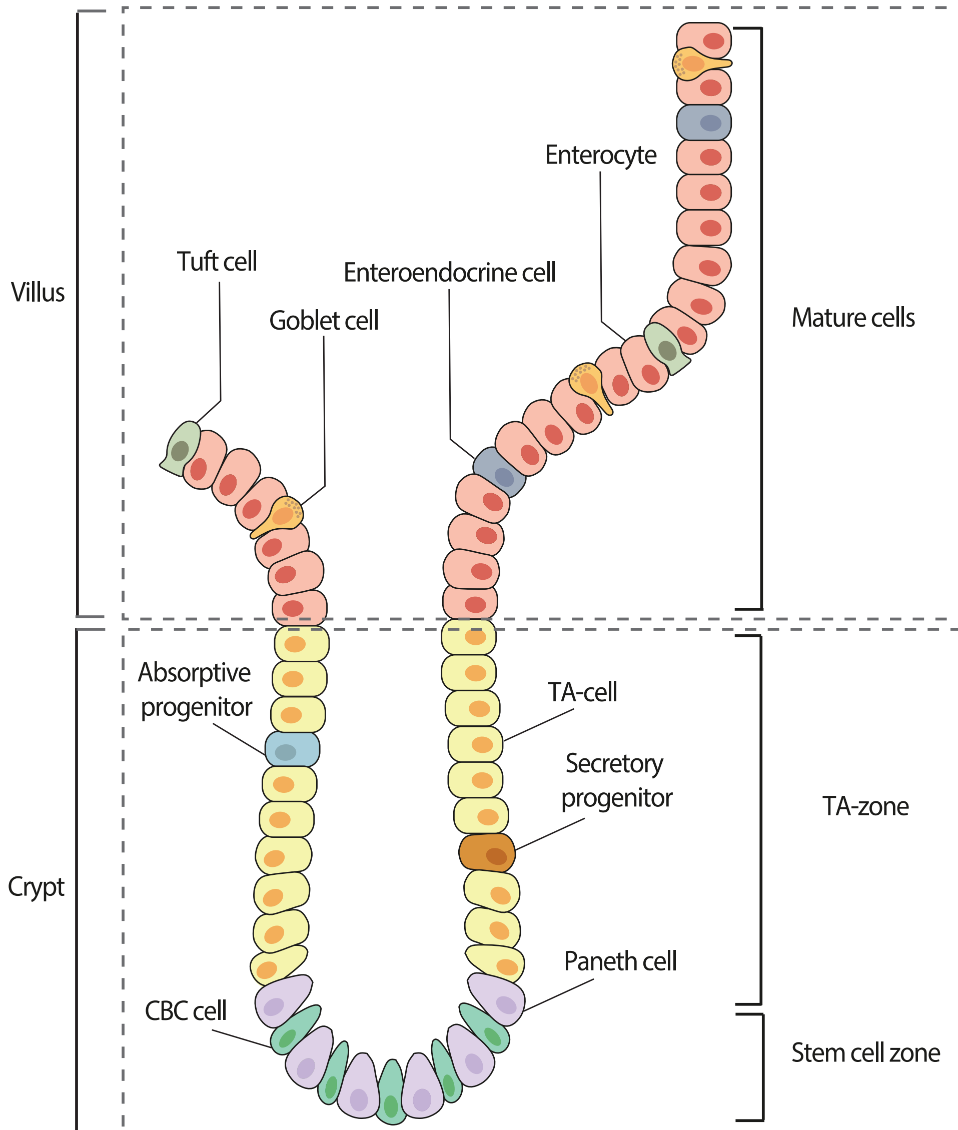

Figure 2. Cell lineages of the intestinal epithelium. The villi of the small intestine consist of mature intestinal epithelial cells. These cells include absorptive enterocytes, mucus-producing goblet cells, enteroendocrine cells, and Tuft cells. The intestinal crypt is composed of a stem cell zone and a transient-amplifying (TA) zone. The stem cell zone is located at the bottom of the crypt, and it consists of crypt base columnar (CBC) cells and Paneth cells. The CBC cells give rise to the intestinal cell lineages. First, CBC cells are differentiated into TA-cells and then into secretory and absorptive progenitors. Secretory progenitors are further differentiated into Tuft cells, Paneth cells, goblet cells, and enteroendocrine cells. Absorptive progenitors are differentiated into enterocytes. Modified from Gehart and Clevers, 2019.

1.2. STEM CELLS AND THEIR PLASTICITY

1.2.1. Crypt base columnar cells

A crucial feature of adult stem cells is their self-renewal capability while generating the constituent cell lineages in specific tissues (Beumer and Clevers, 2020). The CBCs have been the main focus of adult stem cell research since their discovery in the early 1970s (Cheng and Leblond, 1974; Gehart and Clevers, 2019). CBCs can generate cells of all intestinal epithelial cell lineages (Beumer and Clevers, 2020). After the discovery of CBCs, Barker *et al*. (2007) identified Wnt target gene Lgr5 as a specific marker for these cells (Baker *et al*., 2007). The Lgr5+ CBC cells proliferate continuously, and they have high telomerase activity, capable of maintaining undamaged chromosomal ends, leading to a seemingly limitless expansion capacity (Schepers *et al*., 2011). Located at the bottom of the intestinal crypts, the CBC cells are provided with signaling factors from other cells, such as Paneth cells, other epithelial cells, and stromal cells (Beumer and Clevers, 2020). These factors, including EGF, the Wnt signal-enhancing R-spondins, BMP antagonists, Notch ligands, and TGFβ, maintain the stemness of the CBC cells and regulate the fate of the cells differentiating from them (Beumer and Clevers, 2020).

1.2.2. +4 cells

Already at late the 70s, Potten suggested that, at position +4, just between the stem cells and the TA-zone in the crypt, reside the DNA label-retaining cells (LRCs), so-called +4 cells, which retain DNA-labels to their daughter cells via non-random segregation of their DNA strands (Potten *et al.* 1978, Potten *et al.* 2002). Several genes, such as *Bmi1, Tert, Hopx,* and *Lrig1,* have been suggested as markers for the +4 LRCs (Sangiorgi and Capecci, 2008; Breault *et al.* 2008; Montgomery *et al.,* 2011; Takeda *et al*., 2011; Powell *et al*., 2014; Wong *et al.,* 2012). However, also the Lgr5+ CBC cells have been reported to express the mentioned genes, and the expression patterns of these marker genes are notably more extensive than just +4 cells (Wong *et al.,* 2012; Muñoz *et al*., 2012; Wang *et al.,* 2013; Grun *et al*., 2015). The LRCs have been reported to cycle rarely and to function as reserve stem cells for Paneth cells during homeostasis (Marshman *et al.,* 2002; Potten *et al.* 2002; Gehart and Clevers 2019). Depletion of LGR5+ CBC cells with cytotoxic doxorubicin led to dedifferentiation of LRCs to LGR5+ stem cells (Buczacki *et al*., 2013). Furthermore, LRCs show resistance to radiation, and they can give rise to rapidly proliferating LGR5+ CBCs after irradiation-induced epithelial damage (Yan *et al*., 2012). The true nature of the

+4 cells remains controversial, yet their role in intestinal epithelial damage repair is significant (Wong *et al.,* 2012; Muñoz *et al.*, 2012; Wang *et al.,* 2013; Grün *et al.*, 2015).

1.2.3. Intestinal stem cell plasticity

The CBC cells are susceptible to epithelium-damaging insults, such as irradiation and toxins. Thus, the regeneration of intestinal epithelium after such damage can not depend on CBC cells. (Dekaney *et al.*, 2009; Tian *et al.*, 2011; Yan *et al.*, 2012). Depletion of Lgr5+ cells after damage leads to loss of intestinal crypts, inhibiting regeneration of the epithelium. (Dekaney *et al.*, 2009; Tiam *et al.*, 2011; van Es *et al.*, 2012) Despite this, Lgr5+ cells can be re-detected shortly after the injury, suggesting an alternative source for Lgr5+ stem cells (Metcalf *et al.*, 2014). Indeed, it has been shown that +4 cells, as well as lineage-committed progenitors, can be mobilized back into the empty stem cell niche and function as Lgr5+ CBC cells (Takeda *et al.*, 2011; Tian *et al.*, 2011; Van Es *et al.*, 2012 Yan *et al.* 2012). Even further differentiated cells, such as Prox1 and Bmi1 marked secretory cells, and Alpi+ absorptive progenitors can revert to stem cell state after epithelial damage (Muñoz *et al.*, 2012; Yan *et al.*, 2012; Tetteh *et al.*, 2016). Thus, the plasticity of stem cells and the dedifferentiation capacity of intestinal epithelial cells provide a crucial backup system to maintain the integrity of the epithelium.

1.2.4. Competition in the intestinal stem cell niche

The intestinal stem cells should be relatively well-protected from many carcinogenic compounds and mechanical stresses at the bottom of the intestinal crypt, yet they are still susceptible to mutations due to their high renewal rate. The stem cell competition at the crypt bottom protects the intestinal epithelium by limiting the capacity of a damaged cell to produce offspring (Gehart and Clevers, 2019). Since the cell divisions in the crypt have been convincingly shown to be symmetrical, the limited space at the bottom of the crypt creates competition between daughter cells with equal division potential, eventually leading to either dominance or loss of an individual clone (Lopez-Garcia *et al.*, 2010; Snippert *et al.*, 2010). Due to the constant flow of proliferating cells and the pressure created by the Paneth cells, a clone needs to quickly proliferate to maintain its position at the stem cell niche, as the cells with slower proliferation potential are pushed out from the crypt bottom and forced to differentiate (Gehart and Clevers, 2019). Furthermore, the cells are committed to differentiation as they exit the stem cell zone, and the expression of *Lgr5* gradually decreases as

the expression of the differentiation markers increase (Muñoz *et al.,* 2012; Ristma *et al.*, 2014).

Stem cells with oncogenic mutations often gain decreased proliferation fitness based on their delayed chromosomal segregation, irregular cell cycle, and increased apoptosis, due to which they are ultimately displaced from the stem cell niche (Gehart and Clevers, 2019). Thus, the cells propagating a tumor are required to obtain the capacity to divide outside of the stem cell niche before they are expelled from the stem cell zone. Under these circumstances, only the mutations that give the cells independence and enhance their proliferation capacity, such as activating mutations in the Wnt signaling pathway in 93% of the CRCs, are considered as possible “first hit” mutations in intestinal tumorigenesis; these can be found already in early intestinal adenomas (TCGA, 2012; Vermeulen *et al.*, 2013; Snippert *et al.*, 2014). Furthermore, a recent study indicated that *Apc*-mutant intestinal stem cells secrete Wnt antagonists, such as NOTUM, thus acting as supercompetitors by suppressing self-renewal of the neighboring wild type (WT) intestinal stem cells (Flanagan *et al.*, 2021; Van Neerven *et al.*, 2021). This results in the differentiation and outgrowth of the WT stem cells from the crypt, promoting fixation of the crypt to a mutant state (Flanagan *et al.*, 2021; Van Neerven *et al.*, 2021).

1.2.5. Intestinal cancer stem cells

Intestinal cancer is most commonly initiated by Wnt-signaling-activating mutations, such as *Apc* mutation in Lgr5+ stem cells, which gives rise to intestinal adenomas (Barker *et al.*, 2009). In addition, LRIG1-, BMI-, and PROM1-expressing cells could propagate early intestinal adenoma formation after aberrant activation of the Wnt signaling pathway (Sangiorgi *et al.*, 2008; Zhu *et al.*, 2009; Powell *et al.*, 2014). Thus, “cancer stem cells” have been considered to consist of a small population of tumor-initiating cells, being responsible for the proliferation and growth of the tumor (Barker *et al.*, 2009). Recently, however, additional studies have indicated that even further differentiated cells can give rise to an intestinal tumor, and deletion of *Apc* in TA-cells led to the formation of microadenomas, yet the progression to large adenomas was rarely detected (Barker *et al.*, 2009). In fully differentiated epithelial cells, aberrant Wnt signaling activity does not seem to be sufficient for the formation of an adenoma, yet further activation of NFκB signaling in these cells resulted in rapid tumor growth, pointing out the significant role of inflammation in intestinal tumorigenesis (Schwitalla *et al.*, 2013). Nevertheless, an inactivating mutation in *APC* leads to formation of abnormally positioned crypts along the villi, called

ectopic crypts, marked by the transcription factor msh homeobox 1 (MSX1), that progress to intestinal tumors (Horazana *et al.*, 2019).

2. SIGNALING PATHWAYS IN HEALTHY INTESTINAL EPITHELIUM AND TUMORIGENESIS

2.1. WNT SIGNALING PATHWAY

The Wnt signaling can be divided into three different pathways: canonical Wnt pathway, also known as the Wnt/β-catenin signaling pathway, the noncanonical planar cell polarity pathway, and the noncanonical Wnt/calcium pathway (Lojk and Marc, 2021). The canonical Wnt/β-catenin signaling pathway is the most studied and the best understood, and my thesis is focused on the canonical Wnt signaling cascade.

2.1.1 A brief history of Wnt signaling

During embryonic development and tissue maintenance, the Wnt pathway is one of the central intracellular signal-transducing cascades contributing to countless biological processes, and aberrant activation of the Wnt signaling pathway has been connected to a wide range of human pathologies, such as several types of tumors. The fly *Wingless* (*wg*) gene was first identified in *Drosphila melanogaster,* in which it controls segmentation during larval development, (Sharma, 1973; Nüsslein-Volhard and Wieschaus, 1980). The proto-oncogene *Wnt1* was first identified in mouse mammary tumors in the early 80s, and shortly after that, it was shown to be a homolog for *wg* (Nusse and Varmus, 1982; Risewijk *et al.*, 1987). The following studies identifying *porcupine, dishevelled*, and *armadillo* (β-catenin) uncovered a complex signaling cascade, later known as the canonical Wnt signaling pathway (McMahon and Moon, 1989; Siegfred *et al.*, 1992; Noordermeer *et al.* 1994; Peifer *et al.,* 1994). The role of Wnt signaling in carcinogenesis began to clarify, when a mutation of the *APC* gene was found to lead to a hereditary cancer syndrome, familial adenomatous polyposis (FAP) (Kinzler *et al*., 1991; Nishisho *et al.*, 1991), and shortly after that, the APC tumor suppressor protein was shown to associate with β-catenin in the cytoplasm (Rubinfield *et al*., 1993; Su *et al.*, 1993). In 1996, a significant advancement in Wnt signaling research occurred when the TCF/LEF transcription factors were shown to act as nuclear Wnt signaling mediators (Behrens *et al.*, 1996; Molenaar *et al.*, 1996). During the last few decades, the functions and components of the Wnt signaling pathway have been specified, and new features are constantly

being discovered. For example, despite its discovery as a mediator of transcriptional reprogramming of Wnt-activated intestinal cancer cells, the function of *Lef1* in intestinal tumorigenesis was only recently further analyzed. *Lef1* was shown to act as a tumor suppressor in mouse intestinal adenomas by restricting dedifferentiation of the adenoma cells to a crypt-progenitor phenotype and by inhibiting the formation of tumor stem cell niches (Heino *et al.*, 2021).

2.1.2. Basics of the Wnt signaling pathway

Wnt ligands are lipid-modified, poorly soluble proteins that bind to their receptors; frizzled and low-density lipoprotein receptor-related protein 5 (LRP5)/LRP6, forming heterodimeric complexes between the two (Beumer and Clevers, 2021) (Figure 2). In the absence of these complexes, cytoplasmic β-catenin undergoes proteolysis directed by the so-called β-catenin destruction complex, consisting of APC, AXIN, Casein kinase 1 alpha (CK1a), and Glycogen synthase kinase 3 beta (GSK3b) (Figure 2). Here, β-catenin is phosphorylated by GSK3b and then ubiquitinated by the E3 ubiquitin ligase b-transducin repeat-containing protein (b-TrCP), targeting β-catenin for proteasomal degradation (Clevers and Nusse, 2012). Meanwhile, in the nucleus, TCF/LEF transcription factors are complexed with histone deacetylases (HDACs) and Groucho, which inhibits transcription of Wnt target genes. The binding of Wnt ligands to their receptors Frizzled and LRP5/6 recruits Dishevelled (Dvl) proteins to the plasma membrane, which inhibit the formation of the cytoplasmic β-catenin destruction complex, leading to accumulation of β-catenin into the cytoplasm. β-catenin then translocates into the nucleus, where it replaces Groucho, associates with TCF/LEF transcription factors and their co-activators, such as Pygopus (PYG), B-cell CLL/lymphoma 9 protein (BCL9), and CREB-binding protein (CBP)/p300, and activates the expression of Wnt target genes, which are involved in several cellular processes, such as cell cycle regulation. (Kramps *et al.*, 2002; Zhen *et al.*, 2017)

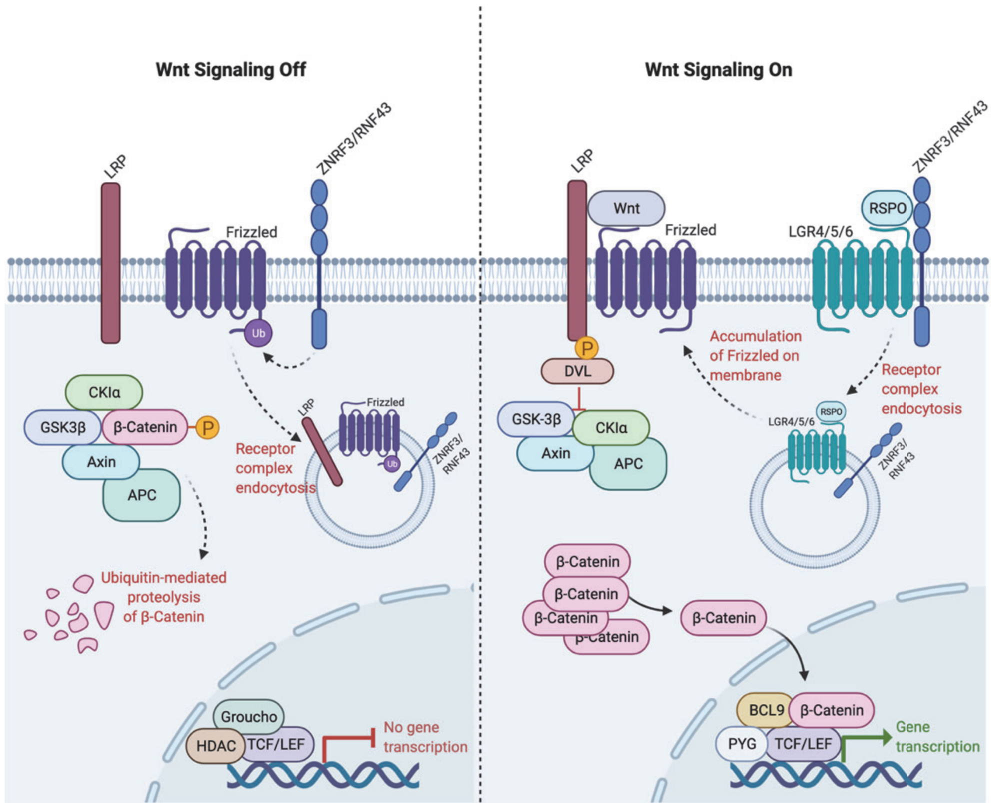

Figure 3. Canonical Wnt/β-catenin signaling pathway. When the Wnt ligands are absent, CK1α, GSK3β, Axin and APC form a complex and phosphorylate β-catenin which leads to ubiquitin-mediated proteolysis of β-catenin in the cytoplasm. In the nucleus, Groucho and HDAC complex with TFC/LEF and suppress their ability to activate transcription of the Wnt target genes. As Wnt ligand binds to FZD receptor, LRP is phosphorylated, followed by recruitement of Dvl to the plasma membrane, which leads to failure of the formation of the β-catenin destruction complex, and the same happens as APC is mutated. Because of this, β-catenin accumulates in the cytoplasm and eventually translocates into the nucleus, where it complexes with TCF/LEF, BCL9, and PYG, leading to activated transcription of Wnt target genes. In cells with active Wnt signaling, LGR receptors reside on the plasma membrane. RSPOs bind to LGR receptors, thus suppressing the function of ZNRF3 and RNF43 E3 ubiquitin-protein ligases, which leads to accumulation of Frizzled receptors at the cell surface and enhances Wnt signaling activity. Modified from Aros *et al.*, 2021. Created with BioRender.com.

2.1.3. R-Spondin proteins as modulators of Wnt signaling

The R-spondin (RSPO) family members are essential Wnt signal enhancers in multiple adult tissues and stem cells, including the intestine (Kim *et al.*, 2006). The RSPO family includes four members, RSPO1-4, which share 40-60 % of overall amino acid sequence homology. The RSPOs are composed of two successive N-terminal furin-like cysteine-rich domains, a thrombospondin domain, and a basic C-terminus (**Figure 4**) (Kim *et al.*, 2008). Although four members of the RSPO family have the potential to enhance Wnt/β−catenin activity, RSPO2 and 3 are more potent than RSPO1, and the activity of RSPO4 is relatively low (Kim *et al.*, 2008).

The E3 ubiquitin ligases Ring finger protein 43 (RNF43) and Zinc and ring finger protein 3 (ZNRF3) associate with LRP5/6 and Frizzled receptors, targeting them for lysosomal degradation whereby they inhibit Wnt signaling (Koo *et al.*, 2012). Whenever Wnt ligands are present, RSPOs can bind to any one of the LGR homologs LGR4/5/6 on the plasma membrane, leading to the formation of a protein complex that includes ZNRF3/RNF43, RSPO, and LGR4/5/6 (**Figure 3**) (Hao *et al.*, 2012; Kim *et al.*, 2008; de Lau *et al.*, 2011; Zebisch *et al.*, 2013). This prevents the function of RNF43 and ZNRF3 and inhibits the phosphorylation of Frizzled or LRP5/6, which leads to recruitment of Dvl (Kim *et al.*, 2008; de Lau *et al.*, 2011). Consequently, the formation of the β-catenin destruction complex is inhibited, resulting in amplified Wnt/β-catenin signaling (Kim *et al.*, 2008; de Lau *et al.*, 2011). Furthermore, LRP5/6 levels at the cell surface are regulated by the secreted Dickkopf 1-2 (DKK1-2) proteins, which are considered to inhibit Wnt signaling by inducing internalization of LRP5/6 via the formation of a complex with Kremen receptors, thus limiting the cell responses to canonical Wnt ligands. RSPOs have been shown to relieve this inhibition of the Wnt pathway by disrupting the DKK1/Kremen-mediated LRP6 internalization (Binnerts *et al.*, 2007).

In the healthy mouse intestine, RSPOs promote the proliferation and regeneration of the intestinal epithelial stem cells, leading to drastically deeper crypts, longer villi, and larger and longer intestines (Kim *et al.*, 2006). Furthermore, deletion of RSPO receptors *Lgr4* and *Lgr5* in conditional mouse models inhibits the RSPO-mediated Wnt-enhancement, resulting in significantly reduced proliferation of the intestinal stem cells and eventually, regression of the entire intestinal epithelium (de Lau *et al.*, 2011). The mesenchyme underlying the intestinal epithelium has been identified as an essential source of RSPOs, which provides

the intestinal stem cells with crucial signals for the maintenance and regeneration of the epithelium (Stzepourginski *et al.*, 2017)

Although the function of RSPOs in the maintenance of healthy intestinal epithelium is clear, the role of RSPO/LGR5 signaling in intestinal tumorigenesis is controversial. One study indicated that in hCRC, promoter hypermethylation could downregulate the expression of RSPO2 (Wu *et al.*, 2013). Furthermore, the RSPO2 expression level was negatively correlated with tumor size, differentiation, and metastasis (Wu *et al.*, 2013). In some hCRC cell lines, RSPO2 overexpression was shown to inhibit Wnt/β-catenin signaling and suppress proliferation by interacting with LGR5 and stabilizing ZNRF3 (Wu *et al.*, 2013). Another study reported that upon RSPO1 stimulation of two hCRC cell lines, LGR5 activated TGFβ signaling by forming a heteromeric complex with TGFβ type II receptor, thus enhancing the growth-suppressing and pro-apoptotic effects of the TGFβ/SMAD signaling pathway and reducing the growth of the tumor cells *in vitro* (Zhou *et al.*, 2017). On the other hand, endogenous RSPO gene fusions that result in chimeric oncoproteins have been shown to drive intestinal tumorigenesis in a Wnt-dependent manner *in vivo* (Han *et al.*, 2017). In some tumors, the *RSPO2* gene was shown to undergo genetic fusion with the gene encoding eukaryotic translation initiation factor 3 subunit E (*EIF3E*), whereas the *RSPO3* gene was most commonly fused with the gene encoding the protein tyrosine phosphatase receptor type K (*PTPRK*) (Seshagiri *et al.*, 2012). Such Wnt-enhancing fusions were found in approximately 10 % of hCRC tumors that did not obtain concomitant mutations in *APC* or *CTNNB1*, and they were suggested to promote colon tumor progression (Seshagiri *et al.*, 2012; Han *et al.*, 2017). In addition, the role of RSPOs appears to be significant in other cancers. In acute myeloid leukemia (AML), for example, the RSPO3-LGR4-interaction has been shown to act as a driver of the disease (Salin *et al.*, 2020). RSPO3 suppresses differentiation and enhances proliferation of the primary AML blasts, and RSPO3-specific antibody-mediatd inhibtion of RSPO3-LGR4 interaction restrics self-renewal and promotes differentiation of the patient-dervied AML xenografts without affecting normal hematopoietic stem cells (Salik *et al.*., 2020). Considering the evidence for RSPO promoter hypermethylation and reduced expression, chimeric RSPO proteins that drive tumorigenesis in some hCRCs, the role of RSPOs in intestinal tumorigenesis remains controversial.

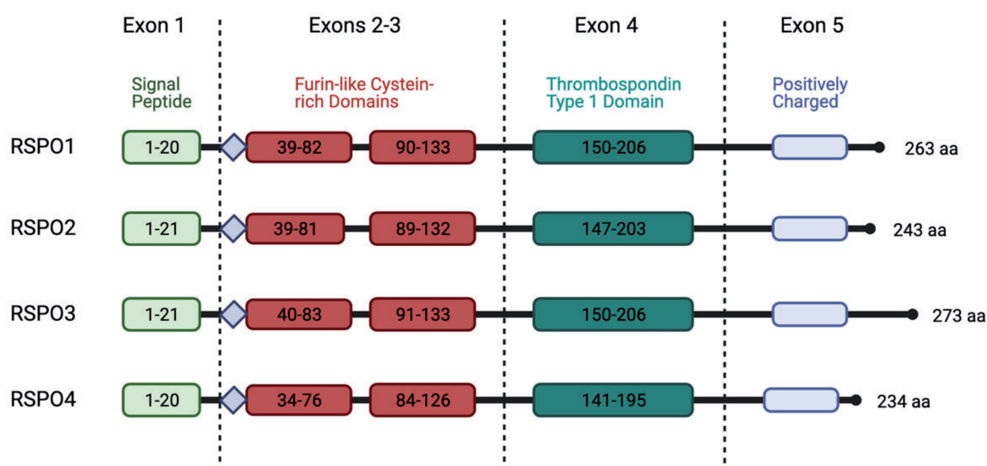

Figure 4. Structure of the RSPO proteins. Each member of the RSPO protein family consists of a signal peptide, two furin-like cysteine-rich domains, a thrombospondin type 1 domain and a positively charged C-terminal domain. The furin-like cysteine-rich domain sequences show 40-60% amino acid sequence homology between the family members. Modified from Kim *et al.*, 2008. Created with BioRender.com.

2.1.4. Wnt signaling in intestinal tumorigenesis

Aberrant activation of the Wnt signaling pathway is involved in tumorigenesis in the majority of CRC cases. In over 80% of CRCs, Wnt signaling is activated via loss-of-function mutations of the *APC* gene (The Cancer Genome Atlas (TCGA), 2012). Hereditary mutations of *APC* lead to a dominantly inherited autosomal disorder termed FAP. The FAP patients develop tens to hundreds of benign polyps in the colon and small intestine, and some of these eventually progress to intestinal cancer if left untreated (Groden *et al.*, 1991). Activating mutations in β-catenin (*CTNNB1*) and *FZD* genes and inactivating mutations in *AXIN2*, Dickkopf (*DKK*) family members*, LRP5*, *TCF7L2*, and *SOX9* have also been detected in hCRC (TCGA, 2012, see also **Figure 3**).

As previously described, the Wnt signal is mediated by a complex system of TCF/LEF transcription factors in the nucleus. The TCF/LEF family contains four members: TCF1, LEF1, TCF3, and TCF4, which are encoded by the *TCF7, LEF1, TCF7L1*, and *TCF7L2* genes, respectively. Alternative splicing of these members can generate isoforms that bind to or antagonize β-catenin (Hovanes *et al.*, 2001). In the healthy intestinal epithelium, TCF1 is expressed at the crypt

bottom, whereas TCF4 is expressed along the crypt-villus axis; lack of *Tcf4* in neonatal mice leads to a complete depletion of the proliferative crypts (Korinek *et al.*, 1998; Van Es *et al.*, 2012). During intestinal tumorigenesis, TCF1, TCF4, and LEF1 transcripts increase (Hovanes *et al.*, 2001). Interestingly, LEF1 is the only family member expressed exclusively in the intestinal adenomas instead of healthy intestinal epithelium (Hovanes *et al.*, 2001). Surprisingly, deletion of *Lef1* in *Apc* mutant mice leads to a massively increased number of intestinal adenomas, suggesting that LEF1 behaves like a tumor suppressor during the progression of intestinal tumors (Heino *et al.*, 2021).

The Prospero-related homeobox-1 (*PROX1*) gene encodes a transcription factor evolutionally highly conserved among the vertebrates (Elsir *et al.*, 2012). PROX1 contains a unique homeodomain in the C-terminus with no direct sequence homology with other homeodomains, distinguishing it from other homeobox family members (Elsir *et al.*, 2012). The PROX1 far upstream enhancer possesses a binding site for TCF/LEF transcription factors, thus being directly regulated by the Wnt/β-catenin signaling pathway (Petrova *et al.,* 2008). PROX1 has been shown to drive dysplasia and malignant phenotype of hCRC by regulating cell polarity, cell-cell interactions, and cell adhesion (Petrova *et al.,* 2008). In the healthy intestinal epithelium, PROX1 is expressed mainly in enteroendocrine cells, which have been shown to function as reserve stem cells, yet the deletion of *Prox1* has no impact on homeostasis of the healthy intestinal epithelium (Petrova *et al.*, 2008; Yan *et al.*, 2017). However, most hCRC cells express PROX1, and deletion of *Prox1* in mouse intestinal epithelium decreases the growth of intestinal adenomas (Petrova *et al.*, 2008). In addition, PROX1 silencing suppresses the growth of Wnt-high hCRC metastases in immunodeficient mice (Ragusa *et al.*, 2014). Furthermore, PROX1 increased tumor initiation and proliferation by acting on tumor cells expressing LGR5 (Wiener *et al.*, 2014). Thus, PROX1 appears to be a critical factor in the development of Wnt-dependent intestinal tumors.

2.2. TGFB PATHWAY

2.2.1. TGFβ pathway in healthy intestinal epithelium

Secreted growth factors of the TGFβ superfamily are ubiquitously expressed in several tissues, and they are crucial for several cellular processes, including embryogenesis, gastrulation, organogenesis, cell differentiation, migration, adult tissue self-renewal, and apoptosis (Weiss and Attisano, 2012). The TGFβ family includes over 30 members, such as TGFβs, Activins, Nodals, Growth and

Differentiation Factors (GDFs), Bone Morphogenic Proteins (BMPs), and Anti-Müllerian Hormone (AMH) (Weiss and Attisano, 2012). In the TGFβ signaling cascade, these ligands bind to their specific, dimeric transmembrane TGFβ type II (TGRβR2) serine-threonine kinase receptors, which recruit the dimeric TGFβ type I receptor (TGFβR1), leading to the formation of a hetero-tetrameric complex and phosphorylation of TGFβR1 (**Figure 5**) (Wrana *et al.*, 1992). The activated receptor complex signals by phosphorylating SMADs transcription factors (Sancho *et al.*, 2004). Based on the different functions of SMADs, they can be divided into receptor-regulated R-SMADs (SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8), common SMAD (SMAD4), and inhibitory SMADs (SMAD6 and SMAD7) (Sancho *et al.*, 2004). The activated TGFβR1 phosphorylates R-SMADS, and the phosphorylation is regulated by the endosomal proteins zinc-finger FYVE domain-containing protein 9 (ZFYVE9, also known as SARA) and endofin (David and Massague, 2018). SMAD4 associates with activated R-SMADs and translocates to the nucleus, where the complex regulates the TGFβ target genes (Sancho *et al.*, 2004). Inhibitory SMADs compete with R-SMADs for binding to TGFβR1 to repress TGFβ signal transduction (Sancho *et al.*, 2004). In addition, activated TGFβ receptors transduce signals by interacting with mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinases (ERKs) and p38 MAPK, Rho GTPases, Ras, and the phosphatidylinositide 3-kinase/Akt (PI3K/AKT) pathway (Zhang, 2017). In the healthy intestinal epithelium, TGFβs are secreted mainly by the stromal cells, and they are considered tumor suppressors regulating cell cycle arrest and apoptosis of the intestinal epithelial cells (Shi and Massague, 2003; Jung *et al.*, 2017).

BMPs bind to type II BMP receptors, resulting in dimerization of type I and II BMP receptors and phosphorylation of rSMADs. The rSMADs then form heterodimeric complexes with SMAD4 and translocate into the nucleus, where they regulate the target gene expression. BMPs regulate the differentiation of the intestinal stem cells together with WNT signals in the intestinal crypt. Stem cell-maintaining WNT proteins and RSPOs are secreted mainly by intercrypt and intervillus mesenchymal cells and Paneth cells at the bottom of the intestinal crypts; thus, WNT signal transfer is based mainly on direct cell-to-cell contact. This leads to a gradual decrease of WNT signals when the cells move further up along the crypt epithelium. On the other hand, mesenchymal cells also secrete differentiation-inducing BMPs which counteract the proliferation-promoting WNT signals. Thus, BMP antagonists, such as Noggin, are secreted by the mesenchymal sells, forming a gradient opposite to the WNT gradient. At the crypt bottom, the BMP antagonists prevent BMP-mediated singnaling, resulting

in increased BMP activity upwards from the crypt bottom. These gradients are essential in regulating the differentiation and fate of the intestinal stem cells, thus being responsible for the maintenance of homeostastis of the intestinal epithelium. (Gehart and Clevers, 2019)

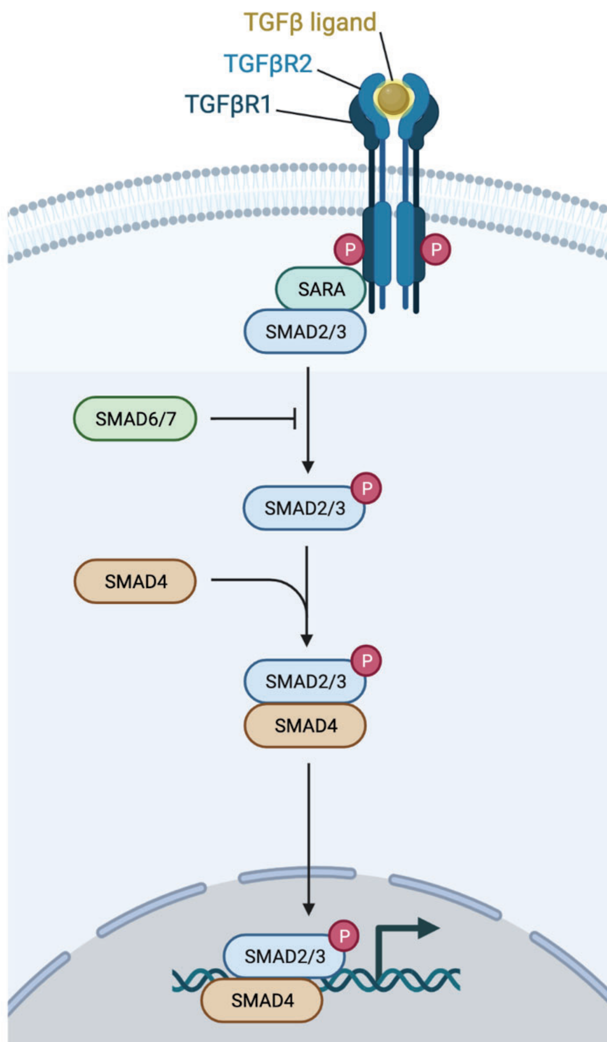

Figure 5. Simplified overview of the TGFβ signaling pathway. The binding of a TGFβ ligand to its specific TGRβ type II receptors on the plasma membrane leads to the recruitment of the TGFβ type 1 receptors and the formation of a hetero-tetrameric complex. In addition, TGFβ type 1 receptors are phosphorylated and activated, which is regulated by SARA. The activated TGFβR1 phosphorylates R-SMADs, which is further associated with SMAD4. The R-SMAD/SMAD4 complex is then translocated into the nucleus, inducing transcription of the TGFβ target genes. Inhibitory SMADS, SMAD6, and SMAD7 can suppress TGFβ signaling by competing with R-SMADS for TGFβR1 binding. Modified from Shi and Massague, 2003. Created with BioRender.com.

2.2.2. TGFβ signaling in intestinal tumorigenesis

Aberrant TGFβ signaling has been discovered in numerous pathologies, such as CRC, and loss-of-function mutations in the TGFβ components, most commonly in *TGFBR2* or *SMAD4*, have been found in 27 % of the non-hypermutated and 87% of hypermutated CRCs (Batlle and Massague, 2012; TCGA, 2012). An exceptionally high frequency of *SMAD4* mutations occurs in hCRCs with a poor prognosis (Lampropoulos *et al.*, 2012). In the early phases of intestinal tumorigenesis, TGFβ functions as a tumor suppressor, inhibiting proliferation and inducing apoptosis of the pre-malignant cells (Batlle and Massague, 2019). As the tumor progresses and additional mutations accumulate, the cells become insensitive to the growth-inhibiting effect of the TGFβ (David and Massague, 2018). Instead, in the more advanced tumors, the TGFβ pathway starts to promote tumor progression. Notably, in the presence of a KRAS mutation, the CRC cells become resistant to TGFβ-induced apoptosis mediated by downregulation of Bcl-2-like protein 11 (Bim) (Wiener *et al.*, 2014). In addition, TGFβ induces SMAD-independent signaling pathways, such as MAPK, ERK, PI3K/AKT, and Wnt pathways, especially during epithelial to mesenchymal (EMT), enabling carcinoma cell invasion (Derynck *et al*., 2014). One of the mechanisms by which TGFβ promotes malignancy works via the mesenchymal cells as an intermediate, whereby TGFβ stimulates interleukin 11 (IL-11) expression in cancer-associated fibroblasts (CAF) in the tumor stroma, and this, in turn, promotes epithelial tumor cell growth and invasiveness (Calon *et al*., 2012). In general, the TGFβs promote CRC invasion, angiogenesis, and metastasis (David and Massague, 2018). As in advanced tumors, the TGFβs secreted by the tumor-associated stromal cells promote tumorigenesis and immune evasion by inhibiting T-cell recruitment to the tumors (Tauriello *et al*., 2018). Thus, inhibition of TGFβs has been attempted as a novel treatment strategy in advanced CRC.

2.3. OTHER SIGNIFICANT PATHWAYS

2.3.1. KRAS and MAPK/ERK pathway

Oncogenes of the rat sarcoma (RAS) family, KRAS, HRAS, and NRAS were first identified in the 60s in rat sarcoma viruses, and their homologs were later detected to be a part of the normal human genome (Harvey, 1964; Kirsten and Mayer, 1967; Chang *et al.*, 1982). After that, the role of RAS in different human cancers has been clarified. RAS proteins are small GTPases located at cellular membranes, and their aberrant activation has been shown to contribute to

increased proliferation and progression of a variety of tumors (Gimple and Wang, 2019). Activating mutations in the RAS family members are found in about 50% of hCRCs, KRAS being the most significant with a 37% share (TCGA, 2012; Gimple and Wang, 2019). In normal cells, activated forms of receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and integrins can activate the RAS signaling cascade (Gimple and Wang, 2019). The normal KRAS protein transduces signals from, e.g., the epidermal growth factor receptor (EGFR), activating different cell growth, promoting downstream cascades, such as PI3K and the MAPK/ERK pathways (Gimple and Wang, 2019). The binding of EGF to its receptor activates RAS by leading to an exchange of the RAS-bound GDP to GTP, which stimulates downstream phosphorylation and activation of RAF, MEK, and eventually MAPK/ERK (Gimple and Wang, 2019). The MAPK/ERK complex then translocates into the nucleus, leading to activation of RAS target genes, such as *c-MYC, c-FOS,* and *c-JUN* (Gimple and Wang, 2019). In the *Apc*-mutant intestinal tumors, *Kras* mutation was shown to increase resistance to growth-suppressing effects of the TGFβ via activation of the p38 of the MAP kinase family (Wiener *et al.*, 2014). Furthermore, driver mutations in the MAPK/ERK pathway members have been found in about 60% of the hCRCs (TCGA, 2012).

From the clinical perspective, the KRAS mutations play a significant part when choosing treatment strategies since mutation in KRAS leads to resistance to EGFR tyrosine kinase inhibitors, such as cetuximab and panitumumab, which are nowadays considered as first-line chemotherapy agents in the treatment of advanced CRC and would otherwise have high curative efficacy (Li *et al.*, 2020).

2.3.2. p53 pathway

The p53 protein is a crucial tumor suppressor of the human cells, and its mutations occur in various cancers (Beckerman and Prives, 2010). In healthy, homeostatic cells, the p53 activity is suppressed by an E3 ubiquitin ligase, mouse double minute 2 homolog (MDM2) which targets p53 to degradation (Haupt *et al.*, 1997; Honda *et al.*, 1997). In cells under stress, the suppression of p53 is relieved, which alters gene expression and can lead to cell cycle arrest, autophagy, apoptosis, or cell senescence (Beckerman and Prives, 2012).

In hCRCs, loss-of-function mutations of *TP53* were detected in 60% of non-hypermutated tumors (TCGA, 2012). Mutations of p53 are associated with progressed CRCs, and they are rarely present in intestinal adenomas (Iacopetta *et al.*, 2006). Due to its loss of function, the strict regulatory impact of p53 on the

cells is lost, and the cell growth escapes out of control, leading to aggressive progression of the tumor. In addition to inactivating mutations of *TP53*, some gain-of-function modifications have been suggested to enhance malignant progression, invasion, and metastasis by reinforcing stemness, modulating chromatin states, and increasing resistance to treatments (Muller *et al.*, 2013; Xu *et al.*, 2014; Zhu *et al.*, 2015; Solomon *et al.*, 2018).

2.3.3. Notch pathway

The Notch pathway is critical in regulating cell fate, including cell differentiation, proliferation, and apoptosis in numerous tissues (Hori *et al.*, 2013). Notch signaling involves Notch 1-4 transmembrane receptor proteins that consist of the Notch extracellular domain (NECD) and the transcriptionally active Notch intracellular domain (NICD), plus their ligands Delta-like 1, 3, and 4 (Dll1, Dll3, Dll4), and Jagged (JAG). Notch ligands induce cleavage of the extracellular domain by an ADAM metalloprotease, which enables a further cleavage of the Notch transmembrane domain by γ-secretase, leading to the release of NICD into the cytoplasm (Hori *et al.*, 2013). NICD then translocates into the nucleus, where it interacts with DNA-binding proteins and transcriptional co-activators to induce the expression of Notch target genes (Hori *et al.*, 2013). In the healthy intestine, the Notch signaling pathway regulates intestinal stem cell differentiation (Fre *et al.*, 2009). However, in the CRC cells, it has been shown to increase stemness and drive tumorigenesis (Reedijk *et al.*, 2008). Aberrant Notch signaling and Notch target gene upregulation has been detected in different stages of intestinal cancer, and Notch signaling has been shown to interact with the Wnt signaling pathway during the intestinal tumorigenesis (Reedijk *et al.*, 2008; Fre *et al.*, 2009; Jin *et al.*, 2017; Högström *et al.*, 2018).

3. MOLECULAR SUBTYPES OF COLORECTAL CANCER

In their “Voglogram”, Fearon and Vogelstein presented already in 1990, a stepwise sequence of mutations in CRC; these affect genes that control cell proliferation and apoptosis, such as *APC*, *KRAS*, and *Tp53*. This adenoma-carcinoma progression sequence was adopted to describe the progression of benign intestinal neoplasms to adenocarcinomas. Mutations in the DNA mismatch repair (MMR) genes resulting in microsatellite instability (MSI) enhanced critical mutations in the CRC progression. Then, around a decade ago, further integrated epigenetic and genetic analysis of extensive hCRC tumor

collections resulted in CRC classification to major subtypes characterized by distinct molecular profiles (Shen *et al.*, 2007). During such studies, concordant methylation of the 5’-CG-3’ (CpG) dinucleotides in the promoter regions of multiple genes revealed a phenotype called the CpG island methylator phenotype (CIMP), in which specific genes are silenced by promoter hypermethylation (Shen *et al.*, 2007). Based on CIMP and MSI, a classification of tumors into three major molecular subtypes was suggested, along the chromosomal instability (CIN) phenotype, MSI phenotype, and the CIMP phenotype (Jass, 2007). However, the pathways are not mutually exclusive, as the tumors often show features of several pathways. Furthermore, the hereditary CRC syndromes, covering around 5% of the CRC cases, represent distinct subtypes (Al-Sohaily *et al.*, 2012).

3.1. THE CIN PHENOTYPE

Around 70 % of sporadic CRCs are initiated via the conventional CIN pathway, which includes the most common mutations in *APC*, followed by additional mutations in, for example, *KRAS*, *SMAD4*, *BRAF*, and *PIK3CA* (Fearon, 2011). These mutations increase the chromosomal instability, often leading to a disrupted DNA damage response, failure of chromosomal segregation during mitosis, telomere instability, and loss-of-function mutations in checkpoint genes, such as *Tp53*, which further promote tumor progression (Pino and Chung, 2010). The CIN tumors frequently show microsatellite stability and low levels of CIMP, plus a relatively good response to 5-fluorouracil (5-FU) based chemotherapy (Schmitt and Greten, 2021).

3.2. THE CIMP PHENOTYPE

The CIMP pathway is responsible for 20 % of CRCs (Dienstmann *et al.*, 2017). CIMP-positivity is defined by methylation of at least three of the *IFG2*, CACNA1G, *NEUROG1*, *RUNX2*, and *SOSC1* marker genes (Laird *et al.*, 2006). The CIMP+ tumors can be further divided into two subclasses: the $\text{CIMP}^{\text{high}}$ tumors often include mutations of *BRAF* and methylation of *MLH1* (see below), whereas the CIMP^{low} tumors are commonly associated with *KRAS* mutations (Shen *et al.*, 2007). Distinct characteristics of the CIMP+ phenotype include a proximal location of the tumor in colon ascendens, female gender, and old age (Al-Sohaily *et al.*, 2012). The tumors are often poorly differentiated, of singet finger or mucinous histology, and resistant to 5-FU based adjuvant chemotherapy (Nosho *et al.*, 2008). The precursors of these tumors are typically flat or

minimally elevated sessile serrated adenomas (SSA), why they are often missed in endoscopy (Al-Sohaily *et al.*, 2012).

3.3. THE MSI PHENOTYPE

The MSI pathway is the driving force in about 10 % of CRCs (Al-Sohaily, 2012). This molecular type includes defects in genes involved in DNA mismatch repairs (MMR), such as mutL homologue1 (MLH1), MLH3, mutS homolog 2 (MSH2), MSH3, and MSH6. Loss of MMR control leads to accumulation of errors, such as shot insertions or deletions and single-base mismatches, during the DNA replication cycles, eventually resulting in occurring microsatellite repeats of one to six base pairs in the DNA sequence and hypermutability of the genome (Dienstmann *et al*., 2017). Typical features of MSI tumors include occurrence in older women, location in the ascending colon proximal to flexura lienalis, increased lymphocyte infiltration resembling a Crohn's disease-like reaction, and poorly differentiated and of mucinous phenotype (Jass, 2004). These tumors are prone to resistance to 5-FU and cisplatin-based chemotherapies (Al-Soheily, 2012).

4. TUMOR MICROENVIRONMENT IN COLORECTAL CANCER

The tumor microenvironment (TME) is composed of extracellular matrix (ECM) and tumor-surrounding non-neoplastic cells, such as fibroblasts and cancer-associated fibroblasts (CAFs), neuroendocrine, adipose, and immune cells, and cells of the vascular and lymphatic networks (Li *et al*., 2020). The TME exchanges signals with the tumor cells, providing them with growth factors, cytokines, chemokines, and matrix remodeling, and inflammation-associated enzymes, which support the malignant progression and affect therapy responses. Thus, the tumors have recently been recognized as a kind of whole organs composed of cells of mesenchymal and hematopoietic origin plus non-cellular components (Pereira *et al.*, 2021).

The significance of the components of the TME distinct from the actual cancer cells has become increasingly evident during the past years. For example, CAFs activated by the tumor cells affect tumor progression by remodeling ECM, by enhancing tumor cell proliferation via secretion of growth factors, induction of angiogenesis, production of immunosuppressive cytokines, and recruitment or inhibition of inflammatory cells (Pereira *et al*., 2021). Endothelial cells (ECs)

receive angiogenic signals, such as vascular endothelial growth factor (VEGF), from the TME, which enhances the neovascularization in the tumors, enabling more efficient transport of oxygen and nutrients to the cancer cells. Immune cells, such as tumor-associated macrophages (TAMs), can enhance or inhibit tumorigenesis (Swann *et al.*, 2008).

Inflammation and cellular immunity have been shown to play an important role in CRC development. Chronic tissue inflammation has been considered a cancer risk factor and inducer of mutations and growth-supporting cytokines, angiogenic factors, and growth factors (Hanahan and Weinberg, 2011; Landskron *et al.*, 2014). Among these pro-inflammatory factors are interleukin 1 b (IL-1b), IL-6, and tumor necrosis factor alpha (TNF-α). TGFβ, the Janus kinases/signal transducer and activator of transcription proteins (JAK/STAT), and Wnt pathways are involved in regulating inflammation in tumor tissue and its pro-tumorigenic impact in CRC (Pereira *et al.*, 2021).

Modifying the stromal landscape has been considered as a potential target for cancer therapeutics. For example, the TGFβ pathway has been suggested to play a pivotal role as a landscaper of the tumor microenvironment (Gruel *et al.*, 2020). This was first evidenced in Peutz-Jeghers hamartoma-like lesions in Lkb1 deficient mouse intestine by Katajisto *et al.*, who found that Stk11-deficient mutant mesenchymal cells produced less TGFβ, and defective TGFβ signaling to epithelial cells promoted epithelial proliferation (Katajisto *et al.*, 2008). Peutz-Jeghers-type mutations in the Stk11/Lkb1 genes thus provide an example of "landscaping" mutations that occur in cells of the surrounding tissue stroma; these can create a microenvironment that facilitates tumorigenesis (Katajisto *et al.*, 2008).

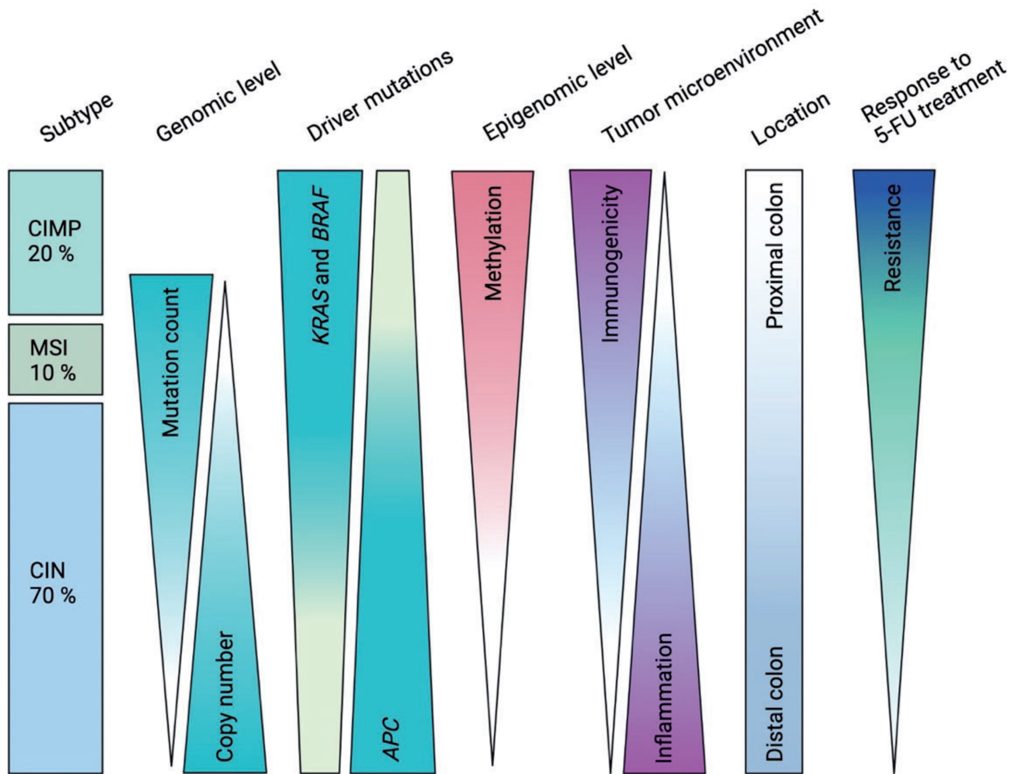

Figure 6. Features of different subtypes of CRC. A schematic representing differences between distinct molecular subtypes of hCRC. CIMP represents about 20% of the cases with *KRAS* or *BRAF* driver mutations, high methylation rate, high immunogenicity, resistance to 5-FU chemotherapy, and tumors more often located in the proximal colon. MSI subtype includes about 10% of the cases, harbors mutations in *KRAS* and *BRAF* genes, has high mutation count and methylation rate, high immunogenicity, often resistance to 5-FU, and tumors that are usually located in the proximal colon. The most common subtype is CIN with a lower mutation count but higher copy number, often mutated *APC,* lower methylation rate, signs of inflammation, relatively good response to 5-FU, and tumors located in the distal colon. Modified from Dienstmann *et al.*, 2017.

5. MODELS OF INTESTINAL TUMORIGENESIS

Mouse models of intestinal tumorigenesis have a crucial role in CRC research. Even though different *ex vivo* and *in vitro* technologies, and most recently normal tissue and tumor organoids, have been developed to investigate the mechanisms

underlying intestinal tumorigenesis and CRC, they cannot recapitulate many of the mechanisms operating in cancer patients *in vivo*. Furthermore, genetically engineered mouse models offer a possibility for preclinical studies.

5.1. *APC$^{MIN/+}$* MODEL

Because sporadic colorectal cancers often involve an early mutation in the *APC* tumor suppressor gene, most of the genetically engineered mouse models developed to recapitulate intestinal tumorigenesis are centered on *APC* mutation. The most widely used model for this purpose is the multiple intestinal neoplasia (Min) model (*Apc$^{Min/+}$*) which carries an autosomal dominant mutation in one of the *Apc* alleles (Moser *et al.*, 1995). In humans, such hereditary mutation would lead to familial adenomatous polyposis, as previously mentioned. Originally, this mouse model was generated using *N*-ethyl-*N*-nitrosourea mutagenesis, which led to a loss-of-function mutation in the *Apc* gene at codon 850, causing a truncation of the APC protein. As the mouse ages, a spontaneous loss-of-heterozygosity occurs in the other *Apc* allele, leading to the development of multiple intestinal adenomas and colonic polyps. Eventually, most mice die at about four months of age because of intestinal obstruction or bleeding and anemia.

Despite the popularity of the *Apc$^{Min/+}$*model, there are challenges in the use of this model. While most gastrointestinal tract tumors in humans occur in the colon, most tumors in *Apc$^{Min/+}$*mice develop in the small intestine. Furthermore, while untreated human FAP patients eventually develop invasive colon cancer, the benign polyps extremely rarely progress to malign tumors in mice (Jackstadt and Sansom, 2016). However, the introduction of suitable CRC progression mutations, such as *Kras, Tp53,* and *Smad4*, via the CRISP/Cas9 technology, for example, leads to invasive cancer also in the *Apc$^{Min/+}$*mice (Jackstadt and Sansom, 2016).

5.2. CONDITIONAL MODELS

5.2.1. Cre-LoxP technology

Site-specific recombination is a natural part of genome replication, differentiation, and movement of mobile genetic elements in several bacteria, bacteriophages, and yeasts. It is based on the ability of site-specific recombinase enzymes to recognize specific DNA sequences and induce recombination between two copies of these sequences. For genetic engineering, these recognition sites can be modified and placed into specific loci, and the expression

of the recombinase enzymes can be conditionally activated in particular cells by transferring them under inducible promoters. (Strachan and Read, 2019).

Cre-LoxP technology is a wide-used example of a site-specific recombination system. It is based on a recombinase enzyme encoded by the *cre* (cyclization recombinase) gene of the bacteriophage P1 and its specific recognition sequence called *loxP* (locus of x-over, P1), which is a 34 bp sequence consisting of an eight base pair (bp) core sequence surrounded by two 13 bp inverted and palindromic repeats (Strenberg and Hamilton, 1981; Sauer and Henderson, 1989). The Cre recombinase recognizes two similar *loxP* sequences and recombines them, excising the intervening DNA sequence (Sauer and Henderson, 1989). If the *loxP* sites are placed in the same DNA molecule in a similar orientation, the Cre-mediated recombination leads to the deletion of the intervening DNA. Furthermore, if the *loxP* sequences are present in the same DNA molecule in opposite directions, recombination results in inversion of the intervening sequence (Guo *et al.*, 1999). Additionally, *loxP* sites can be in separate DNA molecules, enabling chromosomal rearrangements (Smith *et al.*, 1995). To create a mutant mouse model with controlled DNA modifications, two components are required; a Cre-driver mouse strain in which Cre recombinase expression is regulated by a specific promoter targeting the cell type of interest, and a *loxP* flanked (floxed) DNA containing mouse strain, in which the *loxP* sites are in the gene/genes of interest (Rajewsky *et al.*, 1996). By crossing these strains, required modification can be obtained in specific tissue at a particular time point.

Inducible Cre-*loxP* systems, such as tamoxifen and tetracycline-inducible models, were developed to attain more specific genetic alterations. With these models, the Cre recombinase expression can be induced in specific cells at precise timepoints (Metzger *et al.*, 1995; Feil *et al.*, 1996). In a tamoxifen-dependent model, the Cre protein has been fused with an estrogen receptor that contains a mutation in the ligand-binding domain, forming a fusion protein called CreER recombinase, tamoxifen (CreERT) (Metzger *et al.*, 1995; Feil *et al.*, 1996). Under normal conditions, this fusion protein is present in the cytoplasm, bound and suppressed by heat shock protein 90 (HSP90) (Kang *et al.*, 1994). The binding of tamoxifen or 4-OHT to the ligand-binding part disrupts the interaction between HSP90 and CreERT, leading to translocation of CreERT into the nucleus, where it interacts with *loxP* sites and induces their recombination, resulting in the desired alteration in the DNA molecule (Mezger and Chambon, 2001). For more efficient recombination, Feil *et al.* (1997) introduced Cre-ERT2 recombinase, which is at least 3 to 4-fold more sensitive to its ligand than Cre-ERT.

5.2.2. Apc deletion

Even though the tumor initiation in the *Apc*$^{Min/+}$ model is dependent on mutation in both *Apc* alleles, thus strongly mimicking the natural tumor initiation of the intestine, the reproducibility of the experiments remains a challenge. Furthermore, as already mentioned, most of the adenomas in *Apc*$^{Min/+}$ mice develop in the small intestine instead of the colon, unlike in human CRC. Because a homozygous mutation of *Apc* has proven to be lethal in mice (Moser *et al*., 1995), a time- and site-specific model was required for intestinal tumorigenesis. Shibata *et al*. (1997) generated an inducible model for mouse intestinal and colorectal tumorigenesis, known as *Apc*$^{580S/580S}$, in which the *loxP* sites were inserted into *Apc* exon 14. Cre-mediated recombination in these mice leads to a frameshift mutation at codon 580, resulting in truncated and dysfunctional protein product and tumorigenesis in cells homozygous for the *Apc* mutation (Shibata *et al*., 1997).

Crossing the *Apc*$^{fl/fl}$ strain with various Cre recombinase mice yields different phenotypes. For example, intestinal stem cell-specific Cre or intestinal epithelial cell-specific Cre can be used. Furthermore, using *in situ* gene editing by rectal enemas of AAVs or tamoxifen, or targeted Cre activation by colonoscopy-guided injections of tamoxifen or AAVs to the colorectal mucous membrane, colorectal adenoma formation can be obtained (Shibata *et al*., 1997, Roper *et al.*, 2017, Roper *et al.*, 2018).

5.2.3. Intestinal epithelial cell–specific Cre

The *Villin* gene encodes an actin-bundling protein localized in the apical brush border of the gastrointestinal epithelium (Madison *et al.*, 2002). Villin is expressed both in differentiated enterocytes and immature, undifferentiated intestinal epithelial cells at the bottom of the crypts throughout the small intestine, colon, and rectum, with an ascending gradient from the crypt bottom to the tip of the villus. Its global expression in the GI tract potentially targets intestinal epithelial cells using the Cre-*loxP* technology (Robine *et al.*, 1997; Pinto *et al.*, 1999; Janssen *et al*., 2002; El Marjou *et al.*, 2004). To target genes of epithelial cells of the mouse digestive tract, El Marjou *et al.* (2004) developed a *Villin-CreERT2* mouse model by inserting the Cre-ERT2 recombinase sequence under the control of a 9 kb regulatory region in the murine *Villin* gene. Tamoxifen treatment of the mice induced global Cre expression in the digestive tract that persisted for at least 60 days after the induction, providing a powerful strategy for gene targeting in intestinal epithelial cells (El Marjou *et al.*, 2004).

In experiments employing the Villin-CreERT2 to deletor, one should consider that Villin expression is decreased at least in progressed hCRC (Arango *et al.*, 2012)

5.2.4. Lgr5-EGFP-Ires-CreERT

The Wnt target gene Lgr5 is expressed in intestinal CBCs and human colon cancer cells, providing a stem cell marker in the intestine and colon (Barker *et al.*, 2007). To identify and visualize live CBC cells and investigate their stem cell properties, Barker *et al.* (2007) created an *Lgr5-EFGP-IRES-CreERT2* strain by inserting an enhanced green fluorescent protein (EGFP) cassette at the site of the first ATG codon of the *Lgr5* gene. The EGFP cassette was further combined with the internal ribosomal entry site (IRES) sequence, enabling the co-expression of the Lgr5 gene and the EGFP under the control of the same promoter (Brennan and Robertson, 1999; Barker *et al.*, 2007). The *Lgr5*-regulated CreERT2 recombinase sequence enabled stem cell-specific genetic alterations in the LGR5+ cells using the Cre-*loxP* technology (Barker *et al.*, 2007).

5.3. INTESTINAL ORGANOIDS

For decades, intestinal epithelium and CRC studies were mainly performed using *in vivo* models and *in vitro* cell cultures. The first steps in developing applicable *ex vivo* systems were taken by primary cultures of rat intestinal crypts on collagen-coated plates yet subculturing of these systems was not yet obtained (Evans *et al.*, 1992). This system required mesenchymal cells, and it was further developed into an intestinal sphere-like organoid culture with an air-liquid interface and underlying stromal elements, which depended on Wnt growth factors, such as RSPO1 (Ootani *et al*., 2009). Meanwhile, Sato *et al*. elaborated an organoid culture technique with single Lgr5+ mouse intestinal stem cells embedded in Matrigel and cultured in the presence of RSPO1, Noggin, and EGF, resulting in crypt-villus-like domains including all differentiated cell types (Sato *et al*., 2009). These culture techniques have been developed further by involving the entire tissue microenvironment in the air-liquid interface with a complex histological tissue architecture including stroma, tumor parenchyma, and functional tumor-infiltrating lymphocytes present (Neal *et al.*, 2018).

5.4. MODELING ADVANCED INTESTINAL TUMORS

Since tumors in the *Apc* mutant mouse models are often both benign histology and anteriorly displaced in the small intestine compared to distal and malignant

tumors in hCRC, further development of mouse models has been required to obtain relevant results regarding colorectal cancer. Mice carrying engineered mutations in genes related to advanced hCRC, such as *Tp53* (Hallberg *et al.*, 2000), *Kras* (Sansom *et al.*, 2006), *Smad4* (Takaku *et al.*, 1998), *Tgfbr2* (Munoz *et al.*, 2006), and *Pten* (Marsh *et al*. 2008), with or without concomitant *Apc* mutation, have helped model invasive tumors, even some with metastasis. In addition, transplantation models have been developed for investigation of metastatic tumors; either subcutaneously injected xenografts using hCRC cell lines or colonoscopy-guided transplantation of genetically engineered organoids, tamoxifen, or AAV-Cre into mouse colonic submucosa have proven to be helpful in modeling spread CRC (Jackstadt and Sansom, 2015; Roper *et al.*, 2017). Furthermore, chemically induced mutagenesis with or without inflammation in WT mouse colon by treatment with 1,2-dimethylhydrazine (DMH), azoxymethane (AOM), AOM + dextran sulfate sodium (DSS), or DMH + DSS are used for inducing tumorigenesis in the distal colon, however, the obtained mutations cannot be precisely defined which limits the applications of the model (Tanaka *et al.*, 2003; Neufert *et al.*, 2007).

6. GENE TRANSFER USING ADENO-ASSOCIATED VIRAL VECTORS

6.1. BASICS OF THE AAV TECHNOLOGY

While the sequences of the monogenic diseases have been unraveled, the idea of correcting mutations of single genes responsible for significant conditions has driven the development of gene transfer strategies. The recent rapid progress in the ability to sequence entire genomes and to identify genes behind certain diseases has boosted the goal of gene therapy elaboration; the most recent example is the CRISPR-Cas technology for gene correction.

Adeno-associated viruses are non-enveloped viruses containing a single-stranded DNA genome of 4.8 kb. They belong to the parvovirus family, and their ability to invade cells and replicate is dependent on co-infection with other viruses, most commonly adenoviruses. The WT adeno-associated virus genome consists of three genes: *Rep* (replication), *Cap* (capsid), and *aap* (assembly), which encode at least nine proteins because of alternative splicing, three promoters, and different translation sites. Recombinant AAVs (rAAV) are protein-based nanoparticles that do not contain essential elements of the viral DNA but only the parts to enable genetic engineering by transferring genetic information into the

nucleus of a target cell (Naso *et al.*, 2017). In the absence of the viral genome, the transgenes encoded within the rAAV form circular concatemers that commonly remain as episomes in the nucleus of the targeted cells. Such episomal DNA that is not integrated into the host genome is diluted as the cell replicates, followed by an eventual loss of the transgene and its expression, thus making the transgene expression dependent on the host cell turnover rate (Naso *et al.*, 2017).

The recombinant AAVs have been considered to possess remarkable potential as gene therapy vectors, and they have been widely used in clinical-stage experiments. Due to the possible generation of recombinant AAV particles without any viral genes, containing only the DNA sequences of interest may be one of the safest gene transfer strategies; however, the transfer of stabilized RNA has recently become a challenger (Naso *et al*., 2017).

6.2. AAV SEROTYPES AND THEIR CHARACTERISTICS

Different AAV serotypes possess unique characteristics in their preference to target cells for transfection. For example, AAV1, AAV2, AAV5, AAV7, and AAV9 target well hepatocytes, and AAV3, AAV4, AAV6, and AAV8 target cardiac muscle cells. Furthermore, AAV4 and AAV6 can efficiently target the lungs, and AAV9 can transfect neurons and skeletal and cardiac muscle cells (Zincarelli *et al.*, 2008). By selecting mutagenized AAVs, more specific genotypes have recently been generated (Ran *et al.*, 2020).

The rAAVs typically enter the host cells by interacting with carbohydrates, their sialic acid, heparan sulfate, or galactose on the surface of the target cells. Different preferences in binding carbohydrates determine the AAV serotype's ability to enter the host cells. (Agbandje-McKenna *et al.*, 2012; DiMattia *et al.*, 2012) AAV9, for example, prefers binding to galactose to enter the host cell, which may give rise to AAV9's remarkable ability to cross the blood-brain-barrier and transfect efficiently skeletal and cardiac muscle (Zhang *et al.*, 2011; Bell *et al.*, 2012; DiMattia *et al.*, 2012; Wang *et al.*, 2014). Furthermore, secondary receptors, such as fibroblast/hepatocyte growth factor (AAV2), epidermal growth factor (AAV6), and platelet-derived growth factor (AAV5), have been identified to contribute to rAAV target cell specificity. (Naso *et al.*, 2017)

AIM OF THE STUDY

Since the Wnt target PROX1 is involved in progression of intestinal adenomas, we were interested in studying the effects of endogenous and exogenous Wnt signals on PROX1 expression during intestinal tumorigenesis. For modulation of intercellular Wnt signals in the intestinal adenoma microenvironment, we first tested RSPO1 which acts as a rheostat of the Wnt signaling strength in normal intestinal epithelium. This led to an interesting finding that we then pursued in mechanistic detail. As a result of this endevoir, we revealed a novel mechanism to inhibit the growth of *Apc* mutant adenomas in mice.

MATERIALS AND METHODS

1. MATERIALS

Table 1. Mouse models

Model	Description	Reference
$Apc^{Min/+}$	Heterozygous mutation of the *Apc* gene, leading to a truncated protein as the second allele becomes mutated	Moser *et al.,* 1995
$Apc^{flox/flox}$	Conditional deletion of the *Apc* gene	Shibata *et al.,* 1997
Lgr5-eGFP-IRES-Cre^{ERT2}	Tamoxifen-inducible Cre recombinase enzyme under control of the promoter of the *Lgr5* gene	Barker *et al.,* 2007
Prox1-Cre^{ERT2}	Tamoxifen-inducible Cre recombinase enzyme under control of the promoter of the *Prox1* gene	Srinivasan *et al.,* 2007
Villin-Cre^{ERT}	Tamoxifen-inducible Cre recombinase enzyme under control of the promoter of the *Villin* gene	El Marjou *et al.,* 2004
$Rosa26^{LSL}$*-TdTomato*	Inducible red fluorescent protein reporter under the *Rosa26* locus	Madisen *et al.,* 2010
Apc580S	Conditional mutation of one allele in the *Apc* gene	Shibata *et al.,* 1997
$Kras^{LSL\text{-}G12D/+,}$	Inducible activating point mutation of the *Kras* gene	Jackson *et al.,* 2001

Table 2. Primary antibodies

Antigen	Description	Provider
APC C-terminus	Rabbit monoclonal	Abcam, #ab154906
APC N-terminus	Rabbit polyclonal	Abcam, #ab245442
β-catenin	Mouse monoclonal	BD Biosciences, #610153
Cleaved caspase-3	Rabbit monoclonal	Cell signaling, #9661
EdU	Alexa Fluor 488 conjugated	ThermoFisher, # C10337
FLAG	Mouse monoclonal	Sigma, F1804
GFP	Rabbit polyclonal	Torrey Pines Biolabs, #TP401
Glutamine synthetase	Mouse monoclonal	BD Biosciences, #610517
Human IgG	Rabbit polyclonal	Dako, P0214
p21	Rat monoclonal	CNIO
PROX1	Goat polyclonal	R&D Systems, #AF2727
pSMAD3	Rabbit monoclonal	Abcam, #52903
RFP	Chicken polyclonal	Rockland Immunochemicals #600-901-379

Table 3. Reagents

Reagent	Provider
4 % paraformaldehyde	Sigma, #P6148
4-hydoxytamoxifen	Sigma, #H7904
5-ethynyl-2´-deoxyuridine	Invitrogen, #A10044
Advanced DMEM/F12 medium	Gibco, #12634010
B27 supplement	Gibco, #17504001
Benzonase	ChemCruz, #sc-202391
Bovine serum albumine	Biowest, #P6154
Collagenase H	Roche, #11074032001
Collagenase type I	Worthington, #LS004196
Corn oil	Sigma, #8001-30-7
Dispase II	Sigma, #04942078001,
DNase	Fermentas, #EN0521
Eosin Y (yellowish)	Merck #1.15935.0100
Fetal bovine serum	Biowest, #S1810-500
Hanks' balanced salt solution	Gibco, #14175-053

High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific, #4368814
Intesticult	StemCell Technologies, #06005
LGK974	Peprotech, #1241454
Matrigel	Corning, #35623
Maxima SYBR Green/ROX qPCR Master Mix	Thermo Fisher Scientific, #K0221
Mayer's Hemalum Solution	Merck, 1092492500
N-acetyl cysteine	Sigma, #A7250
N2 supplement	Gibco, #17502048
NucleoSpin RNA isolation kit	Macherey-Nagel, #740955.50
PEG300	Sigma, #8.07484
Penicillin and streptomycin	Lonza, #DE-17-602E
Pertex	Histolab, 00811-EX
SB431542	Tocris, #1614
Tamoxifen	Sigma, #T5648
Trypsin-EDTA	Thermo Fisher Scientific, #25200056
Tween-80	Sigma
Vectashield with DAPI	Vectashield, #VEC.H-1000

Table 4. AAVs

AAV	**Description**
hRSPO1-hFC-AAV9	Human RSPO1 protein with conjugated Fc region from human immunoglobulin G
mRspo1-mFc-AAV9	Mouse Rspo1 protein with conjugated Fc region from mouse immunoglobulin G
hRSPO1-FLAG-AAV9	Human RSPO1 protein with conjugated FLAG tag
hRSPO2-hFC-AAV9	Human RSPO2 protein with conjugated Fc region from human immunoglobulin G
hRSPO3-hFC-AAV9	Human RSPO3 protein with conjugated Fc region from human immunoglobulin G
Dkk1-FLAG-AAV9	Mouse Dkk1 protein followed by conjugated FLAG tag
FLAG-Dkk1-AAV9	FLAG tag -conjugated mouse Dkk1 protein
S2-AAV9	Empty AAV9 control vector

Table 5. Primers

Primer	Sequence
Gapdh fw	ACAACTTTGGCATTGTGGAA
Gapdh rev	GATGCAGGGATGATGTTCTG
Prox1 fw	GCTATACCGAGCCCTCAACA
Prox1 rev	ATCCAGCTTGCAGATGACCT
Lgr5 fw	CCTGTCCAGGCTTTCAGAAG
Lgr5 rev	CTGTGGAGTCCATCAAAGCA
Myc fw	CTGTACCTCGTCCGATTCCA
Myc rev	TGCCTCTTCTCCACAGACAC
Notum fw	GGTTCCTGGACAACAAGCAG
Notum rev	TCTCCTTCCTTGAACTGGCG
Axin2 fw	TTCAAGAGAAGCGACCCAGT
Axin2 rev	TTTCTTACTCCCCATGCGGT
Sox9 fw	GACTCCCCACATTCCTCCTC
Sox9 rev	CCCCTCTCGCTTCAGATCAA
Lef1 fw	GCCTGTTTATCCCATCACGG
Lef1 rev	ATAGCTGGATGAGGGATGCC

2. METHODS

Recombinant AAV vector construction, production, and transgene expression

The coding regions of the hRSPO1-FC, mRspo1-Fc, RSPO1-FLAG, hRSPO2-FC, hRSPO3-Fc and FLAG-Dkk1 were cloned into the psub-CMV-WPRE AAV9 expression vector. The AAV9s were produced using a two-plasmid production system as previously described (Grimm *et al.*, 1998; Pajusola *et al.*, 2005; Anisimov *et al.*, 2009). Empty AAV9 vector without an expression cassette insert was used as a control. Serum samples were collected from the AAV9-treated mice at euthanasia, and 2 μL of the serum was electrophoresed and analyzed by Western blotting using anti-human IgG or anti-FLAG antibody. Images were captured with the Odyssey Fc imaging system (LI-COR).

Production of recombinant hRSPO1-Fc protein

The open reading frame of the recombinant hRSPO1-FcHis6 fusion protein was transferred to the pEFIRESp-vector, and the protein was produced in the Chinese hamster ovary (CHO) cells grown in suspension culture and purified by HisTrap Excel chromatography (Cytiva) according to the manufacturer's instructions.

Organoid cultures

$Apc^{Min/+}$ mouse intestinal crypts were isolated and cultured in the IntestiCult mouse organoid growth medium as previously described (Sato *et al.*, 2009; Wiener *et al.*, 2014). To activate gene deletion, organoid cultures from $Apc^{fl/fl}$; *Lgr5-eGFP-IRES-Cre*^{ERT2} mice were treated with 300 nM 4-hydoxytamoxifen (4-OH-Tam) for 24 hours. For the selection of homozygous *Apc* loss, the organoids were cultured in growth factor-deficient medium containing Advanced DMEM/F12 medium, N2 supplement, B27 supplement, 1 μM N-acetyl cysteine, 10 mM HEPES, glutamine, and penicillin/streptomycin (Shibata *et al.*, 1997; Barker *et al.*, 2009). As indicated in experimental setup graphs, the organoid cultures were treated with 10 μg/mL hRSPO1-Fc or 10 μM SB431542 or both for 1, 3, or 6 days.

To determinate the replating efficiency, clusters of 4-5 cells were obtained by extensive trypsinization of the organoids. The clusters were then counted and embedded into Matrigel in equal numbers. The number of organoids/well were counted using a microscope after 3–6 days of subculture.

For analysis of cell proliferation, organoids were incubated with 5-ethynyl-2′-deoxyuridine (EdU) for 2 hours, fixed, and detected using Click-iT EdU Alexa Fluor 488 Imaging kit according to manufacturer's instructions.

In vivo experiments

The National Animal Experiment Board at the Provincial State Office of Southern Finland (ESAVI/6306/04.10.07/2016) approved all the animal experiments. Mice were housed following the guidelines and recommendations of the Federation of European Laboratory Animal Science Association in individually ventilated cages with enrichment materials. The $Apc^{+/+}$ and $Apc^{Min/+}$ mice used were of the C57BL/6 background (Jackson Laboratories).

$Apc^{Min/+}$; $Rosa26^{LSL\text{-}TdTomato}$ mice (Jackson Laboratories) were crossed with $Prox1\text{-}Cre^{ERT2}$ mice for *Prox1* lineage tracing (Harvey *et al.*, 2005). The mice were given a single 2 mg dose of tamoxifen dissolved in 100 μL of corn oil, administered by oral gavage, to activate *Prox1* lineage tracing.

Mice were treated with a single intraperitoneal (i.p.) injection of 1 μg of EdU diluted in 100 μL of 0.9 % saline 4 hours before euthanasia to label proliferating cells.

The mice were closely monitored and weighed every 3 days during the experiment for the survival analysis. Bodyweight loss >15 % or detection of bloody stool or melena were defined as termination criteria.

$Apc^{Min/+}$ mice were treated with daily i.p. injections of 10 mg/kg of SB431542 diluted in 10% DMSO, 40% PEG300, 5% Tween-80, and 45% saline for TGFβ receptor kinase inhibition. The $Apc^{Min/+}$ mice were treated with the porcupine inhibitor LGK974 diluted in 5% DMSO, 30% PEG300, and 10% Tween-80 in H_2O administered by oral gavage (10 mg/kg once a day) for two consecutive days to inhibit Wnt ligand secretion.

Experiments with *Kras* mutant mice were performed according to UK Home Office regulations (license 70/8646), and a local ethical review committee reviewed the experiments at the University of Glasgow. 6-12-week-old male and female Apc^{580S}; $Villin\text{-}Cre^{ER}$; $Kras^{LSL\text{-}G12D/+}$ mice of C57BL/6J background were treated with intraperitoneal tamoxifen injections (80 mg/kg) for two consecutive days. Tissues were harvested four days after the induction.

For each *in vivo* experiment, 10-12 mice were used with approximately equal numbers of female and female mice of matching age in each experimental group. All the *in vivo* experiments were repeated at least twice.

Tissue processing and immunohistochemistry

After termination, mouse livers and intestines were collected. The intestines were extensively washed with PBS, cut into three pieces of equal length, placed on a filter paper, cut longitudinally, and opened luminal side upward. The collected tissues were fixed overnight in 4 % paraformaldehyde (PFA) in +4°C. Tissues were then extensively washed with PBS, dehydrated in a tissue sample processor (Sakura Tissue-Tek), embedded into paraffin, and cut into 5 μm sections.

For histological analysis, the tissues were subjected to hematoxylin & eosin (H&E) staining as follows: the sections were deparaffinized and rehydrated, the nuclei were stained with Mayer's hemalum solution diluted 1:10 in water for 1 min at room temperature, the slides were extensively rinsed with tap water, incubated in eosin for 1 min in room temperature, dehydrated, and mounted with Pertex.

RNAscope *in situ* hybridization for the detection of *Notum* (472548) was performed using RNAscope 2.5 LS Reagent Kit–BROWN (Advanced Cell Diagnostics) on a BOND RX autostainer (Leica) according to the manufacturer's instructions.

For immunohistochemical or histological stainings, the sections were deparaffinized and rehydrated. For immunohistochemical staining, the sections were treated with heat-induced antigen epitope retrieval buffer in a microwave oven at 850 W for 5min and at 450 W for 10 min. The sections were blocked with TNB (0.1 M Tris-HCl, 0.15 M NaCl, 0,5 % BSA) to decrease the background stain for one hour at room temperature. The slides were then incubated with indicated primary antibodies diluted in TNB at +4°C overnight, extensively washed with TNT (0.1 M Tris-HCl, 0.15 M NaCl, 0.05 % Tween20), followed by incubation with secondary antibodies diluted in TNB for one hour at room temperature, washed again with TNT, and mounted with DAPI-containing Vectashield. For EdU detection, the Click-iT EdU Alexa Fluor 488 Imaging kit was used according to the manufacturer's instructions. For counterstaining of the nuclei, Vectashield with Dapi was used as a mounting medium.

Images were captured using a Zeiss Axioimager upright epifluorescence microscope with 10x EC Plan Neofluar (NA 0.3), 20x Plan-Apochromat (NA 0.8), and 40x EC Plan Neofluar (NA 1.3) objectives. Original, unprocessed files were analyzed with the Fiji (Fiji Is Just ImageJ) software.

RNA extraction and qPCR

The NucleoSpin RNA isolation kit was used according to the manufacturer's instructions for the total RNA isolation from organoid and adenoma samples. The High-Capacity cDNA Reverse Transcription Kit was used for reverse-transcription of RNA into cDNA according to the manufacturer's instructions. The Maxima SYBR Green/ROX qPCR Master Mix and a BioRad real-time PCR instrument were used for quantitive reverse transcription PCR (RT-qPCR). For normalization of mRNA levels, *Gapdh* primers were used. The sequences of the primers used are listed in **Table 5.**

Single-cell RNA sequencing and data analysis

$Apc^{Min/+}$ organoids were cultured with RSPO1-Fc fusion protein or vehicle for one day and then processed for single-cell isolation according to the previously published method (Högström *et al.*, 2019). Briefly, organoids were dissociated in a solution containing Hanks' balanced salt solution, 1 mg/mL collagenase type I, 1 mg/mL collagenase H, 4 mg/mL dispase II and 1000 U/mL for 30 min at 37°C with gentle shaking, followed by 10 min incubation with Trypsin-EDTA and filtered to obtain single cells. The single cells were transferred into 0.04% BSA-HBSS for analysis.

Intestinal adenomas of $Apc^{Min/+}$ mice were collected and incubated in a dissociation solution (collagenase I 1 mg/mL, collagenase H 1 mg/mL, dispase II 4 mg/mL, DNase 4 µg/mL) for 30 min at +32°C. The suspension was further incubated with Trypsin-EDTA for 15 min at +37°C, gently pipetted to obtain a single-cell suspension, filtered, and transferred into 0.04% BSA-HBSS for analysis.

The 10x Chromium Single-Cell 3′RNA-sequencing system (10x Genomics, Pleasanton, CA, USA) was used for single-cell sequencing with the Reagent Kit v2 according to the manufacturer's instructions. Briefly, the cells were loaded into Chromium Single-Cell Chip v2 (10x Genomics, Pleasanton, CA, USA), followed by generation of Gel beads in EMulsion (GEM) aiming at 5000 cell captures per sample. This was followed by cDNA purification, amplification (12

cycles), and library construction (sample index PCR 14 cycles) as instructed by the manufacturer. The sample libraries were sequenced on an Illumina NovaSeq 6000 sequencer using read lengths of 26bp (Read 1), 8bp (i7 Index), 0 bp (i5 Index) and 91bp (Read 2), resulting in an average of following reads per cell: 37 547 (*Apc*$^{Min/+}$ *in vivo* D1-2 Ctrl), 47 141 (*Apc*$^{Min/+}$ *in vivo* D1 RSPO1), and 38 871 (*Apc*$^{Min/+}$ *in vivo* D2 RSPO1), 105 926 (*Apc*$^{Min/+}$ *in vivo* D4 Ctrl), 85 650 (*Apc*$^{Min/+}$ *in vivo* D4 RSPO1), 58 414 (*Apc*$^{Min/+}$ organoids Ctrl), and 131 291 (*Apc*$^{Min/+}$ organoids RSPO1). Chromium single-cell 3' RNA-sequencing barcodes and read data were demultiplexed and converted to FASTQ files. Aligned reads and gene-cell matrices were generated using Cell Ranger v 2.1.1 mkfastq and count pipelines (10x Genomics, Pleasanton, CA, USA). Reads were aligned to mouse reference genome mm10. The Seurat R package 3.1.1 was used for quality control, filtering, and data analysis (Butler *et al.*, 2018). Cell filtering was performed based on unique feature counts and the percentage of mitochondrial genes. Cells with more than 15% (*Apc*$^{Min/+}$ *in vivo* D1-2, D4) or 6% (*Apc*$^{Min/+}$ organoids) of mitochondrial genes were filtered out. The expression matrix was further filtered by removing genes expressed in less than five cells and cells with less than 500 distinct expressed genes. The final dataset consisted of the following number of cells: 1 593 (*Apc*$^{Min/+}$ *in vivo* D4 Ctrl), 1 978 (*Apc*$^{Min/+}$ *in vivo* D4 RSPO1), 2 977 (*Apc*$^{Min/+}$ organoids Ctrl), 2 434 (*Apc*$^{Min/+}$ organoids RSPO1), 3 532 (*Apc*$^{Min/+}$ *in vivo* D1-2 Ctrl), 3 761 (*Apc*$^{Min/+}$ *in vivo* D1 RSPO1), and 2 851 (*Apc*$^{Min/+}$ *in vivo* D2 RSPO1). A principal component analysis (PCA) was performed by comparing two samples per dataset to identify shared correlation structures, and the dimensions were aligned using dynamic time warping. Furthermore, clustering was performed using uniform manifold approximation and projection (UMAP), setting the resolution to 0.5. KEGG pathway enrichment analysis with Enrichr was performed for scRNA-seq data (Chen *et al.*, 2013; Kuleshov *et al.*, 2016). The scRNA sequencing data can be accessed in Gene Expression Omnibus with access number GSE146139.

Statistical Analysis

The satistical data is presented as mean+SD. The two-tailed unpaired t-test in the GraphPad Prism 7.0 software was used for the statistical comparison of two experimental groups. The Ordinary one-way ANOVA, multiple comparisons in the GraphPad Prism 7.0 software was used for statistical comparison of three or more experimental groups. Results with $P<0.05$ were considered statistically significant, and the significance was marked by *$P<0.05$, **$P<0.01$, and ***$P<0.005$.

RESULTS AND DISCUSSION

I. EXPRESSION OF R-SPONDIN1 IN *APC*$^{MIN/+}$ MICE SUPPRESSES GROWTH OF INTESTINAL ADENOMAS BY ALTERING WNT AND TRANSFORMING GROWTH FACTOR BETA SIGNALING

Systemic RSPO1 expression reduces Wnt/β-catenin signaling and adenoma cell proliferation in mice

Systemic expression of RSPO1 has been shown to enhance Wnt/β-catenin signaling in LGR5+ intestinal stem cells (Kim *et al.*, 2008). Furthermore, PROX1 has been shown to promote proliferation and tumor initiation in intestinal stem cells expressing LGR5 (Wiener *et al.*, 2014). Although it was known that the *Apc*$^{Min/+}$ tumors should be unresponsive to exogenous Wnt signals, we wanted to know if PROX1 expression in the intestinal epithelium and adenomas can be affected by exogenous RSPO1. To induce systemic expression of RSPO1, we generated an AAV-vector encoding dimeric human RSPO1 protein fused with the Fc region of human IgG to enhance the stability and solubility of the RSPO protein and to enable the detection of the fusion protein. For *in vitro* validation of the vector function, WT mice were injected with a single dose of $1x10^{12}$ AAV particles (vp) containing RSPO1-Fc or no payload. The mice were terminated at several different time points. Expression of the fusion protein was confirmed in their sera using Western blotting analysis and in liver sections by immunostaining for human Fc. Because the AAV9 serotype transfects well hepatocytes, whose zonation in the liver is driven by Wnt signaling (Benhamouche *et al.*, 2006), we confirmed the biological activity of the transgene by immunostaining for glutamine synthetase in the liver sections. Further confirmation of the biological activity was also apparent by the finding of increased intestinal crypt depth and villus length after the AAV-RSPO1 treatment. (**Work I, Supplementary Figure 1**)

To study the effect of systemic RSPO1 expression in mouse intestinal adenomas, we treated 17-week-old *Apc*$^{Min/+}$mice with AAVs encoding RSPO1-Fc or control vector and terminated the mice and analyzed the guts four days after that. The diameters of the macroscopic small intestinal adenomas, but not their numbers, were significantly increased in RSPO1-Fc-treated mice compared to the control group. Unexpectedly, however, the RSPO1-Fc treatment seemed to suppress Wnt

signaling in the adenomas since the further analysis of the tumors using immunofluorescent staining showed reduced nuclear β-catenin and PROX1 positive areas were remarkably reduced after the RSPO1-Fc treatment. The mice had been injected with EdU four hours before their termination to label the cells with active DNA synthesis. In the tumor sections, the number of EdU+ proliferating cells within the adenoma areas was significantly lower in the RSPO1-Fc treated than the control-treated group, whereas the proliferation of the normal intestinal epithelial cells was increased by the RSPO1-Fc treatment. These striking results indicated that RSPO1-Fc treatment rapidly suppresses Wnt signaling and proliferation of the intestinal adenoma cells, simultaneously promoting the expansion of the healthy intestinal epithelial cells. (**Work I, Supplementary Figure 2**)

The RSPO1-Fc treated tumors were subjected to single-cell mRNA sequencing (scRNAseq) to validate the reduced Wnt signaling activity. The subspace alignment was performed among the cells, and the nonlinear dimensionality reduction using uniform manifold approximation and projection (UMAP) was conducted after that. Unsupervised hierarchical clustering of the scRNAseq data in UMAP resulted in 11 epithelial cell clusters and the number of cells in the cluster showing high expression of Wnt target genes, such as *Prox1*, *Lgr5*, *Notum*, *Lef1*, *Tcf4,* and *Nkd1*, was significantly reduced by the RSPO1-Fc treatment. Furthermore, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the data showed down-regulation of the Wnt pathway activity in the RSPO1-Fc treated adenomas, supporting our previous data. (**Work I, Figure 1 and Supplementary Figure 2**)

Systemic RSPO1 expression reduces the number and dysplastic features of mouse intestinal tumors

To further observe the effect of systemic RSPO1 expression on intestinal adenoma growth and progression, we treated 12-week-old *Apc*$^{Min/+}$mice with RSPO1-Fc-AAV or Ctrl-AAV and analyzed the mice at 1, 4, and 6 weeks thereafter. Again, the average diameter of the tumors in the small intestine was decreased after one week of RSPO1 expression, but the total small intestinal tumor burden was not affected. Strikingly, however, a remarkably lower number of intestinal tumors was detected in the mice four and six weeks after the RSPO1-Fc-AAV injection than in the control groups. This finding supported our previous data indicating reduced Wnt signaling activity and tumor cell proliferation in intestinal adenomas after the RSPO1 treatment and suggested that RSPO1 would act as a tumor suppressor in intestinal tumorigenesis. (**Work I, Figure 1**)

Next, we sought to analyze the effect of prolonged systemic RSPO1 expression on Wnt signaling activity in intestinal tumors. Immunofluorescence analysis of the RSPO1 treated tumor sections indicated that nuclear β-catenin+ adenoma areas were luminally displaced and further from the crypt bottom than in mice injected with the control vector. Furthermore, we detected remarkably smaller and fewer areas positive for both PROX1 and nuclear β-catenin after four and six weeks of the RSPO1 treatment, although the PROX1+/β-catenin+ area percentage was not altered. Moreover, significantly less tumor cell proliferation was detected within the nuclear β-catenin+ adenoma areas one, four, and six weeks after the RSPO1 treatment when analyzed by EdU-labeling, whereas the proliferation rate of the nuclear β-catenin negative, normal intestinal epithelial cells was increased. These findings further suggested the initial increase in tumor diameter was based on the increased proliferation of the surrounding normal intestinal epithelium. (**Work I, Figure 1**)

To evaluate the impact of RSPO1 treatment on the histology of the intestinal adenomas, the tumor sections were stained with hematoxylin and eosin and analyzed by a gastrointestinal pathologist in a blinded manner. All the samples in the control group were diagnosed as mildly dysplastic, broad-based tubular adenomas with branching crypts and irregular stroma. The nuclei of the cells were relatively well polarized, elongated, hyperchromatic, and contained inconspicuous nucleoli. After one week of RSPO1 expression, tumors were less dysplastic and presented less crypt branching than the ctrl tumors. Tumors from four and six-week timepoints were diagnosed as hyperplastic intestinal polyps containing straight, elongated glandular structures. Their nuclei were ovoid, lacked atypia, showed only mild serration, and abutted the epithelial basement membrane. Based on these findings, RSPO1 treatment gradually decreased dysplastic features in the intestinal adenomas in the $Apc^{Min/+}$mice. (**Work I, Figure 2**)

To exclude the possibility that the human Fc protein domain affected the phenotypic conversion following the systemic RSPO1 expression, we generated an AAV vector encoding monomeric RSPO1 protein fused with a FLAG tag and injected 15-week-old $Apc^{Min/+}$ mice with the vector. When the mice were analyzed one and four weeks thereafter, a similar phenotype was detected as with AAV-RSPO1-Fc. The nuclear β-catenin+ adenoma area was decreased, and cell proliferation outside the adenomas was increased. Interestingly, however, the reduction of the tumor burden in the RSPO1-FLAG treated mice did not reach statistical significance compared to the control group, implying that the dimeric

RSPO1-Fc form is more effective in the repression of intestinal tumor growth. (**Work I, Supplementary Figure 3**)

To clarify whether the loss of the intestinal adenomas is due to the overgrowth of the normal intestinal epithelium or regression of β-catenin+ adenoma cells back into normal intestinal epithelial cells, we used 16-week-old *Apc*$^{Min/+}$ mice carrying an inducible *Rosa26*$^{LSL\text{-}tdTomato}$*; Prox1-Cre*^{ERT2} cell lineage tracer allele. In these mice, fluorescent tdTomato expression can be induced in PROX1 positive cells by using tamoxifen injection. To label the PROX1+ cells, the mice were given tamoxifen by oral gavage, treated with RSPO1-Fc-AAV or control vector two days later, and analyzed one week after that. Notably, fewer tdTomato-expressing areas were detected in the intestinal adenomas of the RSPO1-treated mice. Moreover, in comparison with the control group, the tdTomato+ areas in the intestines of the RSPO1-treated mice were luminally translocated further from the crypt bottoms and underlined by a layer of highly proliferating, β-catenin negative intestinal epithelial cells. (**Work I, Figure 2**) To confirm the mutational status of *Apc* in the remaining cells that were negative for nuclear β-catenin, the tumor sections were stained for the C-terminal part of the APC protein, which is deleted together with the Axin and β-catenin binding domains by the *ApcMin/*+ mutation. Only the apical borders of the polarized epithelial cells negative for nuclear β-catenin showed staining, whereas the β-catenin positive adenoma areas remained without signal. Furthermore, staining for the N-terminal region of APC, which is present even in the APC^{Min} mutant protein, indicated Apc expression throughout the intestinal epithelium and the adenoma areas. Based on these findings, we concluded that the RSPO1 treatment suppresses the growth of the β-catenin positive cells, while enhancing the growth of healthy intestinal epithelial cells. Thus, the adenomas are replaced by the adjacent WT intestinal epithelium, which gradually repels the adenomas by pushing them towards the lumen. (**Work I, Supplementary Figure 4**)

Prolonged expression of RSPO1 suppresses intestinal tumorigenesis and improves survival of the tumor-bearing mice

Next, we sought to assess the effect of long-term RSPO1 treatment on intestinal tumors in the *Apc*$^{Min/+}$ mice by treating 8-week-old mice with RSPO1-Fc or control AAV-vectors. When analyzed ten weeks after the AAV injection, a drastic decrease in the number and size of the intestinal adenomas was detected in the RSPO1-treated mice. Moreover, no β-catenin+ or PROX1+ areas were seen in these remaining intestinal adenomas. Surprisingly, however, no significant difference was detected in the number or size of colonic tumors

between the two groups. We were also interested to learn whether RSPO1 treatment would increase the survival of the tumor-bearing *Apc*$^{Min/+}$ mice, so we repeated a similar experimental setup in another set of 8-week-old mice. The mice were closely monitored until they met the humane termination criteria. RSPO1 treatment increased the lifespan of the *Apc*$^{Min/+}$ mice by approximately ten weeks, and when terminated, the RSPO1 treated mice had a remarkably lower small intestinal tumor burden than the control mice. The few RSPO1 treated mice that had met the termination criteria during the experiment were mainly euthanized due to weight loss or rectal bleeding caused by colonic tumors, which appeared unresponsive to RSPO1 treatment. (**Work I, Figure 3**)

RSPO1 suppresses Wnt signaling and induces the TGFβ/SMAD pathway in mouse adenoma cells

To study the mechanisms underlying the intestinal tumor regression *ex vivo*, we isolated intestinal adenoma organoids from *Apc*$^{Min/+}$ mice. I found a previous study suggesting that activation of LGR5 by exogenous RSPO1 could directly activate TGFβ signaling by binding to TGFβR2 in two hCRC cell lines and thereby inhibit cell growth and increase apoptosis (Zhou *et al.*, 2017). To analyze if this is the mechanism behind our findings, we cultured *Apc*$^{Min/+}$ mouse intestinal adenoma organoids in a medium containing RSPO1-Fc with or without TGFβ receptor kinase inhibitor (TGFβRi) SB-431542 for one, three, or six days. We detected decreased frequency of formed organoids after the addition of RSPO1-Fc, and this was rescued by the TGFβRi. (**Work I, Figure 4**) Similar results were obtained using tamoxifen-induced *Apc*$^{fl/fl}$*; Lgr5-eGFP-IRES-Cre*^{ERT} organoids (**Work I, Supplementary Figure 7**). At the mRNA level, we detected suppression of several Wnt targets, such as *Prox1* and *Notum*. In the immunofluorescence analysis of the organoids, reduced PROX1 expression was observed after three days of RSPO1 treatment, and the TGFβRi rescued the phenotype. Surprisingly, however, *Lgr5* and *Myc* gene transcripts were upregulated six days after the RSPO1 treatment in the remaining organoids, most probably due to the presence of some residual normal intestinal epithelial cells lacking the *Apc* mutation. However, the possibility of elevated *Lgr5* expression being able to provide a survival advantage for *Apc* mutant cells cannot be excluded entirely since high LGR5 expression level has been suggested to correlate with poor prognosis and metastatic features in hCRC (Tsuji *et al.*, 2014). In the Western blotting analysis of the organoids, increased SMAD2 phosphorylation was detected in the RSPO1 treated organoids. Upregulation of several gene transcripts regulated by the TGFβ/SMAD pathway was also found in the scRNAseq analysis of the organoids treated with RSPO1 for 24 hours. This

ex vivo data strongly suggested that the growth inhibition of adenoma cells caused by RSPO1 treatment could be mediated by the TGFβ/SMAD pathway. (**Work I, Figure 4**)

Involvement of the TGFβ/SMAD pathway in the *in vivo* phenotype resulting from RSPO1 treatment was evaluated by immunohistochemical analysis of the tumor sections using antibodies against TGFβ targets phospho-SMAD3 and its downstream cell cycle kinase inhibitor p21. After four days of RSPO1 expression, the tumors showed increased phosphorylation of SMAD3 and increased expression of p21 protein, consistent with activation of the TGFβ/SMAD pathway. In the scRNAseq data from the RSPO1 treated tumors, we observed higher expression of transcripts encoding several TGFβ/SMAD targets, and similar data was obtained using the KEGG pathway enrichment analysis when comparison was done with control-treated tumors. Based on these results, we concluded that the growth suppression of intestinal adenomas after RSPO1 treatment *in vivo* is due to the concurrent enhancement of the TGFβ/SMAD pathway. (**Work I, Figure 5**)

Induced RSPO1 expression results in a rapid wave of apoptosis in mouse adenomas

Because the TGFβ/SMAD pathway has been implicated in the regulation of apoptosis, we were interested to learn if RSPO1 treatment can induce apoptosis in the $Apc^{Min/+}$ intestinal tumors. When we analyzed tissue samples from the mice on days 1-4 after the AAV injections, we observed a significant increase of apoptotic cells in the RSPO1 treated tumors already one day after the injection, and the rate remained higher than in the control group on days 2 and 3, returning back to the control level on day 4. Further analysis of the scRNAseq samples from the *in vivo* tumor samples showed increased expression of genes that encode pro-apoptotic proteins, such as *Trp53, Cdkn1c, Bcl2l1, Bax, Bad*, and *Pycard*. Several pathways involved in apoptosis were upregulated in the KEGG pathway enrichment analysis. When the RSPO1 treated mice were concomitantly treated with TGFβRi, the apoptosis rate was restored to the same level as the control group. (**Work I, Figure 6**)

Further analysis of the scRNAseq data on cells harvested after one day of RSPO1 treatment revealed increased expression of several Wnt targets simultaneously with the increased apoptosis. However, the expression of these targets was decreased during the following days, likely because of the dropout of the "Wnt-high" cells after the initial increase in the apoptosis of adenoma cells. When the

mice were simultaneously treated with RSPO1-Fc-AAV and the Wnt inhibitor LGK-974, the reduced proliferation and increased apoptosis within adenoma areas were not affected. In contrast, enhanced proliferation of the adjacent normal intestinal epithelial cells was inhibited. These observations indicated that the impact of RSPO1 on intestinal adenoma growth is independent of Wnt ligands and mainly occurs via activation of the TGFβ/SMAD pathway, leading to enhanced apoptosis and eventual regression of the intestinal adenomas. (**Work I, Supplementary Figure 8-9**)

Mouse tumor cells expressing a mutant Kras are resistant to growth suppression by systemic RSPO1 expression

Our lab has previously shown that the oncogenic mutant *Kras* rescues *Apc* mutant intestinal organoids from apoptosis induced by the TGFβ/SMAD pathway (Wiener *et al.*, 2014). In advanced CRC, the growth promoting TGFβ signals are mainly provided by the stromal cells (Batlle and Massague, 2019). We considered that genetic alterations occurring during intestinal tumor progression, such as KRAS-activating and TGFβ inactivating mutations, may protect CRC cells from the growth-suppressing effects of RSPO1 treatment. To evaluate this, we treated tamoxifen-inducible $Apc^{fl/fl}$;$Kras^{G12D/+}$;$Villin\text{-}Cre^{ERT}$ KRAS-mutant mice with RSPO1 and analyzed the mice four days thereafter. In line with our previous findings, increased phosphorylation of SMAD3 and expression of p21 were repressed in the presence of mutant *Kras*, indicating that KRAS eliminates the suppression of adenoma growth caused by RSPO1 treatment. (**Work I, Supplementary Figure 10**)

Discussion

Our data thus suggests that systemic delivery of Wnt ligand, RSPO1, efficiently impedes intestinal tumorigenesis by inducing TGFβ/SMAD-mediated apoptosis and growth suppression in tumor cells, concomitantly inhibiting Wnt signaling activity. Simultaneous enhancement of proliferation of the healthy intestinal epithelial cells provides the healthy epithelium with growth advantage, which disrupts the competitive advantage of the adenoma cells, leading to an eventual regression of the existing tumors and inhibition of tumor initiation. *In vivo,* these events result in remarkably longer survival times of the tumor-bearing mice.

Intestinal adenomas in $Apc^{Min/+}$ mice most frequently develop in the small intestine. These mice only occasionally carry colonic tumors, whereas, in humans, intestinal cancer is most abundant in the colon and rectum. We thus

cannot assume that our results faithfully represent human adenomas. The RSPO1-treated mice were terminated in our survival experiments because of colonic tumors nonresponsive to RSPO1-mediated growth inhibition. The exact mechanisms that make colonic tumors resistant to RSPO1 remain to be further investigated. For example, RSPO receptors may differ between colonic epithelial cells and small intestinal epithelial cells. In further studies, DNA sequencing of the remaining colonic *Apc^{Min}* tumors could be performed to track possible additional mutations that could make these tumors resistant to the RSPO1 treatment. For further investigation of the RSPO1 effect on mouse colonic tumors, a colonoscopy-guided injection of tamoxifen or AAV-Cre (in the inducible models) or transplantation of genetically engineered organoids into mouse colonic submucosa could provide an option (Roper *et al.*, 2017). Since the mice were treated with adeno-associated viral vectors and human RSPO1-Fc protein, the involvement of immunological responses in our findings cannot be excluded entirely either.

Consistent with our previous finding of an increased sensitivity of *Apc* mutant intestinal epithelial cells to pro-apoptotic effects of the TGFβ, we showed that RSPO1 suppresses explicitly the growth of intestinal adenoma cells by activating TGFβ/SMAD signaling, whereas growth of the adjacent WT epithelium was not inhibited. If considered in the light of the "just-right Wnt" hypothesis (Fodde and Tomlinson, 2010), the upregulation of several Wnt target genes shortly after the RSPO1 treatment could sensitize the adenoma cells to apoptosis. Yet, proliferation, apoptosis, and Wnt target gene expression levels began to decline only after a few days of RSPO1 treatment, and the addition of Wnt inhibitor in combination with RSPO1 had no impact on apoptosis in the adenoma cells, suggesting that the initial increase in Wnt activity does not contribute to increased apoptosis in the adenomas.

RSPOs are agonists of the Wnt signaling pathway that, in the normal intestine, stimulate CBC cell proliferation by stabilizing β-catenin (Kim *et al.*, 2005; Binnerts *et al.*, 2007; Kim *et al.*, 2008; de Lau *et al.*, 2011), and they have been shown to enhance Wnt activity by binding to LGR4-6. This promotes their interaction with the E3 ligases RING finger protein 43 and zinc and RING finger 3 (RNF43/ZNRF3) encoded by Wnt-regulated genes, creating a negative feedback loop of Wnt signaling activity (de Lau *et al.*, 2014). Regulation of this feedback loop may be necessary in several models where the competition of normal and mutant epithelium is critical. Remarkably, a subtle reduction of Wnt ligand secretion using a porcupine inhibitor led to the accelerated fixation of *Apc*-deficient cells at the crypt bottom and enhanced tumorigenesis (Huels *et al.*,

2018). In contrast, RSPO1 promoted Wnt signaling in the WT intestinal stem cells, followed by their gain of growth advantage over the *Apc* deficient adenoma cells, which led to tumor regression.

Recently, the concept of biased competition between healthy intestinal epithelial cells and the adenoma cells was investigated further. The impact of the Wnt antagonist *Notum* was analyzed in our study in collaboration with Flanagan *et al*. (2021), as well as in another parallel research on the contribution of *Notum* to the fixation of *Apc* mutant cells in the intestinal crypts and the formation of intestinal adenomas (Flanagan *et al.*, 2021; Van Neerven *et al.*, 2021). It was suggested that in healthy intestinal epithelial cells, stemness and Wnt activity are actively suppressed by Notum and that this suppression is lost upon the loss-of-function of *Apc*, providing the *Apc* mutant cells a remarkable growth advantage over the healthy epithelium (Flanagan *et al.*, 2021; Van Neerven *et al.*, 2021). Furthermore, the *Apc* mutant tumors were shown to secrete Notum to suppress the growth of the adjacent cells (Flanagan *et al.*, 2021). This intriguing concept is well in line with our results since we detected reduced Notum expression in the RSPO1-treated tumors. This indicates that in addition to the proliferation enhancement of the healthy intestinal epithelial cells due to RSPO1 treatment, they would gain even a further growth advantage over the adenoma cells, as the inhibitory secretion of the Wnt inhibitor Notum by the adenoma cells was suppressed. All these data combined provide a convincing novel concept for future cancer treatment strategies.

In a healthy individual, the mutational burden in colorectal crypts increases by age (Lee-Six et al., 2019). The clonal evolution of colorectal crypts in a healthy epithelium was elucidated using laser capture microdissection and whole-genome sequencing of individual crypts obtained from patient samples (Lee-Six et al., 2019). All classes of somatic mutations were discovered in hundreds of normal colorectal epithelial cells, and around 1 % of crypts in a healthy 50-60-year-old individual were found to carry common colorectal cancer driver mutations in genes such as AXIN2, CDKN1B, TP53, and RNF43 (Lee-Six et al., 2019). Interestingly, even though the colon contains approximately 10 million crypts, only 5 % of people develop CRC during their lifetime, indicating that only an extremely small proportion of the mutated crypts progress into a macroscopic adenoma (less than 1:375 000) or an invasive carcinoma (less than 1:3 000 000) within the subsequent decades (Stamp et al., 2018; Lee-Six et al., 2019). The factors restricting the mutated clones from outcompeting their WT neighbors remain unknown, but carcinogens in food most probably increase the risk of CRC development. Furthermore, inflammation may play an essential role in this context since the usage of the anti-inflammatory drug aspirin significantly

reduces the risk of CRC in the elderly (Guo et al., 2021). There appears to be an equilibrium between the cells in the intestinal crypt restricting the growth of the stem cells with driver mutations, which could be disturbed if some early microneoplasms were to be suppressed. Thus, further knowledge regarding these mechanisms is required before the competitive set-up at the intestinal stem cell level should be interfered.

Endogenous RSPO gene fusions resulting in chimeric oncoproteins have been identified as Wnt-dependent drivers of intestinal tumorigenesis in vivo (Han et al., 2017). The RSPO2 gene was shown to fuse most commonly with the EIF3E gene, whereas the RSPO3 gene was most commonly fused with the PTPRK gene (Seshagiri et al., 2012). Overall, these fusions were found in approximately 10 % of hCRC tumors (Seshagiri et al., 2012). Interestingly, however, the EIF3E-RSPO2-fusion organoids did not survive without exogenous RSPO1, whereas the PTPRK-RSPO3 fusion organoids were independent of RSPO1 (Han et al., 2017). These fusions have been associated with the serrated pathway of colorectal tumorigenesis (Sekine et al., 2016). Moreover, no RSPO1 fusions were found in the studied tumors (Seshagiri et al., 2012), emphasizing the distinct molecular features of different RSPO family members in intestinal tumorigenesis: while RSPO2 and RSPO3 could be potent drivers of intestinal tumors, RSPO1 seems capable of suppressing intestinal adenomas. However, the fusions of RSPO2 and RSPO3 were mainly studied in advanced CRC, which leaves open the possibility that the effects of RSPOs could turn from tumor suppressive to pro-tumorigenic as the tumors progress.

According to current knowledge, the role of the tumor microenvironment in intestinal tumorigenesis is remarkable. In my studies, we did not perform specific analyses regarding the tumor microenvironment, stromal components, or tumor-infiltrating leukocytes. However, the Wnt/β-catenin signaling pathway has been associated with inflammation and modulation of the immune responses; for example, a pro-inflammatory cytokine TNF-α can activate β-catenin in intestinal crypts via enhancement of NFκB and TNF-α (Schwitalla et al., 2013; Bradford et al., 2017). Furthermore, in cultured gastric cancer cells, high Lgr5 expression was associated with the immunosuppressive environment, and the co-culture of these cells with regulatory T-cells upregulated Lgr5 expression via the TGFβ signaling pathway (Liu et al., 2019). In addition, a high Lgr5 level in combination with an increased number of regulatory T-cells in the patient samples was associated with poor prognosis in gastric cancer (Liu et al., 2019), underlining the significant crosstalk between the immune response and the activated Wnt signaling pathways during the tumorigenesis. Taking these into account, further

evaluation of the effects of RSPO1 on the tumor microenvironment and immune response would be required.

II. EXPLORATION OF RSPO1 EFFECT IN COLORECTAL TUMORS INDUCED BY A CARCINOGEN PLUS AN INFLAMMATORY AGENT

Since we found that RSPO1 treatment had little effect on tumors with a *Kras* mutation, we sought to further investigate the effect of RSPO1 in tumors with several unspecific mutations. We collaborated with Dr. Saara Ollila to model mouse sporadic and inflammation-associated colorectal tumorigenesis. The WT mice were first injected with the mutagenic azoxymethane (AOM) and then administered with dextran sodium sulfate (DSS) in drinking water during three consecutive periods. They were injected with RSPO1-Fc-AAV or Ctrl-AAV on day 28 after the start of the AOM treatment, as shown in **Figure 7A**. The weights of the mice were closely monitored, and they were terminated on day 70. The effect of RSPO1 was evident from the finding that the average body weight loss in the RSPO1 treated group was significantly lower than in the control group (**Figure 7B**). However, the average number or size of the colonic tumors was not affected by the RSPO1 treatment, indicating that they are resistant to RSPO1-mediated growth suppression. Further analysis of the RSPO1 treated tumors showed fewer PROX1+ cells, reduced cell proliferation, and increased apoptosis within the β-catenin positive tumor area (**Figure 7C-E**). These results indicate that despite the tumor resistance to RSPO1-mediated growth suppression, the tumor-bearing mice benefited from RSPO1 treatment. This benefit could be due to increased growth of the healthy intestinal epithelium, decreasing overall intestinal damage. Because tumorigenesis in this model involves untargeted mutagenesis plus inflammation, the mutational status of the tumor cells should be highly heterogenic, complicating mechanistic deductions. This should be kept in mind when evaluating the results obtained from experiments performed with the AOM-DSS model.

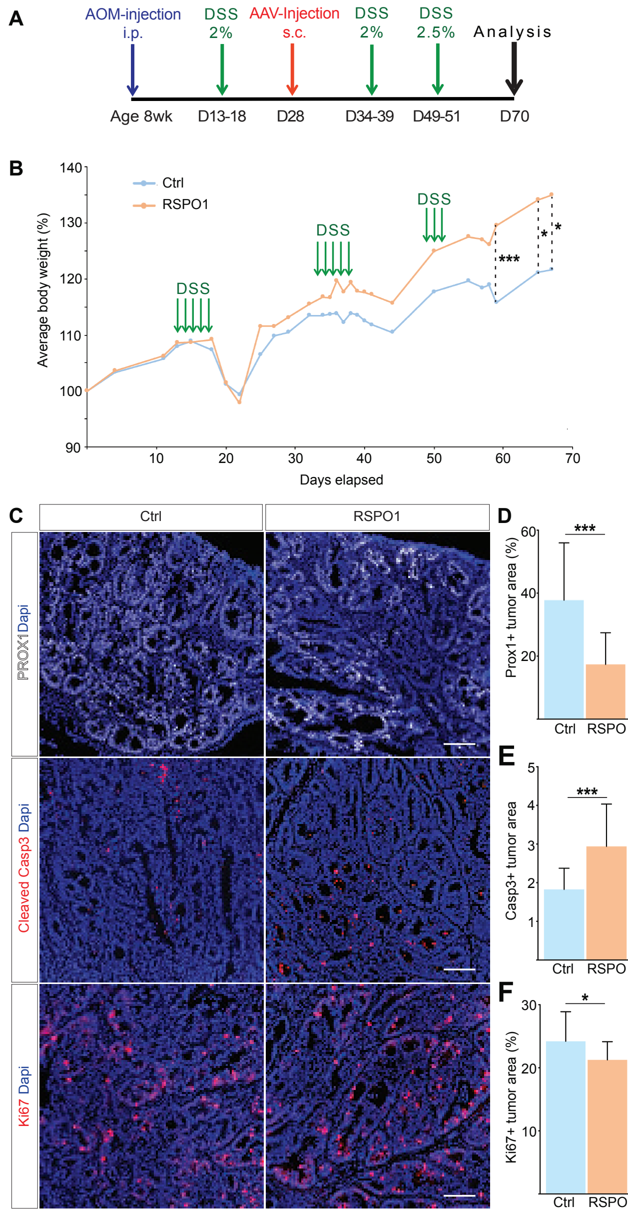

Figure 7. RSPO1 expression in AOM-DSS treated mice suppresses Wnt signaling and proliferation and induces apoptosis in colonic tumors. (*A*). 8-week-old WT mice were injected intraperitoneally with AOM, treated with 2% DSS in their drinking water for five days, followed by subcutaneous injections of RSPO1-Fc-AAV or Ctrl-AAV ($1x10^{12}$ vp) on day 28. Two more cycles of DSS treatment were given during days 34-39 and 49-51 and the mice were analyzed on day 70. (***B***). The average relative body weights of the RSPO1-treated vs. the control mice. (***C***). Colonic tumors in the mice were stained and quantified for PROX1, (*C, D*), cleaved Caspase-3 (*C, E*), and Ki67 (*C, F*) in the nuclear β-catenin positive area. Scale bar: 100µm. Data are presented as mean + standard deviation (SD). Student unpaired t-test, *P <0,05, ***P < 0,001. Unpublished data of ML and SO.

III. PRELIMINARY ANALYSIS OF THE EFFECTS OF RSPO2 AND RSPO3 ON ADENOMA DEVELOPMENT

Because the R-spondin family members RSPO2 and RSPO3 are more potent in enhancing Wnt signaling than RSPO1 and RSPO4 (Kim *et al.*, 2008), we were interested in comparing their effects on intestinal tumorigenesis. We generated AAV-vectors encoding RSPO2-Fc and RSPO3-Fc fusion proteins, which we injected into 15-week-old $Apc^{Min/+}$ mice (**Figure 8A**). When analyzed one or four weeks later, the total number or average diameter of the intestinal tumors was not significantly affected by RSPO2 or RSPO3 (**Figure 8B** and data not shown). In immunofluorescence analysis of the tumor sections, PROX1 expression level and β-catenin positive adenoma areas were similar between the RSPO2 and RSPO3 treated tumors and control samples (**Figure 8D-E**). Interestingly, however, the RSPO2-treated tumors showed significantly more proliferation in the β-catenin positive adenoma areas, whereas cell proliferation outside of the tumor areas was not affected. RSPO3 treated samples showed less proliferation in the adenoma areas and more proliferation outside of them when compared with the control samples (**Figure 8D, F**).

These results suggest differences in the potential of RSPO family members to regulate intestinal tumorigenesis. However, the results need to be confirmed, and the mechanisms behind any distinct functions remain to be further investigated. One possible explanation could be the domain structure variation between the family members, which shows only 40-60 % amino acid sequence conservation (Kim *et al.*, 2008). The two furin-like domains of the R-spondins are essential for the efficient enhancement of Wnt signaling, whereas different domains specify the individual activity of each RSPO family member (Kim *et al.*, 2008, De Lau *et al.*, 2012). The possibility that a binding site for the LGR5/TGFβR2 heteromeric complex is exclusively present in RSPO1 would require further structural evaluation of the RSPO homologs in this specific context.

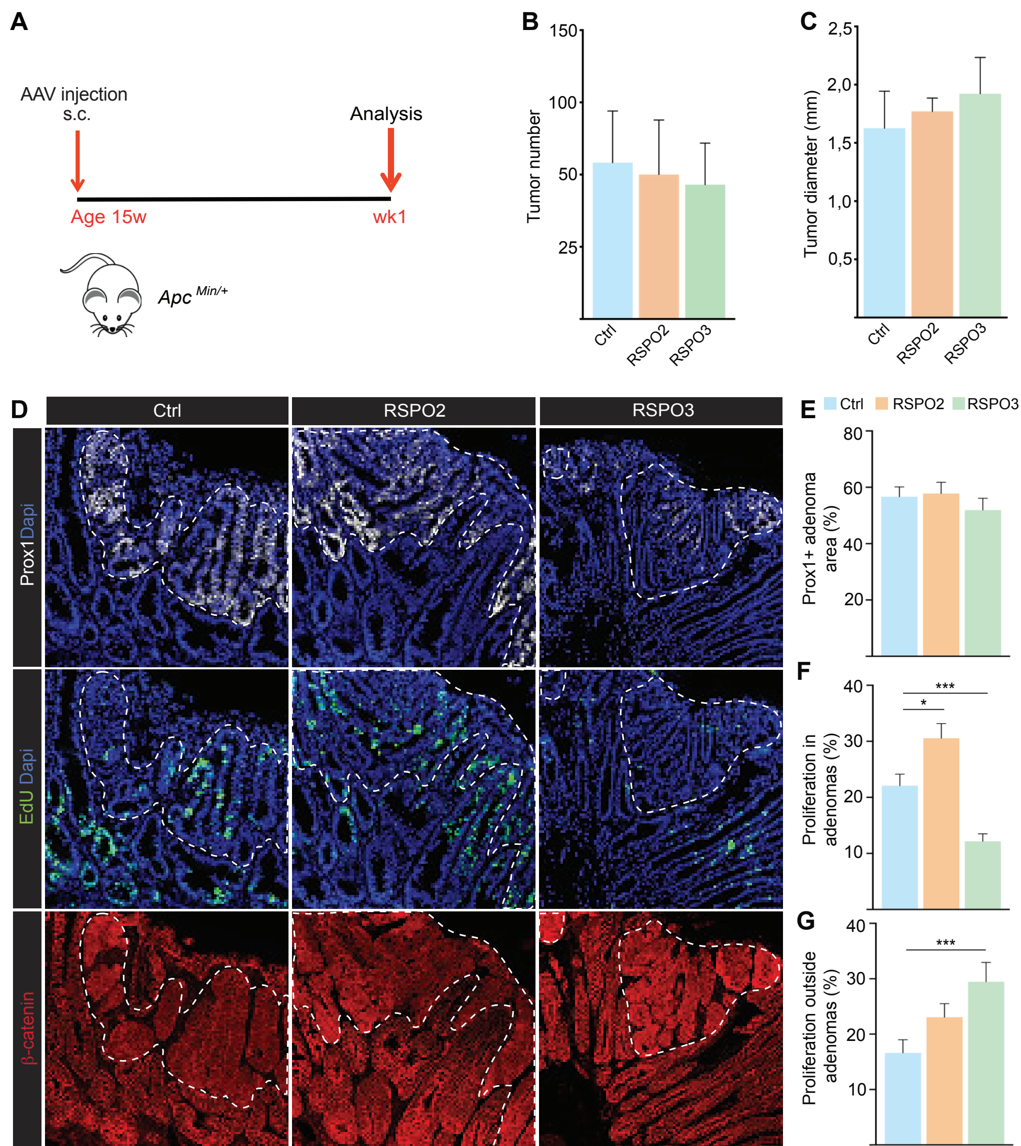

Figure 8. RSPO family members have a distinct impact on intestinal adenomas $Apc^{Min/+}$ mice. (***A***). 15-week-old $Apc^{Min/+}$ mice were injected subcutaneously with RSPO2-Fc-AAV, RSPO3-Fc-AAV, or Ctrl-AAV (1x10^{12} vp) and analyzed one week after that. (***B***). The number of macroscopic intestinal tumors in different groups. (***C***). Average size on macroscopic tumors of the small intestines of the mice. (***D***) Immunofluorescent staining for PROX1 and β-catenin and detection of EdU in the tumor sections. (***E***). Relative PROX1+ area within the nuclear β-catenin positive adenoma area. (***F***). Relative EdU+ area within nuclear β-catenin positive area. (***G***). Relative EdU area within the β-catenin positive adenoma area. Data are presented as mean+SD. Ordinary one-way ANOVA, multiple comparisons, *P <0,05, ***P < 0,001. Unpublished data.

IV. COMBINATION OF RSPO1 WITH INHIBITION OF MYC-MEDIATED APOPTOSIS

High expression levels of the Wnt target gene MYC increases the sensitivity of cancer cells to apoptosis by activating the AMP-activated protein kinase (AMPK) and the AMPK activator and the diabetes drug metformin synergizes with inhibitors of the anti-apoptotic BCL-2/BCL-X_L protein (BH3) family (Pelengaris *et al.*, 2002; Nieminen *et al.*, 2007; Haikala *et al.*, 2019). Treatment of mice modeling MYC-high breast cancer with a combination of metformin and BCL-2 specific inhibitor venetoclax induced apoptosis in the tumors, increased survival of the tumor-bearing mice, and promoted immune cell infiltration and activity in the tumor tissue (Haikala *et al.*, 2019). Since elevated Myc levels were detected in the RSPO1-treated mouse intestinal adenoma cells *ex vivo* and *in vivo*, I analyzed if the combination of the apoptosis sensitizers with RSPO1 would increase apoptosis in RSPO1-treated tumors even further and if this would lead to the total eradication of the tumors. 17-week-old *Apc*$^{Min/+}$ mice were injected with a single dose RSPO1-Fc-AAV or Ctrl-AAV, treated with metformin+venetoclax (Met+Ven) combination for four consecutive days, and then analyzed (**Figure 9A**). Compared to the control group, a significant reduction cell proliferation rate was detected in *Apc*$^{Min/+}$ adenomas treated with either RSPO1 or Met+Ven separately, yet no additional reduction was achieved using their combination (**Figure 9B-C**). The increase in proliferation of normal intestinal epithelial cells by RSPO1 was not affected by the inclusion of Met+Ven (**Figure 9B, D**). Met+Ven treatment without RSPO1 had no significant effect on apoptosis of tumor cells, but a substantial increase in apoptosis was obtained by RSPO1+Met+Ven, unlike in the healthy intestinal epithelium (**Figure 9B, E-F**), suggesting that elevated Myc expression in the tumors by RSPO1 sensitizes tumor cells for the pro-apoptotic effects of Met+Ven. Although further experiments would be needed to determine whether a complete tumor eradication can be achieved, the preliminary data seems promising. Since both metformin and venetoclax are widely used in the clinic, these results could be translationally significant.

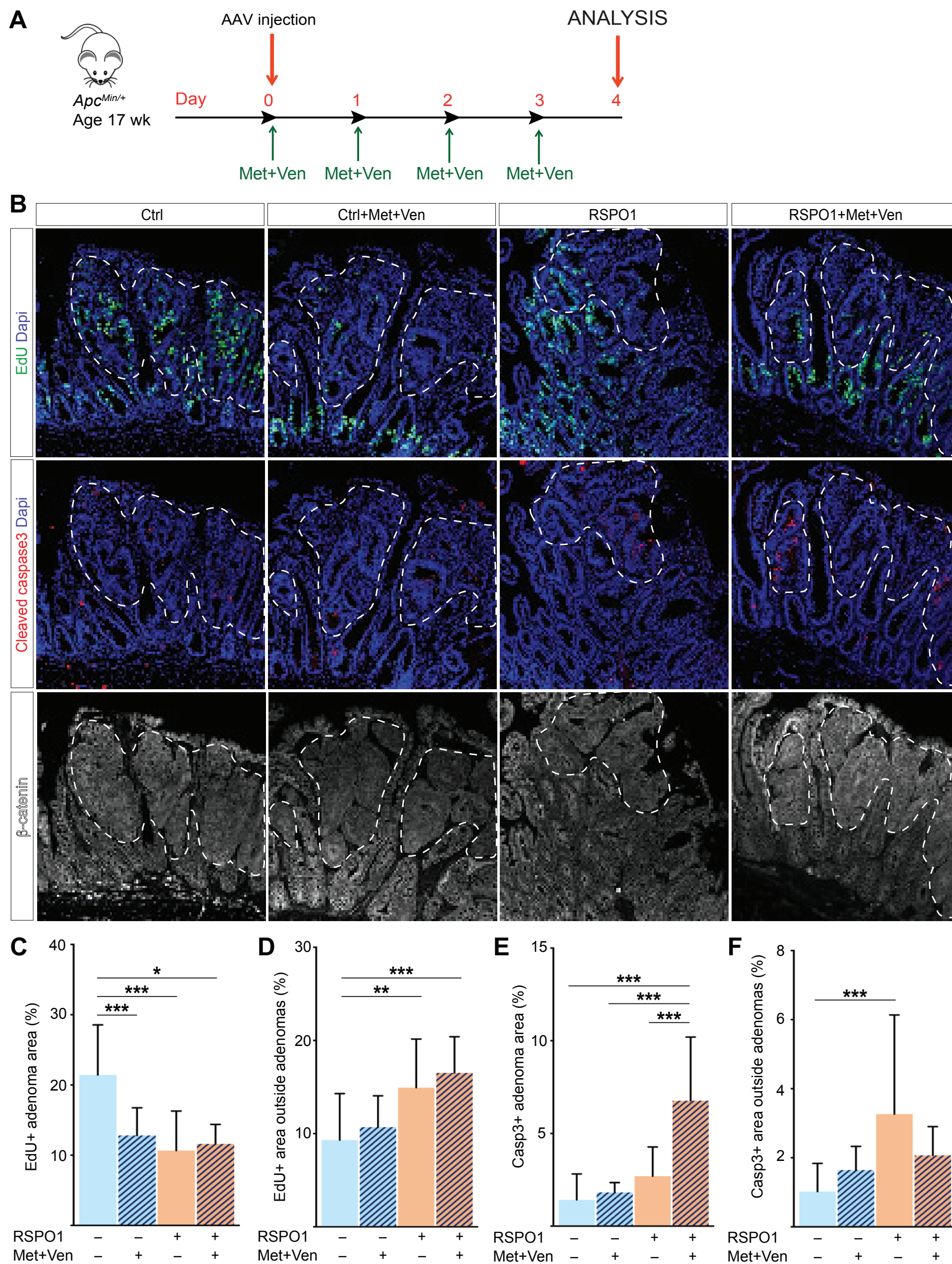

Figure 9. Combination of RSPO1 with apoptosis-sensitizers metformin and venetoclax results in a radical increase in apoptosis in $Apc^{Min/+}$ mouse intestinal adenomas. (*A*). 17-week-old $Apc^{Min/+}$ mice received RSPO1-Fc or Ctrl-AAV ($1x10^{12}$ vp) and were treated with Met+Ven or vehicle for four consecutive days. The mice were analyzed on day four. (***B***). Immunofluorescent staining for cleaved caspase-3, β–catenin, and detection of EdU in the adenoma sections, (***C***). Quantification of relative EdU+ area within adenoma areas. (***D***). Quantification of relative EdU+ area outside adenomas. (***E***). Quantification of the relative cleaved caspase-3+ area within adenomas. (***F***). Quantification of the relative cleaved caspase-3+ area outside adenomas. Data are presented as mean + SD. Student unpaired t-test, *P <0,05, **P<0,01, ***P < 0,001. Unpublished data.

CONCLUSIONS AND FUTURE PROSPECTS

CRC is one of the most common cancers in the western world, causing a large proportion of cancer-related deaths. According to previous knowledge, most CRCs are initiated via a stepwise sequence of mutations inactivating tumor suppressors and activating oncogenes. After the discovery of intestinal stem cell-specific markers, the contribution of stem cells to intestinal cancer initiation, progression, and prognosis has been extensively studied. This research has uncovered the involvement of growth factors and signaling pathways that drive the proliferation of intestinal stem cells and maintain the limitless growth capacity of cancer cells. In the present study, we defined a novel role for RSPO1, which was previously known as a potent enhancer of Wnt signaling and intestinal stem cell growth.

Our data indicate that RSPO1 can disrupt the competition between the healthy intestinal epithelial cells and adenoma cells by activating TGFβ/SMAD signaling and apoptosis pathways and by concomitantly promoting the proliferation of the adjacent healthy intestinal epithelial cells. Because of the mutation in the *Apc* gene in $Apc^{Min/+}$ mice, the adenoma cells are sensitized to the growth suppression mediated by the TGFB/SMAD signaling. This provides the normal epithelium a remarkable growth advantage over the adenoma cells, leading to an eventual regression of the adenomas and prolonged survival of the tumor-bearing mice. That a growth factor resulted in a significant increase in survival of the $Apc^{Min/+}$mice was totally unexpected. Our data provide a novel perspective on possible new tumor treatment strategies, in which reprogramming of the competition between the tumor and the healthy tissue could be tried to inhibit adenoma growth. For clinical translation of our results, one could experiment with controlled delivery and intestine-specific expression of the RSPO1. For example, a combination of RSPO1 transfection together with a targeted repair of *Apc* or *CTNNB1* mutations *ex vivo* could be envisaged in organoids from $Apc^{Min/+}$mice, followed by transplantation of the repaired intestinal epithelial cells to the gut. Moreover, various combination treatments involving RSPO1 could be tried. As explained above, Myc expression is increased in CRC, and Myc overexpressing cells are sensitive to apoptosis when exposed to AMPK activating drug metformin plus BH3 mimetic drug Venetoclax. Along with this rationale, we have already done some preliminary experiments, as shown in the results and discussion. On the other hand, one could search for suitable combinations by drug screening studies using clinical cancer drugs or chemical compound libraries in cultures of $Apc^{Min/+}$ intestinal organoids. Innovative off-

the-box development of such strategies could eventually benefit patients suffering from FAP, who can currently be treated only by prophylactic subtotal colectomy, which, understandably, leads to a substantial reduction in the quality of life.

My thesis should provide a novel insight into the interaction of the TGFβ/SMAD and Wnt signaling pathways in the initiation and growth of intestinal tumors and expand the current knowledge regarding the regulation of CRC tumors and stem cells. I show that the abnormal recruitment of the TGFβ/SMAD pathway by heterodimers formed by LGR5 and TGFβRII leads to apoptosis of Apc mutant adenoma cells. This results in growth suppression in the tumors and inhibits the initiation of new adenomas caused by the Apc^{Min} allele. Our results are consistent with the previously published data suggesting that RSPO2 can reduce the growth of two hCRC cell lines via activation of an LGR5-dependent Wnt-inhibiting feedback loop (Wu *et al.*, 2014). Although activating chromosomal rearrangements that target RSPO2 and RSPO3 have been shown to induce Wnt signaling and drive intestinal tumorigenesis, their ability to give rise to tumors alone remains uncertain (Seshagiri *et al.*, 2012; Han *et al.*, 2017). Moreover, based on my preliminary experiments shown in the results and discussion, the effect of RSPO1 is distinct from that of RSPO2 and RSPO3 in the context of intestinal tumor initiation and growth. Altogether, these results highlight the extreme complexity of the Wnt pathway and its interacting signaling pathways in the regulation of intestinal stem cells and tumorigenesis and fuel enthusiasm for further investigations.

According to recent studies, the role of the tumor microenvironment in intestinal tumorigenesis is remarkable. However, I did not focus on the tumor microenvironment, for example, stromal components or tumor-infiltrating leukocytes in our studies, even though the Wnt/β-catenin signaling pathway has been associated with inflammation that could modulate anti-tumor immune responses. For example, the pro-inflammatory cytokine TNF-α can activate β-catenin in intestinal crypts via enhancement of NF-κB and TNF-α expression (Schwitalla *et al.*, 2013; Bradford *et al.*, 2017). We did not either consider RSPO1 treatment combinations with immune checkpoint inhibitors because the mutation load in the $Apc^{Min/+}$ adenomas should be low, and they thus likely would not respond well to immunotherapy. Moreover, it has been shown that $Apc^{Min/+}$ mice usually lack intestinal inflammation and that the $Apc^{Min/+}$ mutation solely does not significantly alter the mouse intestinal immune function (Kettunen *et al.*, 2003), suggesting the modest role of immunity in our experiments.

The incidence of CRC worldwide is expected to increase by 60 % within the next ten years (Arnold *et al.,* 2017). However, the growth in the burden rate seems to be fastest in the low- and middle-income countries, as the incidence, and especially CRC mortality, is decreasing in the Western world (Arnold *et al.,* 2017). These trends provide good indicators that the various types of cancers related to a high quality of life are replacing the infection-related cancers in the fast-developing countries (Arnold *et al.*, 2017). Thus, one of the future challenges would be to enable even the most novel and efficient treatment strategies for the patients in developing countries to decrease the total burden of CRC worldwide. To achieve this goal, extensive research and development will still be required to provide applicable targets and technologies. I hope that my research results presented here would offer valuable possibilities for further studies along these lines.

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