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DECIPHERING CELL MOTILITY AND SPATIAL SENSING OF INTESTINAL CELL TYPES USING AN *EX VIVO* INTESTINAL MODEL

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Cover illustration: The cover features a fluorescently stained section of a decellularized small intestine from a mouse. The prominent large structures represent the domains of the intestinal villi, while the circular openings indicate the entrance to the intestinal crypt structures.

Deciphering cell motility and spatial sensing of epithelial cell types using an *ex vivo* intestinal model

Thesis for Doctoral Degree (Ph.D.)

By

Anna Webb

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To the strongest person I have ever known, my Mother Katherine.

Popular science summary of the thesis

The intestine, often referred to as the gut, has an exceptional capability of renewing itself. Its inner lining, made up of specialized cells called epithelial cells, undergoes renewal every 5-7 days, making it the fastest regenerating organ in the body. This incessant regeneration serves multiple vital purposes. It acts as a defense against harmful viruses, bacteria, and other microorganisms present in the food we consume. Simultaneously, it efficiently absorbs crucial nutrients and water while disposing of waste materials.

The intestine consists of two primary segments: the small intestine and the large intestine. The small intestine features finger-like projections known as villi, as well as smaller pockets termed crypts. Each of these regions harbors distinct cell types, each with a specific role to play. Within the crypts, there is a particular type of cell known as paneth cells. These cells provide essential support to their neighbors, the stem cells.

Stem cells play a crucial role, they have the ability to divide and give birth to new cells. These newborn cells can then journey upwards towards the villi. As these cells ascend, they undergo a transformation. They morph into various cell types, such as goblet cells (responsible for maintaining the gut's lubrication) and enterocytes (the professionals at absorbing nutrients). Upon reaching the top of the villi, these cells exit into the intestinal lumen, creating space for the next generation of cells. Empowering this entire process is a vast structure known as the extracellular matrix, which provides the architectural framework of your gut. It provides the necessary structural support and acts as a repository for essential microenvironmental factors.

While significant strides have been made in understanding this process, there is still much to uncover. Our current focus lies in deciphering the intricacies of cell migration, regeneration, and the aging process within the gut. Additionally, we investigate how cancer cells compete with normal cells for space and location in the tissue.

In **Papers 1** and **2** of this thesis, we first investigate which stem cells are able to stay within the crypt for the longest amount of time, indicating which stem cells are the "best" functioning stem cells. To uncover this, we employed a cutting-edge method that enabled us to observe stem cells inside the intestines of mice over several weeks. The findings unveiled a clear distinction: the intestine boasts twice as many long-lasting stem cells compared to the large intestine. To dig even deeper, we conducted an experiment where the essential components of the small intestine were conserved, including its intricate structures, the villus, and crypts, along with the extracellular matrix that provides vital support. These preserved structures, which we call "scaffolds", were used as a platform to place isolated single stem cells and closely monitor their behavior

under a microscope. Astonishingly, stem cells in the intestine displayed an ability to navigate to the crypt's bottom by themselves, without any assistance from neighboring cells. However, their counterparts in the large intestine could not accomplish this. This unique downward movement of stem cells was orchestrated by a special element present in the extracellular matrix known as Wnt. Wnt functioned as a compass, helping stem cells find their designated home in the intestine.

We expanded our investigation to explore whether neighboring paneth cells also possess this solitary navigation skill. We discovered that paneth cells could indeed descend to the crypt bottom when left to their own devices. However, when we turned our attention to another cell type, the enterocytes, it was a different story. These cells rely heavily on their neighbors for guidance and do not journey independently to the villus. Finally, we delved into how the navigation system of stem cells is affected by aging and the onset of intestinal cancer. We discovered in both these scenarios, that stem cells lost their way and were not able to find their way home, to the crypt bottom.

Together, these two papers show that cells in the gut sense where they should be, by following a sort of map. But in aging and during the initiation of cancer, cells lose their way.

In **Paper 3** we came up with a new approach to understand how the supporting structures, the extracellular matrix, affect the ability of intestinal tissue to heal after injury. We put single stem cells as well as clusters of cells onto the intestinal scaffolds and were able to re-grow the cell lining of the intestine. Using this approach, we discovered that during this re-growing phase, there is a factor within the extracellular matrix called Asporin that gets more activated, assisting in the healing process. Interestingly, when instead investigating an aged intestine, this factor, Asporin, was activated constantly, and was instead slowing down the healing process.

In the final paper, **Paper 4**, we explored how cancer cells in the intestine are able to become dominant and take over, causing growths called adenomas (mini tumors), the first stage of intestinal cancer. We found that these dominant cancer cells produce a factor called Notum. This contributes to cancer cells becoming more competitive through suppressing the abilities of the surrounding normal stem cells, thereby, reducing the competition. Fortunately, we were able to stop the Notum production, which resulted in smaller adenomas, and enhanced the survival of mice.

In summary, our exploration throughout these four papers highlights the remarkable capabilities of the intestine, from maintaining balance to understanding new aspects of aging and intestinal cancer. This thesis reveals new insights into intestinal biology and potential avenues for future research and interventions.

Abstract

The intestine is a highly organized tissue with two distinct regions: the crypt and the villus. When stem cells divide at the crypt bottom, half of their progeny migrates upwards towards the villus, where they differentiate into various cell types, including the abundant absorptive enterocytes. However, the precise mechanisms governing this migration and tissue organization remain poorly understood. In this thesis, novel methodologies, such as long-term intravital imaging and decellularization of mouse intestine, are used to study cell type-specific motility within the tissue architecture. Moreover, work in this thesis probes the mechanisms mediating intestinal regeneration and aging, and the clonal competition during tumor development.

In paper 1, we employ long-term intravital imaging to identify a greater number of long-term functioning intestinal stem cells (ISCs) in the small intestine compared to the colon. We further investigate this phenomenon by combining intravital imaging and the novel *ex vivo* live cell imaging assay to discover that stem cells in the small intestine display downward motility directed by Wnt-ligands.

In Paper 2, the *ex vivo* live cell imaging assay was utilized to investigate active cell migration in several cell types. Our findings reveal that both ISCs and paneth cells possess an intrinsic ability to perceive positional cues embedded in the extracellular matrix (ECM), which guides them to their native location, the crypt. In contrast, enterocytes, lack this capability. Finally, we discovered that during aging ECM loses the signals guiding crypt homing of ISCs, and that the tumor-causing mutations render cells insensitive to ECM signals resulting in loss of crypt homing.

In Paper 3, we introduce an optimized intestinal decellularization protocol and demonstrate its capacity to regenerate the intestinal epithelium from single-seeded stem cells, freshly isolated crypts, or organoids. During regeneration following damage, we discovered mesenchymally produced Asporin, which promotes Tgfβ-signaling and induces fetal-like reprogramming in intestinal tissue. Additionally, we observed that chronic upregulation of Asporin in the aged intestinal tissue hampers tissue repair.

In Paper 4, we elucidate how *Apc*-mutant ISCs gain a clonal advantage over wild-type ISCs. We reveal that *Apc*-mutant ISCs secrete the Wnt-inhibitor Notum, which reduces the stemness and competitiveness of wild-type ISCs. Inhibition of Notum reverted the clonal advantage of *Apc*-mutant cells and reduced tumor burden.

In conclusion, this thesis focused on highlighting the interplay between intestinal epithelial cells and the ECM, particularly the ability of ISCs and paneth cells to sense positional cues embedded in the ECM, guiding them to their native location. Additionally, key mechanisms disrupted during aging and in intestinal cancer are elucidated.

List of scientific papers

Maria Azkanaz*, Bernat Corominas-Murtra*, Saskia I.J. Ellenbroek*, Lotte Bruens*, **Anna.T.Webb*,** Dimitrios Laskaris, Koen C. Oost, Simona J.A. Lafirenze, Karl Annusver, Hendrik A. Messal, Sharif Iqbal, Dustin J. Flanagan, David J. Huels, Felipe Rojas-Rodríguez, Miguel Vizoso, Maria Kasper, Owen J. Sansom, Hugo J. Snippert, Prisca, Liberali, Benjamin D. Simons, Pekka Katajisto, Edouard Hannezo, and Jacco van Rheenen. *Retrograde movements determine effective stem cell numbers in the intestine*. Nature, 2022 July, Volume 608: 548-554.

Anna.T.Webb, Karl Annusver, Agustin Sola Cavajal, Sandra Scharaw, Sharif Iqbal, Maria Kasper, Pekka Katajisto. *Aging and Apc mutations abolish the niche-homing capability of intestinal stem cells*. Manuscript.

Sharif Iqbal, Simon Andersson*, Ernesta Nestaite*, Nalle Pentinmikko, Ashish Kumar, Sawan Jha, Daniel Borshagovski, **Anna.T.Webb,** Nadja Gebert, Emma W. Viitala, Sandra Scharaw, Hjalte L. Larsen, Tuure Saarinen, Anne Juuti, Saara Ollila, Ari Ristimäki, Michael Jeltsch, Alessandro Ori, Markku Varjosalo, Kirsi H. Pietiläinen, Kim B. Jensen, Menno Oudhoff, Pekka Katajisto. *Fetal-like reversion in the regenerating intestine is regulated by mesenchymal Asporin*. (*In revision in Cell Stem Cell*)

Dustin J. Flanagan, Nalle Pentinmikko, Kalle Luopajärvi, Nicky J. Willis, Kathryn Gilroy, Alexander P. Raven, Lynn Mcgarry, Johanna I. Englund, **Anna.T.Webb**, Sandra Scharaw, Nadia Nasreddin, Michael C. Hodder, Rachel A. Ridgway, Emma Minnee, Nathalie Sphyris, Ella Gilchrist, Arafath K. Najumudeen, Beatrice Romagnolo, Christine Perret, Ann C. Williams, Hans Clevers, Pirjo Nummela, Marianne Lähde, Kari Alitalo, Ville Hietakangas, Ann Hedley, William Clark, Colin Nixon, Kristina Kirschner, E. Yvonne Jones, Ari Ristimäki, Simon J. Leedham, Paul V. Fish, Jean-Paul Vincent, Pekka Katajisto & Owen J. Sansom. *NOTUM from Apc-mutant cells biases clonal competition to initiate cancer*. Nature, 2021 June, Volume 594: 430-435.

* These authors have contributed equally.

Contents

List of abbreviations

APC	Adenomatous polyposis coli
Aspn	Asporin
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
EC	Enterocyte
ECM	Extracellular matrix
EM	Electron microscopy
FAP	Familial adenomatous polyposis
ISC	Intestinal stem cell
Lgr	Leucine-rich repeat-containing G-protein coupled receptor
Li	Large intestine
NICD	Notch intracellular domain
PC	Paneth cell
qPCR	Quantitative polymerase chain reaction
SDC	Sodium deoxycholate
SI	Small intestine
TA-cells	Transit-amplifying cells
Wnt	Wingless-related integration site
WT	Wild-type

1 Introduction

1.1 Overview of the intestine

The intestine is the fastest renewing organ in the mammalian body, renewing its epithelial lining every 3-5 days (Cheng and Leblond 1974, Barker, van Es et al. 2007). The epithelial lining of the intestine also serves as an interactive surface between the resources of the external environment and the rest of the body, thereby, it must form a protective barrier against foreign micro-organisms while allowing the transport of small molecule metabolites and protein antigens (Barker, Huls et al. 1999, Vancamelbeke and Vermeire 2017). Digestion and absorption of nutrients occur mostly in the small intestine (subdivided into proximal duodenum, jejunum, and distal ileum), while the colon absorbs water and remaining nutrients, as well as excretes waste products. The epithelium in both parts forms invaginations called crypts, and reflecting its high absorptive capacity, epithelium in the small intestine also forms fingerlike projections referred to as villi, which increase the absorptive surface (**Figure 1**) (Leedham, Brittan et al. 2005).

1.2 Organization and epithelial cell types

Within the crypts of the intestine reside the large, granulated Paneth cells (PCs), and squeezed in between them are the Lgr5+ intestinal stem cells (ISCs). The ISCs divide every day generating new ISCs, which will either remain as ISCs or become transit-amplifying cells (TA-cells) and give rise to differentiated cells of the villus domain (Bjerknes and Cheng 1981, Barker, van Es et al. 2007). These differentiated cells will become either cells of the absorptive lineage such as the highly abundant enterocytes (ECs), or of the secretory lineage such as the mucin-producing goblet cells (Noah, Donahue et al. 2011) (**Figure 1**). Interestingly, paneth cells of the secretory lineage is the only differentiated cell type that is found not in the villus but instead in the crypt. Eventually, all other differentiated cells will shed off into the lumen at the tip of the villi and commit apoptotic cell death (Clevers 2013).

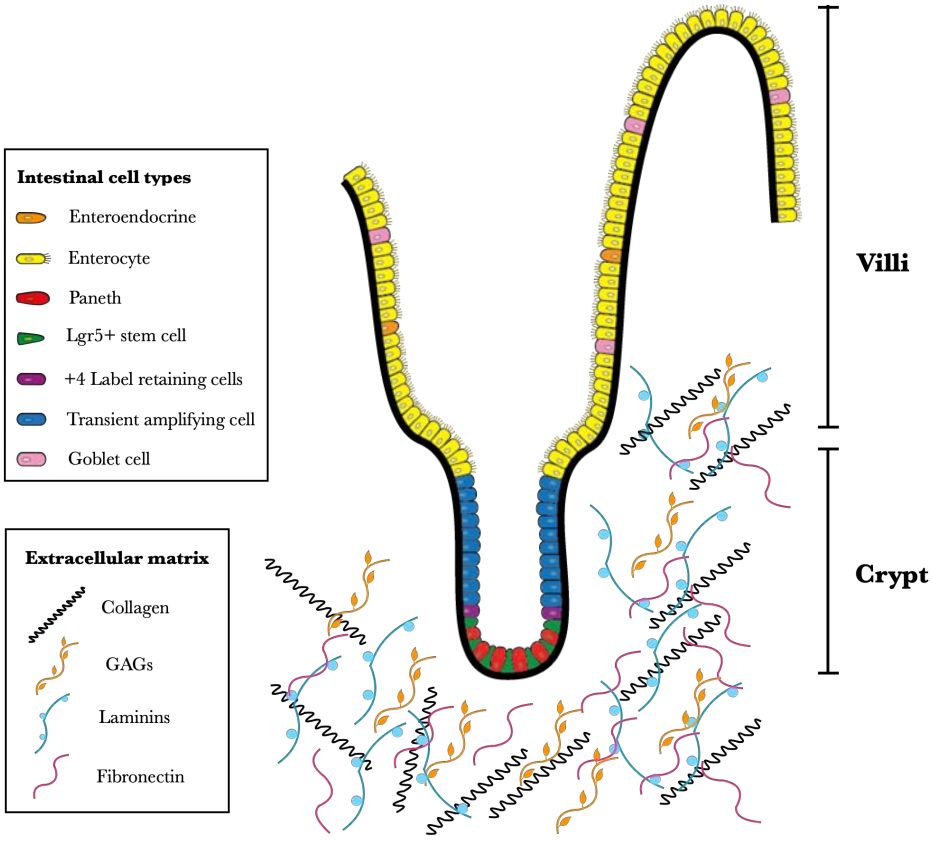


Figure 1. Organization of the small intestine. The Lgr5+ stem cells (green) reside in the crypt pit together with the neighboring Paneth cells (red). These stem cells divide every day giving rise to new cells, which will first migrate, joining the transit-amplifying zone (blue), continue dividing and migrating vertically, and eventually differentiate into the secretory (orange, pink) or absorptive (yellow) cell lineage in the villus domain. Surrounding the intestinal epithelial cells is the supporting extracellular matrix, including collagen, glycosaminoglycans (GAGs), laminins, and fibronectin.

1.2.1 Enterocytes

The absorptive ECs, also known as columnar cells, are highly polarized cells residing in the villus domain of the intestine. This cell type constitutes 80% of all intestinal epithelial cells. Each EC is equipped with an apical brush border consisting of microvilli (Mooseker 1985), significantly increasing their surface area. The microvilli, in conjunction with their glycocalyx tips, serve as a diffusion barrier, preventing direct contact between macromolecules like viruses and bacteria in the lumen and the apical surface of the epithelium. Simultaneously, they facilitate the absorption of nutrients, water, electrolytes, and the transport of ions (Snoeck, Goddeeris et al. 2005).

1.2.2 Paneth cells

PCs are large secretory cells with granules containing antimicrobial peptides, such as lysozyme, cryptdins, and defensins. The contents of these granules can be released into the lumen, as part of the innate immune system, providing a defense against foreign pathogens, and controlling the microbiota (Clevers and Bevins 2013, Barreto E Barreto, Rattes et al. 2022). Paneth cells also play a vital role in supplying ISCs with microenvrionmental factors to maintain their proliferative capacity (see section 4.).

1.2.3 Stem cells

The intestine of a mouse has about 1 million crypts, and each crypt generates around 3000 cells every day (Hagemann, Sigdestad et al. 1970). This impressive renewal capability depends on the actively-cycling Lgr5+ ISCs, which reside at the bottom of each crypt, and are also referred to as crypt base columnar ISCs (Barker, van Es et al. 2007). The stem cell marker leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) is a receptor tyrosine kinase, which is activated by binding of the ligands R-spondins (de Lau, Peng et al. 2014). The binding of R-spondin to the Lgr5 receptor activates the Wnt/β-catenin-signaling pathway which in turn functions to maintain and regulate the survival of ISCs as well as differentiation (Carmon, Gong et al. 2011).

Lgr5+ ISCs divide symmetrically to generate two ISCs of equal potency (Snippert, van der Flier et al. 2010). Each daughter cell will compete for the limited space at the bottom of the crypt pit, where growth factors and interactions with other cells forming the *stem cell niche* maintain their stem cell potency (see section 4.). As both daughter cells are considered to have an equal opportunity of staying in the crypt pit, the selection of long-term stem cell clones follows neutral competition (Snippert, van der Flier et al. 2010). Even though this process was first classified as random throughout the crypt, it was later discovered that the ISCs located further down in the crypt pit do have an advantage in not being relocated from the crypt pit, compared to their competitors (Ritsma, Ellenbroek et al. 2014).

1.3 Lineage definition

Cell lineage determination from stem cell division hinges on signaling molecules within key pathways such as the Wnt- and Notch pathways. Below, the crucial stages in cell lineage determination are outlined.

To commit a TA-cell to a secretory fate such as the paneth-, enteroendocrine-, or goblet cells, a loss of Notch function is required. This is achieved by the Notch target Hes1 repressing downstream, the transcription of the BHLH family member of transcription factors, Math1 otherwise referred to as ATOH1 (Jensen, Pedersen et al. 2000, Yang, Bermingham et al. 2001). For the secretory cell to differentiate into a goblet cell, 3 factors are needed; Gfi1, kruppel-like factor 4, and E47-like factor 3 (Elf3) (Katz,

Perreault et al. 2002, van der Flier and Clevers 2009). If the cell is to become an enteroendocrine cell, Neurogenin 3 is required (Jenny, Uhl et al. 2002), while for paneth cell commitment, the combination of GFi1 and Sox9 is instead needed (Bastide, Darido et al. 2007, Mori-Akiyama, van den Born et al. 2007). Finally, if a TA-cell is to become an absorptive EC, Notch signaling is required. Notch functions to activate Hes1 through the transcription factor Elf3, which together activates downstream the receptor Tgf- βR11 promoter (Jensen, Pedersen et al. 2000, Ng, Waring et al. 2002, Flentjar, Chu et al. 2007).

Interestingly, the order of lineage specification can be reshuffled during tissue regeneration, wherein a significant portion of differentiated cells exhibit the ability to dedifferentiate for the restoration of the ISC pool. This phenomenon encompasses mature paneth cells, Alpi^{+} ECs, and Dll1^{+} secretory progenitor cells (van Es, Sato et al. 2012, Tetteh, Basak et al. 2016, Yu, Tong et al. 2018). Moreover, it is noteworthy that at the site of damage or injury, both ISCs and other epithelial cells can transition into a fetal-like state (Nusse, Savage et al. 2018, Yui, Azzolin et al. 2018, Wang, Chiang et al. 2019). This temporary fetal-like transition facilitates healing and suggests that tissues could employ developmental processes to achieve effective restoration.

1.3.1 Lateral inhibition

Lateral inhibition is a phenomenon within the intestine that plays a crucial role in maintaining a delicate balance between secretory and absorptive cells, while also regulating the population of ISCs. This process takes place between two adjacent cells that have yet to undergo differentiation, such as daughter cells originating from a stem cell or between two TA-cells. In this intricate mechanism, one of the neighboring cells expresses the Notch ligand, Delta, which binds to the Notch receptor in the adjacent cell. This interaction initiates the Notch signaling pathway, resulting in the release of the Notch intracellular domain (NICD). NICD activation, in turn, triggers the activation of Notch repressors, ultimately establishing a Notch-low state within the cell. The cell now characterized by reduced Notch signaling responds by upregulating the production of Notch ligands. These ligands activate the Notch signaling pathway in the neighboring cell, resulting in reduced Notch signaling within that cell. Subsequently, the cell with low Notch signaling further increases ligand production due to the diminished inhibitory signaling from its neighbor. This reciprocal interaction sets in motion a feedback loop where even the slightest disparities in Notch signaling, no matter how small, gradually intensifies. Ultimately, the process creates a Notch high expressing cell and a Notch low expressing cell (Sancho, Cremona et al. 2015).

1.4 Signaling pathways and gradients

Intestinal signaling pathways are of significant importance, as elaborated in section 2. Due to the intricate nature of the signaling landscape, the focus will be directed toward

pivotal pathways closely associated with ISCs; Wnt, BMP, Notch, and Eph-ephrin signaling.

1.4.1 Wnt

The roles and implications of Wnt (Wingless-related integration site) signaling is vast and many, ranging from development to various diseases including cancer, and importantly in maintaining stem cells in various tissues (Holstein 2012, Nusse and Clevers 2017). This conserved signaling pathway plays a crucial role in tissue and organ development in both invertebrates and vertebrates(Cadigan and Nusse 1997). The following year it was discovered that the adult tissue of mice who were TcfL2$^{-/-}$, a key transcription factor in Wnt signaling, lost the proliferative capacity of ISCs (Korinek, Barker et al. 1998). During the upcoming years, more discoveries were made regarding the importance of Wnt in the intestine. For instance, when inhibiting Wnt by Dkk1 loss of secretory cells was prominent (Pinto, Gregorieff et al. 2003). In addition, around the same time, a gradient of Wnt signaling was discovered along the crypt-villus axis, with high Wnt target gene expression at the bottom of the crypt (Kongkanuntn, Bubb et al. 1999, van de Wetering, Sancho et al. 2002). This finding was important, as it is suggested that the Wnt gradient functions to control the spatial organization of the intestinal epithelium (Batlle, Henderson et al. 2002).

1.4.1.1 Canonical Wnt pathway

In the canonical Wnt pathway, β-catenin functions as the key mediator, it will transduce the signal to the nucleus, which will, in turn, trigger the response of specific Wnt targets. This branch of the Wnt pathway will control cell fate decisions in most cells and tissues.

When Wnt ligands bind to the Frizzled (Fzd) receptors, this binding triggers the recruitment of a co-receptor, Low-density lipoprotein receptor-related protein 5 or 6 (Lrp5/6). Together these three components will create a complex referred to as Wnt-Fzd-Lrp. The creation of the complex will lead to the translocation of the β-catenin destruction complex which consists of Axin, Glycogen Synthase Kinase 3 β (GSKβ), APC, and Casein Kinase 1alpha (CK1α) being translocated to the plasma membrane with the assistance of the adaptor protein Dishevelled (DVL). This new location at the plasma membrane leads to the blocking of the destruction complex and thereby, the release of β-catenin. After the build-up of β-catenin in the cytoplasm, it translocates to the nucleus where it induces the transcription of the TCF/LEF target genes. These genes will play an essential role in the regulation of stemness, cell cycle progression, and proliferation (Valenta, Hausmann et al. 2012, Koch 2017).

1.4.1.2 Non-canonical Wnt pathway

The non-canonical Wnt-pathway is β-catenin independent and functions to alter cellular behavior, such as the involvement in cell migration, cell growth, mediating protein

stability, as well as regulating tissue patterning (Green, Nusse et al. 2014, Acebron and Niehrs 2016, Humphries and Mlodzik 2018).

There are two key pathways for non-canonical Wnt signaling, the Wnt/PCP (Planar Cell Polarity) pathway (Veeman, Axelrod et al. 2003) and the Wnt/calcium pathway (De 2011). The role of Wnt/PCP signaling is to regulate the organization of tissue morphogenesis during development. It functions by Wnt ligands binding to the Fzd receptor which in turn activates JNK (Jun N-terminal kinase). This triggers the organization of the asymmetric cytoskeleton as well as coordinates polarization of the cell in the epithelial sheets, through the involvement of various factors such as Fmi, Knyand, and Stbm (Veeman, Axelrod et al. 2003, Humphries and Mlodzik 2018).

The Wnt/calcium pathway's most important function is controlling cell motility. This pathway is more complex, with many branches within the signaling cascade. Compared to the traditional canonical Wnt-signaling, leading to the release and increase of the concentration of β-catenin, here the crucial component is Ca2+. In brief, the release of Ca2+ through G-proteins triggers the activation of key factors in the pathway, including PLC and PKC. Increased Ca2+ concentration will also activate phosphatase calcineurin. This enzyme, in turn, dephosphorylates the transcription factor NFAT, allowing for it to accumulate in the nucleus. In the nucleus, NFAT regulates the transcription of target genes that play essential roles in cell motility (De 2011). Due to the Given the intricate natire of this pathway, further research is required to truly understand the details and interplays within this signaling cascade (De 2011).

1.4.2 Bmp

The family of BMPs (Bone morphogenic proteins) is known for their role in inducing bone formation (Reddi 2005), however, they also have important functions in organ development and maintaining tissue homeostasis (Wang, Green et al. 2014). In the intestine, BMP signal protein is expressed as an opposing gradient to Wnt signaling. BMP has the highest expression in the villi, while at the bottom of the crypt, BMP antagonists are expressed (He, Zhang et al. 2004). The BMP antagonists such as gremlin 1/2, noggin, and ANGPTL2 are supplied by the surrounding mesenchymal niche (Sneddon, Zhen et al. 2006, Kosinski, Li et al. 2007, Horiguchi, Endo et al. 2017). This pathway functions as a negative regulator of crypt formation, while inducing the terminal differentiation stage of mature intestinal cells (Haramis, Begthel et al. 2004, Auclair, Benoit et al. 2007, Qi, Li et al. 2017).

1.4.2.1 BMP signaling

The BMPs are extracellular signaling molecules that are classified as a sub-category of the TGF-β superfamily of proteins. BMPs trigger the canonical Smad-dependent pathway however, are also known to induce various non-canonical pathways. To induce

the signaling pathway, BMP ligands bind to BMPR-1 and BMPR-11 receptors, this causes them to fuse, leading to the phosphorylation of the receptor-bound R-Smads1/5/8. These Smads join with Smad4 and this newly formed complex translocates to the nucleus (Heldin, Miyazono et al. 1997, Wang, Green et al. 2014). Here, the Smad complex aligns with coactivators and corepressors, to regulate targets such as Msx Homeobox genes and the proto-oncogene JunB (Hollnagel, Oehlmann et al. 1999, Miyazono, Maeda et al. 2005).

1.4.3 Notch

Notch signaling has the important task of directing and maintaining cell fate, as mentioned previously (see section 2) (Basak, Beumer et al. 2017). In addition to its role to induce the final differentiation of TA-cells into the secretory cell lineage or the absorptive cell lineage (Jenny, Uhl et al. 2002) (van der Flier and Clevers 2009), it also directly targets the ISCs through one of their markers, Olfm4 (VanDussen, Carulli et al. 2012). Notch can, therefore, function to directly control the maintenance and activity of the ISCs. A clear indication of this was shown when Notch signaling was ablated in the intestine, resulting in a quick loss of ISCs. This demonstrated the dramatic impact of Notch signaling on ISC proliferation and survival (VanDussen, Carulli et al. 2012).

1.4.3.1 Notch signaling

The activation of the Notch pathway is only possible when a cell expressing a Notch receptor is adjacent to a cell expressing Notch ligands, thereby establishing a direct cell-cell contact. The receptors consist of 4 single transmembrane Notch receptors, Notch1-4, and the ligands of 5 single transmembrane Delta/Serrate/Lag2 (DSL) Notch ligands, Jag1 and 2, Dll1, 3, and 4. The binding of the ligand to the receptor triggers a small cascade of proteolytic steps, in which ultimately the NICD through the assistance of gamma-secretase protease activity will be released. NICD translocates to the nucleus where it interacts with DNA-binding proteins including CSL and activates target genes, such as Hes-1 (Guruharsha, Kankel et al. 2012, Gassler 2017).

1.4.4 Ephrin family

The family of Eph receptors and Ephrin ligands are Wnt targets that stretch their influence into many different biological processes. They have been discovered to modulate cellular shape, motility, migration, the spatial organization of cell populations, and proliferation (Poliakov, Cotrina et al. 2004, Pasquale 2005, Batlle and Wilkinson 2012, Niethamer and Bush 2019). Upregulation and deregulation of the Eph receptors and ligands have been identified in most classes of cancer (Ireton and Chen 2005, Noren and Pasquale 2007, Xi, Wu et al. 2012). For instance in colorectal cancer, constitutive activation of the Wnt/β-catenin/Tcf pathway causes the emergence of adenomas,

through the upregulation of EphB expression in the early stages of tumor development (Clevers and Batlle 2006).

1.4.4.1 Eph-ephrin signaling

This family of tyrosine kinases can be divided into 2 categories, the EphA receptors with their corresponding EphrinA ligands, and the EphB receptors with EphrinB ligands (Dodelet and Pasquale 2000). Signaling occurs when there is cell-cell contact followed by the binding of an ephrin ligand to the extracellular domain of the Eph receptor. This results in the autophosphorylation of the intracellular residues tyrosine and serine on the receptor, which will lead to the cytoplasmic tyrosine kinase becoming activated and triggering various signaling cascades such as MAPK, Ras, and ERK signaling (Kalo and Pasquale 1999, Pasquale 2010).

1.4.4.2 Eph and ephrin signaling in the intestine

In the intestine it is believed that Eph and ephrin function to establish the strict organization observed, ISCs and Paneth cells in the very bottom of crypts, TA-cells further away, and finally differentiated cells such as enterocytes and goblet cells in the villi (Batlle, Henderson et al. 2002). Components in the EphB and ephrin-B subfamily function as a repulsive force upon interaction, which maintains the cell organization in the crypt-villus axis. This is achieved by Paneth cells expressing EphB3 and other differentiated cells in the villi expressing ephrinB1. Both are expressed as opposing gradients, EphB3 highest expression at crypt bottoms by Paneth cells and reduces towards the villi domain, while ephrinB1 is highly expressed by differentiated villus cells and diminishes towards the crypt (Batlle, Henderson et al. 2002). What keeps the ISCs in place between the EphB3 expressing Paneth cells, is the ISCs' high levels of EphB2 expression and the lack of EphB2 expression in the Paneth cells. The TA-cells also express EphB2, however, this is reduced the further upwards they migrate. This repulsive migratory force demonstrated as a counter gradient of EphB2/3-ephrinB1 allows the upward migration of differentiated cells to the villi tip, while restricting paneth cells and ISCs to the crypt bottom (Batlle, Henderson et al. 2002, Cortina, Palomo-Ponce et al. 2007, Jung, Sato et al. 2011).

1.5 Stem cell niche

For ISCs to effectively carry out their crucial functions in maintaining the intestinal epithelium, they require a supportive environment, known as the niche. This environment has important tasks; to protect the ISCs from environmental damage, it can influence the number of ISCs as well as the cell lineages, and can greatly affect the regeneration capacity (Lane, Williams et al. 2014).

1.5.1 Overview

The niche surrounding the ISCs, comprises the neighboring cells, the growth factors and cytokines produced by the surrounding cells, and the extracellular matrix (ECM, see section 5). The cell types constituting the niche for the ISCs include the adjacent paneth cells as well as mesenchymal cells such as pericryptal myofibroblasts, fibroblasts, endothelial cells, pericytes, immune cells, neural cells, and smooth muscle cells. The surrounding microenvironment collectively establishes gradients of signaling molecules, including but not limited to, elevated levels of Wnt, EGF, Noggin, and Notch signaling at the crypt bottom (which supports stem cell properties), gradually decreasing toward the villus. Conversely, heightened BMP-signaling, which promotes differentiation, is prevalent in the villus and tapers off towards the crypt. This combined signaling landscape serves to uphold the equilibrium of ISC dynamics (Yen and Wright 2006, Powell, Pinchuk et al. 2011).

1.5.2 Cell types

1.5.2.1 Paneth cells

The close epithelial neighbors of the ISCs, the paneth cells were identified to play a major role in constituting the niche, by supplying ISCs with niche factors such as EGF, TGF-α, Wnt3, and Notch ligand Dll4 to the neighboring ISCs (Sato, van Es et al. 2011). The importance of the proximity of ISCs to Paneth cells was highlighted when ISCs that lost contact with Paneth cells, reduced stemness and began to differentiate into TA-cells (Clevers 2013). Importantly, Paneth cells are generated by ISCs, generating a potential autoregulatory loop.

1.5.2.2 Mesenchymal cells

Within the lamina propria layer, which lies just below the epithelial monolayer of cells, exists the mesenchymal cells embedded in the ECM. The major players among these, are the fibroblasts and myofibroblasts, concentrated around the crypt bottom. The subepithelial myofibroblasts (ISEMFs) are present in the pericryptal regions in the lamina propria and these secrete both Wnt ligands and BMP antagonists, functioning to maintain the ISCs (Roulis and Flavell 2016, Meran, Baulies et al. 2017)**.** Alongside these ISEMFs, reside the smooth muscle cells which also secrete BMP antagonists to assist in maintaining the high Wnt concentration at the crypt bottom (Kosinski, Li et al. 2007). Two distinct cell populations have been chosen for further discussion, owing to their critical roles in preserving the integrity of the ISC niche.

1.5.2.3 PDGFRα cells

Within the intestinal mesenchyme, reside subtypes of PDGFRα-expressing cell types (Greicius, Kabiri et al. 2018, McCarthy, Manieri et al. 2020, Roulis, Kaklamanos et al. 2020),

which together are important in maintaining the native BMP-signaling gradient. The PDGFRαhigh subpopulation of telocytes is predominantly located at the villus base, which functions to provide BMP-ligands, the highest being BMP-5 and BMP-7. While in the mesenchyme, directly under the crypt bottom the BMP antagonist Gremlin 1 producing CD81+ PDGFRαlow trophocytes are found (McCarthy, Manieri et al. 2020).

1.5.2.4 Telocytes

One of the significant mesenchymal cell types that has garnered attention is the rare cell population known as $FOX1^{+}$ telocytes (Shoshkes-Carmel, Wang et al. 2018). These large and flattened cells have been identified in both the small intestine and colon. While they play a role in providing structural support, their crucial function lies in acting as vital suppliers of Wnt ligands *in vivo*, contributing significantly to the support of the intestinal stem cell niche (Shoshkes-Carmel, Wang et al. 2018).

The distribution of telocytes throughout the crypt-villus axis of the intestine, suggests varying functions depending on their specific location. A noteworthy instance occurred in 2020, when researchers combined laser-captured micro-dissection with single-cell RNA sequencing. This effort unveiled a subset of telocytes positioned in the stroma of the villus tip, remarkably expressing Lgr5 (Bahar Halpern, Massalha et al. 2020). The removal of this particular subtype had repercussions, resulting in the loss of EC markers that are associated with ECs shedding into the lumen (Moor, Harnik et al. 2018). Undoubtedly, telocytes play a curcial role in maintaining intestinal homeostasis, However, the full extent is still not fully understood.

1.6 The ECM

The ECM is an important part of the stem cell niche, located beneath the epithelial monolayer of cells. It can be subdivided into two layers, the basal lamina, and the reticular sheet of matrix referred to as the interstitial matrix (Laurie, Leblond et al. 1982). Together, the ECM plays a vital role in providing mechanistic support in the shape of geometry and stiffness. This structural support consists mainly of collagens and laminins, which have the capability of self-building and forming complex structures which are key for maintaining structural stability (**Figure 1**).

Mainly synthesized by the mesenchymal cells, the ECM serves as a reservoir (Tschumperlin 2015), furnishing a diverse range of approximately 300 different molecules, including, integrins, cytokines, chemokines, and growth factors, thus contributing to a highly intricate niche milieu (Harburger and Calderwood 2009, Frantz, Stewart et al. 2010, Speca, Giusti et al. 2012, Gattazzo, Urciuolo et al. 2014). The close proximity between ECM and epithelial cells facilitates the interaction of ECM ligands with cell surface receptors, notably integrins. Consequently, these interactions instigate a plethora of cellular processes, encompassing adhesion, migration, proliferation,

apoptosis, and differentiation (Hynes 2009, Bonnans, Chou et al. 2014, Lane, Williams et al. 2014).

1.6.1 Laminins

The intestinal tissue is marked by its compartmentalization with spatial variations in the niche composition along the crypt-villus axis. This spatial variance is influenced not only by signaling gradients originating from epithelial cells but also by the underlying ECM (Malijauskaite, Connolly et al. 2021). Among the ECM components, laminins play a crucial role, with distinct laminin subtypes identified in both the crypt and villus regions of the intestine. Notably, laminin α1 is expressed along the entire crypt-villus axis, whereas laminin α2 is confined to the crypt region and laminin α5 to the villus region (Leivo, Tani et al. 1996, Orian-Rousseau, Aberdam et al. 1996, Kedinger, Duluc et al. 1998, Glentis, Gurchenkov et al. 2014). Of significant importance, laminin α5 has been identified as a crucial factor in preserving the structural architecture of the villus domain within the small intestine. The absence of laminin α5 resulted in the disruption of villus architecture, resembling the structural pattern observed in the colon (Mahoney, Stappenbeck et al. 2008). To what full extent laminins and other ECM components can directly influence various epithelial cells in the crypt-villus regions has yet to be investigated.

1.6.2 ECM and Aging

The ECM is a dynamic structure, that during aging undergoes degradation and deformation due to various ECM components becoming up-or-down-regulated (Levi, Papismadov et al. 2020). Due to the close relationship between stem cells and their supportive ECM niche, stem cells are directly affected by changes in the environment. For instance, in an aging brain, increased ECM stiffness was detected, which had a direct negative impact on stem cell maintenance (Segel, Neumann et al. 2019). Furthermore, since the ECM is largely produced by the surrounding mesenchyme, the mesenchyme can, therefore, directly influence ISC dynamics (Tschumperlin 2015), and is altered during aging (Krtolica and Campisi 2002, Parrinello, Coppe et al. 2005). Still, much remains unknown concerning the relationship between the ECM and ISC capacity during aging, and warrants further investigation.

1.7 Cellular migration

Controlled cell migration is a fundamental process occurring in various biological events including embryogenesis, wound healing, tissue repair, and regeneration (Li, He et al. 2013, Scarpa and Mayor 2016). In the subsequent section, specific instances of these events will be explored, delving into the pivotal stages that characterize cell migration.

1.7.1 Cell migrational events

Embryonic development encompasses a myriad of processes including germ layer positioning, patterning, and organ morphogenesis, all of which necessitate collective cell migration. This intricate phenomenon relies on the coordination and cooperation among migrating cells, underpinned by crucial course cell-cell interactions. Wound healing too, involves cell migration, particularly in the restoration of epithelial barriers, such as skin wounds. Epithelial cells surrounding a skin wound initiate migration, proliferating as cohesive sheets toward the wound center to achieve comprehensive coverage (Scarpa and Mayor 2016).

Tissue repair introduces another facet of cell migration, notably observed in organs like the lung, transitioning from a quiescent state to that of a highly proliferative state during injury. Progenitor cells within the lung's population undergo proliferation and subsequent differentiation to replace lost cell types caused by injury (Kotton and Morrisey 2014).

Intriguingly, the intestinal epithelium represents a remarkable example of tissue renewal during homeostatic conditions, replenishing its lining every 5 days (see section 1) (Cheng and Leblond 1974, Barker, van Es et al. 2007). This captivating process underscores the exceptional regenerative capacity of the intestine.

1.7.2 Modes of cell migration

Cells have the capability to migrate either individually or collectively (Haeger, Wolf et al. 2015, Paluch, Aspalter et al. 2016). In either scenario, the initiation of cell migration hinges on the cell's ability to perceive cues within its environment, guiding its movement towards or away from these cues. This process encompasses four distinct migration modes, each responsive to varying types of cues: chemotaxis, hapotaxis, durotaxis, topotaxis, and galvanotaxis (SenGupta, Parent et al. 2021).

Chemotaxis involves cells responding to diffusable chemical cues, often released from bacteria or chemoattractants like chemokines and growth factors originating from surrounding endothelial, epithelial, and stromal cells. Cells exhibit directed cell migration when cues are presented in a gradient, while random cell migration occurs without such gradients (Wilkinson 1985, Petrie, Doyle et al. 2009). Hapotaxis, on the other hand, relies on chemical substrates located on cell-underlying surfaces, such as those found in the ECM, including fibronectin, laminins, and collagens. Durotaxis responds to molecular cues and substrate stiffness, prompting cells to migrate toward stiffer substrates. In the topotaxis migration mode, cells sense geometric features or surface topography of the substrate, adapting their shape to fit specific structures and migrating in their preferred direction (SenGupta, Parent et al. 2021).

The final migration mode, galvanotaxis, induces cell migration in response to electric fields. During wound healing, for example, disruptions in the physiological electrical field

lead to cell polarization, generating a new electrical field at the wound site and accelerating cell recruitment (SenGupta, Parent et al. 2021).

1.7.3 Cell migration process

Upon sensing environmental cues (see section 6.1), cells initiate migration by embarking on a series of steps. When a cell is positioned on the ECM, this journey begins with the formation of protrusions at the forefront (the "leading edge) of the cell. This dynamic process is facilitated by the reorganization of actin filaments at the leading edge. Consequently, this polymerization generates a force that propels the cell's plasma membrane forward, resulting in the creation of protrusions characterized as lamellipodia and filopodia. Concurrently, as the leading edge extends, the cell secures itself to the ECM via cell surface receptors, specifically integrins that interact with ECM ligands. These interactions lead to the formation of focal adhesions, robustly anchoring the cell's actin cytoskeleton to the ECM. Through these focal adhesions, contractile forces are conveyed in a myosin-dependent manner evolving (Abercrombie, Dunn et al. 1977, Ananthakrishnan and Ehrlicher 2007).

To facilitate forward movement, the focal adhesions located at the trailing edge (the "rear") of the cell are deliberately dismantled, allowing the cell to detach from the ECM. Ultimately, the cell advances by harnessing contractile forces at the trailing edge and throughout the cell body, including contraction and retraction mechanisms. It is important to note that while cell migration is outlined as a series of sequential steps, the process is inherently dynamic and continuously evolving (Abercrombie, Dunn et al. 1977, Ananthakrishnan and Ehrlicher 2007).

1.7.4 Pushing force in the intestine

The phenomenon in which the progeny from ISCs migrate from the crypt pit and continue migrating vertically upwards until reaching the villus tip (Bjerknes and Cheng 1981, Barker, van Es et al. 2007, van der Flier and Clevers 2009) is thought to be in place due to mitotic pressure within the crypt, generated by the ISCs proliferating. This would in other words give rise to a pushing force from the crypt pit, continuing in the transit-amplifying zone, and up throughout the villi region (Heath 1996, Parker, Maclaren et al. 2017). For a long time, this has been the working theory. However, what can not be explained using this theory, is why treatments involving radiation and mitotic inhibitors, do not stop epithelial migration, even though there is a great loss of cell number in the crypt (N. A. Wright 1984, Kaur and Potten 1986). This would suggest that active migration might exist in the intestine.

1.7.5 Collective migration of Enterocytes

In 2019, a paper was published in which the authors demonstrate active collective migration of ECs in the small intestine (Krndija, El Marjou et al. 2019). Important findings

were made to support their claim. Firstly EdU-pulse-chase assays *in vivo* were performed, using an inhibitor specific to S-phase. Results indicated that the previously observed mitotic pressure is indeed present in crypts, however, restricted to only the lower part of the villi. Secondly, live imaging was performed using gut explants, in which a Villin: CreERT2/mTmG reporter mouse had membrane-targeted green fluorescent protein. This allowed for cell tracking, to follow each cell's possible migration ex vivo. It was evident that cells moved along the villus axis, furthermore, accelerated the closer they approached the villus tip. This data, together with ex vivo speed profiles produced from their developed biophysical model for gut epithelium renewal, indicated active migration in the villus. Finally, the authors developed a mouse model which expressed Life Act- mCherry in the intestinal epithelium, which allowed visualization of F-actin in live cells. Remarkably, ECs were found to have F-actin-rich basal feet protrusions, connected to the underlying basement membrane and which were pointed in the direction of the cell's movement. Finally, these cells were confirmed to have front-back polarity. In addition, the highly rich F-actin protrusions required actin-related protein 2/3 complex to enable active migration.

Given ECs' capability to migrate actively towards the villus domain (as a unit), it implies their responsiveness to microenvironmental cues influencing directional movement. This raises the intriguing prospect that additional intestinal cell types may also exhibit active migratory abilities.

1.8 Colorectal cancer

Colorectal cancer (CRC) stands as the third most prevalent cancer on a global scale. This malignancy can be categorized into three groups based on the source of the cancer-initiating mutation: inherited, familial, and sporadic (Mármol, Sánchez-de-Diego et al. 2017).

Inherited cancers constitute around 5% of all cancer cases and can be further subdivided into two primary categories; those that form polyposis and those that do not. The polyposis group mainly consists of familial adenomatous polyposis (FAP), a condition marked by the presence of a substantial number of malignant polyps within the colon. Conversely, the non-polyposis group is identified by mutations in DNA repair mechanisms, among which Lynch syndrome, is responsible for 2-3% of cases (Lynch and de la Chapelle 2003). Among the 25% of cancers classified as "familial", these stem from inherited mutations. However, they do not fit within the "inherited" classification due to the absence of any inherited cancer variant (Stoffel and Kastrinos 2014).

Constituting the remaining 70% of cases, cancers categorized as "sporadic" emerge from point mutations (Fearon and Vogelstein 1990). In the subsequent sections, the emergence of sporadic colorectal cancers will be explored in greater detail.

1.8.1 Enhanced Wnt signaling in colorectal cancer

Sporadic CRCS arise due to mutations in the Wnt signaling pathway (Fodde and Brabletz 2007). These mutations result in the deletion of the tumor suppressor *APC* or *Axin2* (Powell, Zilz et al. 1992, Miyaki, Konishi et al. 1994), or activation of the proto-oncogene β-catenin (Morin, Sparks et al. 1997, Rubinfeld, Robbins et al. 1997). Consequently, β-catenin accumulates in the nucleus of epithelial cells, binding to TCF/LEF transcription factors and triggering the activation of Wnt target genes (Barker, Huls et al. 1999). This initiates the transformation of intestinal epithelial cells, leading to early-stage cancer (Morin, Sparks et al. 1997), in a ligand-independent manner. Tumor progression is closely associated with further elevated β-catenin expression, followed by sequential mutations in Apc, Kras, Smad4, and TP53 (Fodde, Smits et al. 2001). Furthermore, an occurrence called loss of heterozygosity (LOH) frequently coincides with these mutations. LOH entails the loss of one functional copy of a gene (usually a tumor-suppressor gene) due to genetic occurrences like deletion or mutation, thereby, adding to the progression of tumor formation (Mármol, Sánchez-de-Diego et al. 2017).

1.8.2 APC mouse models for colorectal cancer

For the study of CRC, various genetically engineered mouse models (GEMM) have been established. The very first GEMM of CRC established was the APCmin (multiple intestinal neoplasia) mouse. This was created by applying N-ethyl-N-nitrosourea, which caused a nonsense mutation in the codon 380 of the APC gene (Moser, Pitot et al. 1990). The APCmin mice characteristically develop multiple intestinal neoplasia (multiple tumors). The vast numbers of adenomas form in the small intestine after 120-140 days, caused by LOH (see section 7.1). Due to the high adenoma burden and side-effects caused such as anemia, mice demonstrate high mortality with increasing age (Shoemaker, Gould et al. 1997). Nevertheless, there are limitations associated with this mouse model, primarliy stemming from the delayed onset of adenomas and the confinement of adenoma formation to the small intestine. These are disadvantages when compared to adenoma formation in humans, which typically forms in the colon (Roper and Hung 2012). This model can be used to study the early stages of FAP, however, is not ideal for studying sporadic CRCs.

To more accurately mimic sporadic CRC, the $Apc^{fl/fl}$ or $Apc^{fl/+}$ mouse model can be employed. This model relies on cre-recombinase to excise the targeted genetic region flanked by floxed sites, leading to the reduction or removal of APC function, depending on the targeted alleles. In more detail, the enzyme cre-recombinase cleaves the DNA at the LoxP sites, and the Cre recombination proteins expressed by the cells containing Cre facilitate DNA recombination between the LoxP sites. In the case of $Apc^{fl/fl}$ mice, such recombination results in the deletion of APC, leading to the formation of adenomas in both the small intestine and colon (Shibata, Toyama et al. 1997). This approach can be further tailored to target specific cell or tissue types by utilizing cell-specific Cre-

recombinase expression. An example of this is the Apcfl/fl; *Lgr5-EGFP-IRES-creER*^{T2} mouse model, where the Lgr5 promoter drives the expression of Cre recombinase in ISCs. This model showcases adenoma development in both the small intestine and colon, with a more pronounced occurrence in the small intestine. Indicating a medium to high tumor burden relative to the progression of time (Barker, Ridgway et al. 2009). Employing this model permits a more comprehensive exploration of adenomas originating from individual Lgr5+ ISCs within the intestine. Moreover, it closely mirrors the development of sporadic CRC in humans, making it the favored mouse model for the research presented in this thesis's constituent papers.

1.8.3 Apc$^{-/-}$ stem cells

It is believed that in most cases, the Lgr5+ stem cell serves as the cell of origin for the development of intestinal cancer (Barker, Ridgway et al. 2009) by typically acquiring deletions in the Apc gene, initiating intestinal tumorigenesis. These ISCs without proper APC function, however, display high Wnt signaling, manage to lose niche-dependency and develop adenomas (Barker, Ridgway et al. 2009).

In the context of homeostatic conditions, a stochastic event referred to as neutral drift takes place within crypts. This phenomenon entails a continuous cycle of loss and replacement of functional stem cells situated at the bottom of the crypt (see section 1.2) (Vermeulen and Snippert 2014). When ISCs acquire mutations, such as those affecting the Apc gene, they gain a competitive edge over their neighboring ISCs, securing their position at the crypt pit. Subsequently, with these ISCs with Apc mutants giving rise to their progeny, the advantage is inherited, leading to a shift in the nature of neutral drift. This bias now favors the mutated ISCs, tilting the balance (Vermeulen and Snippert 2014).

This raises the questions: How exactly do ISCs harboring Apc mutations gain this clonal advantage?
Is it possible for these cells to exert an influence on their surrounding microenvironment or potentially impact neighboring cells?

1.9 Organoids, Hydrogels matrixes, and Tissue Decellularization

The stem cell system of the intestine is particularly well-defined due to breakthrough technologies allowing culturing of the intestinal epithelium with relative ease. ISCs can be readily isolated based on their expression of Lgr5, and when presented with a cocktail of growth factors supplementing the stromal-produced niche components (R-Spondin, Noggin, EGF), and a hydrogel matrix such as Matrigel™, ISCs generate so-called organoids (Sato, Vries et al. 2009). These "mini-guts" contain all of the cell types of the intestinal epithelium and present a rudimentary approximation of the crypt-villus organization found *in vivo*.

Owing to their self-organizing ability, organoids are a useful tool for studies investigating intra-epithelial mechanisms (Sato, Vries et al. 2009, Gjorevski, Sachs et al. 2016). However, there are limitations to this system; it is a closed system and does not consist of the native ECM components (Fatehullah, Tan et al. 2016). In more recent years, great strides have been made in creating synthetic scaffold-based systems mimicking that of the *in vivo* intestine (Guiu and Jensen 2022). However, there are limitations to this type of system as well, such as not offering the spatial contexts nor the ratios of the *in vivo* components in the crypt-villus axis (Beaulieu and Vachon 1994, Fatehullah, Tan et al. 2016).

The ECM plays a vital role in regulating ISC fate, therefore, the aim to have a system that recapitulates the *in vivo* intestinal structure and environment is crucial for studying the true natural behavior of ISCs and other intestinal cell types. The approach in which the tissue is decellularized, aiming to use the native ECM, has been tested from various organs (Fu, Wang et al. 2014). However, preserving the native ECM composition and not harming the structure has been challenging.

The creation of a native scaffold extracted from intestinal tissue, which could preserve the intricate architecture of the intestine and the spatial arrangement of ECM components within the crypt and villus regions, holds the potential to unlock a multitude of research inquiries, that have been otherwise challenging to answer.

2 Research aims

The intricate interplay between intestinal epithelial cells and their surrounding microenvironment has been an intriguing topic for many years. This thesis delves into a spectrum of inquiries, spanning clonal dynamics during homeostasis and cancer, active migration of intestinal epithelial cells, and intestinal regeneration. The aims below will guide our exploration.

The specific aims addressed in this thesis are:

- Explore mechanisms that determine the position and number of functionally effective stem cells.

- Investigate whether intestinal cell types demonstrate intrinsic and cell-type-specific migratory behavior in the absence of neighboring cells.

- Investigate how aging and oncogenic events influence stem cell migration.

- Examine the role of the intestinal mesenchyme in tissue repair and its implications in the context of aging.

- Investigate how *Apc*-mutant stem cells gain a clonal advantage over wild-type stem cells in the intestinal crypts.

3 Materials and methods

Each constituent paper includes a comprehensive description of the methods used in this thesis. In this section, I will specifically focus on the primary research tools used in **Papers 1** and **2**.

3.1 Advanced imaging approaches for studying intestinal cell dynamics

Great strides have been made in live-cell fluorescent imaging, and over the last 15 years, it has advanced to the point where intravital microscopy can be conducted in live animals (Pittet and Weissleder 2011). This technology allows researchers to visualize not only individual cells and their behaviors but also cellular, and extracellular components *in vivo*. This capability is harnessed through multiphoton microscopy, which employs near-infrared light (>680nm) to penetrate tissues and excite fluorophores by focusing two or three photons ono a single point. Importantly, this excitation occurs only at the focal point of high laser intensity, minimizing photodamage to surrounding tissues, making it ideal for live animal imaging. Furthermore, in two-photon microscopy, a phenomenon called second harmonic generation (SHG) can take place when two photons combine to form one with half the wavelength. This occurs when the laser interacts with certain structures like collagen or surfaces with refractive index mismatches, resulting in label-free imaging (Larson 2011).

Imaging of the inner organs has been made possible by using an abdominal imaging window, initially, in the small intestine (Ritsma, Steller et al. 2013) and later into the colon (Rakhilin, Garrett et al. 2019). This approach has since been frequently used to investigate various aspects of stem cell dynamics. For instance, to explore stem cell maintenance, intravital live imaging of the intestine was conducted using Lgr5-Confetti mice, allowing for the fluorescent visualization of Lgr5+ ISCs and their progeny to assess the fate of the individual ISCs (Ritsma, Ellenbroek et al. 2014). Subsequently, researchers delved into the specific patterning of ISCs and PCs at the crypt base. They achieved this by selectively ablating single cells within the intestinal crypts using a three-photon microscope equipped with high peak-power lasers. The real-time monitoring of pattern recovery and disruption in aged mice was conducted using a two-photon microscope (Choi, Rakhilin et al. 2018).

In **Paper 1**, we employ intravital imaging to investigate stem cell dynamics, specifically focusing on the number of long-term ISCs in the small intestine and colon. Unlike previous studies that utilized short-term intravital microscopy, our approach allows us to track ISCs and their progeny over the course of many weeks (Ritsma, Ellenbroek et al. 2014).

While multi-photon intravital imaging is a potent tool for probing cell behavior *in vivo,* certain questions still remain challenging to address using this technique. For instance, understanding the interplay of different cell types with the ECM, and if and how individual cells can inherently sense cues in their surrounding environment guiding them to their *in vivo* destinations.

To tackle these queries, we require a system that enables the assessment of individual cell-type behavior within an environment closely resembling the *in vivo* conditions. Crucially, this system should preserve the structural integrity of the intestinal 3D tissue architecture and the spatial composition of ECM components. Additionally, this system would allow for studies and perturbations that would be unfeasible in an *in vivo* setting.

For both **Paper 1** and **Paper 2**, we have devised such a method, building on the optimized decellularization protocol established in **Paper 3**. In **Paper 1**, we combine intravital imaging with our innovative *ex vivo* live cell imaging approach to elucidate the mechanism driving the positioning of the long-term ISCs in the crypt. In **Paper 2**, we utilize this novel method to explore variances in motility behaviors among multiple intestinal cell types and to investigate how an aging ECM and the initiation of colorectal cancer impact the motility behavior of ISCs.

3.2 Ethical considerations

All experimentation involving mice was performed according to the ethical permits listed for each paper. Ethics concerning the usage of animals in research is something that is heavily discussed at Karolinska Institutet and in our laboratory. We follow Karolinska's guidelines and regulations including the 3 R's; replacement, reduction, and refinement. These are key points when we are setting up an experiment or discussing new project ideas.

When discussing the possibility of using a technique or assay that does not require the usage of animals (replacement), it is a difficult thing to implement in these research projects. The research questions we address in this thesis involve studying the interactions and behavior between individual cell types and their surrounding environment. Today there are no *in vitro* systems that can fulfill these conditions and therefore, the usage of mice cannot be excluded. When instead discussing the second R referred to as reduction, we have strived to always reduce the number of animals used for each experiment. To achieve this, when planning experiments we always discuss the lowest number of animals needed, while, still being able to statistically reliably answer our research question.

Finally, addressing the third R, refinement, we always try to optimize all aspects of the experiments. As a rule, everyday monitoring of the condition of the mice is handled by professional caretakers. Furthermore, when planning experiments that could involve

suffering for the mice (i.e. injections), we coordinate our plans with the caretakers and veterinarians in an effort to find the best treatment options and to minimize pain and stress for the animal.

4 Results and conclusions

4.1 Paper 1: Retrograde movements determine effective stem cell numbers in the intestine

In this study, we sought to explore the quantity and spatial distribution of long-term ISCs, also referred to as "effective" ISCs, within the small intestine and colon. These are the cells capable of generating persistent clones over an extended period. To achieve this, we employed a combination of long-term intravital imaging, 2D-biophysical modeling, and an innovative *ex vivo* live cell imaging approach, allowing us to dissect the underlying mechanism driving the abundance of effective ISCs.

4.1.1 Number of effective stem cells in small intestinal crypts is higher than in colonic crypts

Before identifying "effective" ISCs, we conducted an assessment of the total Lgr5+ cells population in both the small intestine (SI) and large intestine (LI). The count of Lg5+ cells within the crypt was consistent in both the SI and LI, ranging from 22 to 24. These cells were categorized based on their GFP expression levels; those with the highest expression, located at the center of the crypt (positions "0" and "1"), were referred to as "center" ISCs while those with intermediate expression at positions "2" and "3" were termed "border" ISCs. Beyond position 3, GFP expression was substantially lower and not included in the Lgr5+ cell count.

To identify which Lgr5+ cells could function as "effective" ISCs, we conducted intravital imaging using the Lgr5eGFP-IRES-creERT2-Rosa26-Confetti mouse model, allowing us to trace Lgr5+ cells and their progeny over an 8-week period. At the 8-week mark, we evaluated clonal retention, which measures the number of clones remaining within the Lgr5+ zone. This was conducted in a spatially defined manner, with clonal retention rate determined for cells at the bottom of crypts and in three other positions upward towards the TA-zone. In the SI, 40.6% of center-derived clones and 15.1% of border-derived clones remained within the Lgr5+ zone. In contrast, in the LI solely center-derived clones remained, at 25.1%. This indicates that despite both crypts having a similar number of Lgr5+ cells, the SI possesses a twofold higher percentage of long-term effective ISCs compared to the LI.

Given the significant disparities in effective stem cell numbers between the SI and the LI, we aimed to understand the underlying reasons, considering both tissues share similar molecular capabilities. To address this, we initiated a quantitative mathematical modeling approach. The model predicted that the probability of long-term clone retention decreases as ISCs are located further from the crypt pit. This prediction was consistent with intra-vital imaging data, indicating that ISCs situated at positions 2 and 3 (the border) in the LI are lost more rapidly than their counterparts in the SI at the same

positions. Using this model, we further predicted distinctions in the fate of border ISCs between the SI and LI, suggesting that mechanistically, differences might arise due to downward directional motility of ISCs within the crypt. To test the prediction, we performed intravital imaging with lineage tracing in which the border and center ISCs were monitored over four days. In the SI, ISCs exhibited noticeable downward directional motility, while this phenomenon was almost absent in the LI.

To elucidate the factors driving this behavior, we initially used a 2D-system in which paneth cells were co-seeded with ISCs. This experiment aimed to investigate if Wnt ligands supplied by paneth cells (Sato, van Es et al. 2011) could influence stem cell motility. The results demonstrated a marked increase in stem cell motility, suggesting a role for Wnt.

4.1.2 Intestinal stem cells demonstrate cell intrinsic motility towards the crypt bottom

To confirm that the observed downwards directional motility was indeed driven by Wnt, we developed a 4D *ex vivo* live cell imaging assay. To closely mimic the intestinal environment, we utilized the protocol for decellularizing the small intestine of mice (dECM), which preserves the crypt-villus structures and at least partially retains factors associated with ECM (as described in **Paper 3**). It is worth noting that in **Paper 1** and **2**, the decellularized small intestine is abbreviated as "dECM", while in **Paper 3**, it is denoted as "iECM". However, it is important to clarify that the same protocol is consistently applied in both cases. Subsequently, we utilized the bare ECM as a platform to seed single ISCs and evaluate their individual behavior. For enhanced visualization of the dECM, it underwent fluorescent staining. Lgr5+ ISCs were then FACS sorted, stained, and seeded onto the dECM for live imaging. Utilizing a Trackmate plugin in the Fiji imaging software, we obtained x, y, and z coordinates for all time points during the live imaging. A custom Python script was employed to organize the data and investigate stem cell motility.

To directly assess the impact of Wnt on stem cell motility, we used the small molecule inhibitor porcupine, LGK974, to inhibit Wnt secretion in live mice. Mice were treated for 5 consecutive days. Subsequently, we extracted and decellularized the SIs. A comparative analysis of ISC motility on control-treated dECMs and LGK974-treated dECMs revealed that ISCs demonstrated reduced Z-motility on LGK974 treated dECMs. This indicates that the presence of Wnt-ligands in the control-treated ECM was responsible for the ISC motility in Z.

In **Paper 1**, we elucidate the quantity and location of effective ISCs in both the SI and the LI. Moreover, by integrating long-term intravital imaging with a novel *ex vivo* live cell imaging assay, we, for the first time, unveil Wnt-driven downward directional motility of ISCs in the SI, during homeostasis.

4.2 Paper 2: Aging and Apc mutations abolish the niche-homing capability of intestinal stem cells.

In this paper, we leveraged our innovative *ex vivo* live cell imaging assay to explore whether other cell types in addition to ISCs exhibit active directional motility. Furthermore, we probed whether the directional motility of ISCs is influenced by aging and intestinal tumorigenesis.

4.2.1 Intestinal cell types have cell type specific moving patterns that reflect their *in vivo* localization

Our methodology involved separately seeding single ISCs, PCs, and ECs onto dECMs. We tracked their movements using *ex vivo* live cell imaging, as detailed in **paper 1**. For FACS-mediated isolation of ECs, we developed a novel protocol. To this end, we severed villus domains form the intestines to enrich for ECs, dissociated villi into single cell preparations, and used cell sorting to isolate multiple candidate populations for analysis of EC markers. Using qPCR, we analyzed expression levels of the EC marker Alpi, ISC marker Olfm4, and differentiation markers for PCs, enteroendocrine cells, and goblet cells (Lyzo, ChgA, Muc2). Based on the analysis of marker ratios, we selected the subpopulations most enriched for ECs for subsequent *ex vivo* live cell imaging.

We found cell types to differ significantly in their motility. ISCs exhibited the highest xy-motility, covering more distance than PCs and ECs. ECs, on the other hand, exhibited the lowest xy-motility among the three cell types studied.

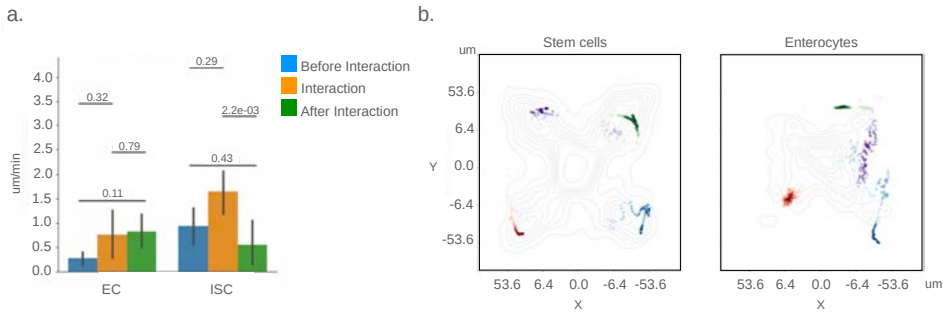
To investigate if these three cell types displayed directional motility in the z-axis (i.e., moving up or down relative to their starting point) we performed a ~4-hour time-lapse, in which we measured the distance each cell traveled per time point compared to its starting point. ISCs and PCs exhibited a downward movement of approximately 2-3 um when starting in both the crypt and villus region.

In contrast, ECs did not display directional motility in the crypt region, but demonstrated a slight upward trend in the villus- fitting the upward motility of villus cells in the intact intestine. Among the cells starting in the small region between the crypt and villus, which we termed the “intermediate” region, ISCs moved approximately 2.5 um downwards, while PCs only moved about 1.5 um, and ECs showed no directional motility. Interestingly, 62.5% of ISCs reached the crypt, whereas only 23.5 % of PCs and 10% of ECs reached the crypt.

4.2.2 Crypt-villus interactions drive cell motility independent of ECM cues

In the intestine, Ephrin-EphB signaling between two cells can induce repulsive motility, resulting in the two cells moving in opposite directions. ISCs express EphB, while ECs express Ephrin, serving as polar opposites in this repulsive signaling pathway (Batlle, Henderson et al. 2002, Holmberg, Genander et al. 2006). To address whether cell-cell

interactions and the Ephrin-EphB signaling may provide changes in the motility and direction of cells, we co-seeded ISCs and ECs onto the same dECM simultaneously and assessed their motility. ISCs exhibited enhanced xy-motility when co-seeded compared to when seeded alone. Interestingly though, both ISCs and ECs covered greater distances in xy compared to when seeded alone. Moreover, the downward directional motility of ISCs was completely abrogated when co-seeded with ECs, and instead, ISCs demonstrated an upward directional motility toward the villus. Furthermore, as shown in our **Supplementary Data Figure 1,** we separately analyzed those ISCs and ECs that were noted to interact during imaging and assessed their motility before, during, and after interaction (5 cell pairs). Our observations revealed that upon interaction, both ISCs and ECs increased xy-motility compared to their pre-interaction state. However, while ECs maintained the enhanced speed also after interaction, ISCs surprisingly reduced their speed to a level lower than observed before interaction.



Supplementary Figure 1. Interacting stem cells and enterocytes gain speed when interacting. Stem cells (ISCs) and enterocytes (ECs) were co-seeded on a decellularized mouse intestine. Live imaging was performed for 4 hours using a spinning disk confocal, obtaining measurements in x, y, and z. Using the Trackmate plug-in in Fiji, data points were obtained, and data were analyzed using Python custom script. Five interacting pairs were identified out of the 62 ISCs and 133 ECs. **a.** Speed of cells in xy. A two-sided Mann-Whitney test was performed, p-value was significant when <0.05. **b.** Distance reached in xy (displacement in xy) during time-lapse. Each color represents a cell's movement in xy during time.

4.2.3 Aging induced ECM changes abrogate directional cues and stem cell crypt-homing

As aging significantly alters the ECM (Levi, Papismadov et al. 2020), and considering the close physical proximity between ISCs and the ECM, it is not surprising that ISCs are directly influenced by the ECM (Yen and Wright 2006, Meran, Baulies et al. 2017) (Pentinmikko, Lozano et al. 2022). Furthermore, reduced Wnt-signaling during aging leads to diminished stemness (Pentinmikko, Iqbal et al. 2019), and in paper 1 of this thesis we found that Wnt-ligands serve as directional cues for ISCs. We therefore sought to investigate whether aging of the ECM could impact the motility of ISCs. Our findings, based on seeding young ISCs (3-6 months) on an old dECM (20-24 months)

and subsequent tracking of their movements, revealed a significant reduction in xy-motility and xy-displacement compared to young ISCs seeded on a young ECM. More notably, the crypt-homing capacity of ISCs that we earlier observed on a young ECM was entirely lost on an old dECM. This suggests that the positional cues that guide ISCs to the crypt bottom on a young dECM, were lacking in the old dECM.

4.2.4 The crypt homing mediated by exogenous Wnt ligands is lost in stem cells with tumor-inducing cell intrinsic Wnt-activation

Considering the drastic effect of an aged ECM (in which Wnt-signaling is reduced) on the crypt-homing capability of ISCs, we aimed to investigate how upregulated Wnt-signaling could influence ISC motility. Wnt-signaling was enhanced in ISCs using two approaches: pharmacologically with the GSK-3 inhibitor CHIR99021 and genetically using the mouse model for colorectal cancer, Lgr5-eGFP-IRES-creERT2. In the latter, the tumor suppressor *Apc* deletion was induced by five daily tamoxifen injections. Employing both approaches and conducting the *ex vivo* live cell imaging assay, we observed a reduction in xy-motility and displacement in ISCs with either Wnt-activation method when compared to untreated ISCs. Additionally, the crypt-homing capacity of ISCs was completely abolished in both treatments compared to untreated ISCs. Surprisingly, the *Apc*-mutant ISCs even exhibited upward directional motility, which was most prominent in the upper part of the crypt where cells can also escape the crypt. The percentage of CHIR99021-treated ISCs that successfully moved from the intermediate region to the crypt was 18.5% while *Apc*-mutant ISCs showed 14%, in contrast to the 62.5 % of WT ISCs demonstrating this ability. When specifically examining the orientation of CHIR99021-treated ISCs relative to the crypt using electron microscopy, these cells either exhibited randomly oriented leading-edge protrusions or no leading-edge at all, whereas majority of WT ISCs interestingly displayed clear leading-edge protrusions directed towards the crypt opening.

Paper 2 illustrates that ISCs and PCs possess an intrinsic ability to perceive cues within the ECM, and can accordingly direct their motility towards the crypt bottom. Highlighting cell type-specific homing within the tissue topology, ECs do not move toward the crypts. Furthermore, our study demonstrates that the ECM cues guiding crypt-homing capability of ISCs are lost during aging, and that oncogenic events can render cells unreceptive to ECM containing homing cues.

4.3 Paper 3: Fetal-like reversion in the regenerating intestine is regulated by mesenchymal Asporin

In this paper, we investigate how factors contained in the ECM influence intestinal tissue repair and whether such factors contribute to the reduced regeneration during aging. To facilitate our investigation, we developed novel system for growing intestinal epithelium on decellularized mouse intestines *ex vivo*.

4.3.1 Decellularized intestinal ECM supports organotypic growth of the epithelium

To investigate how ECM and the factors it reposits could influence the regeneration of intestinal epithelium, we initially optimized the decellularization process of mouse intestines, building upon a previously published protocol from 2006 (Chen, Wei et al. 2016). The primary objective was to decellularize the mouse intestinal ECM (iECM), with the aim of removing all cells while preserving the architecture of the crypt and villi, as well as the spatial composition of the ECM components. Immunofluorescent and hematoxylin+eosin stainings, as well as scanning electron microscopy imaging, revealed intact crypt-villus structures and complete removal of cells. Proteomic analysis with mass spectrometry on the iECM composition indicated that the majority of intracellular proteins were indeed removed, as 15 out of the 20 most abundant proteins we detected were ECM-associated proteins.

To confirm the dECM's viability and its ability to support new epithelial growth, we tested different sodium deoxycholate (SDC) concentrations (ranging from 0.5% to 6%) during the decellularization process. Subsequently, we cultured intestinal organoids and assessed the re-establishment of the intestinal epithelium. Notably, 0.5-1% SDC concentrations facilitated optimal growth, while higher SDC percentages correlated with reduced re-epithelialized crypts at 8 days post-seeding. We then proceeded with seeding intestinal organoids or crypts on the iECMs, both were capable of re-epithelializing the previously bare crypt and villus structures. Furthermore, the co-seeding of single Lg5+ISCs with PCs resulted in successful re-epithelialization of the iECMs, with an ISC-PC patterning at the crypt bottom similar to that observed *in vivo*. Additionally, these newly formed "intestines" could be cultured for over three weeks without passaging, as owing to the open format of the culture, dead cells were shed into media and removed with media changes. The iECMs ability to support and sustain intestinal re-epithelialization and the localized formation of crypts only in former crypt pits strongly suggests that these matrices retain critical ECM components, guiding tissue architecture, cell fate, and proliferation.

4.3.2 Re-epithelialization of decellularized ECM models *in vivo* regeneration

Considering the success in re-epithelializing empty crypts *ex vivo*, we contemplated whether this could serve as a model for studying *in vivo* aspects of re-epithelialization following damage. Our investigation revealed that the epithelial cells seeded on the iECMs expressed *Sca1 and Clu*, both of which are markers associated with a fetal-like state (Yui, Azzolin et al. 2018, Ayyaz, Kumar et al. 2019), while reducing the expression of the stem cell marker Lgr5. Interestingly, by the 6th day after seeding, the seeded epithelial cells had reduced their expression of Sca1, and the expression of Lgr5 was restored. These findings suggest that the restoration of the intestinal epithelium on iECM could mimic the dynamics of *in vivo* repair.

4.3.3 Mesenchymally produced Asporin induces a regenerative fetal-like state in the epithelium via Tgfβ-signaling

Utilizing both the iECM system and intestinal organoids, we identified the importance of Tgfβ-signaling in the re-epithelization of the iECM. Additionally, our mass-spectrometry of the iECM highlighted Asporin (*Aspn*) as a candidate mediating the Tgfβ-signaling.

Aspn was previously known for its role in the Tgfβ-signaling pathway as an inhibitor of downstream signaling (Maris, Blomme et al. 2015). However, our findings indicated a contrasting role for Aspn in promoting Tgfβ-signaling. *In-situ* hybridization experiments further confirmed the expression of *Aspn* in the mesenchyme, specifically within the pericryptal mesenchymal cells located beneath the crypt cells. Intriguingly, *Aspn* was found to trigger a fetal-like regenerative state by enhancing Tgfβ-signaling in ISCs via the CD44 receptor.

4.3.4 Timely regulation of mesenchymal Asporin is required for epithelial regeneration

To investigate the role of Aspn in the regenerative process following intestinal damage, we developed a mouse model that allowed for the specific deletion of Aspn in the intestinal mesenchyme. To achieve this, we first generated a mouse carrying the conditional allele of Aspn, *Aspn*^{lox}. This mouse strain was then crossed with *Twist2-Cre* mice (Šošić, Richardson et al. 2003), resulting in the generation of *Twist2-Cre; Aspn*$^{lox/lox}$ mice. These mice were subjected to 5-FU treatment *in vivo*. Deletion of Aspn in the mesenchyme compromised the necessary fetal-like conversion in the early stages of the regeneration, and resulted in a delayed recovery process, characterized by reduced body weight, shorter villi, and a diminished population of proliferating cells. These observations underscore the crucial role of Aspn in facilitating the regeneration of the intestinal epithelium.

In our final line of investigation, we delved into the influence of Aspn during regeneration within an aged intestine. Mass spectrometry analysis and *in situ* hybridization of aged intestines unveiled chronically high levels of Aspn. In line with the high Aspn levels, old mice exhibited a significantly higher count of Sca1+ expressing cells after 5-FU than young mice. As the intestinal epithelium in old mice has lower Wnt-signaling activity due to expression of the secreted Wnt-inhibitor Notum (Pentinmikko, Iqbal et al. 2019), we postulated that the inability of the old epithelium to revert back to adult-like state from the regenerative fetal-like state may reflect a role of Wnt-signaling in the process. We administered small molecule Notum inhibitor ABC99 to aged mice prior to the 5-FU treatment, and noted a reduction in the intensity of Sca1-expressing cells during the recovery phase. Jointly the findings indicate that, in addition to regulation of Aspn levels, Wnt-signaling indeed contributes to the cell-state reversion after damage is repaired.

Paper 3 demonstrates the utility of employing decellularized mouse intestinal tissues to investigate the influence of the mesenchyme-produced factors on intestinal regeneration following damage. In our study, we identify Aspn as a key regulator that promotes Tgfβ-signaling through the CD44 receptor on ISCs, inducing a fetal-like reprogramming of the intestinal tissue. Furthermore, our findings reveal an upregulation of Aspn in the aged intestines, contributing to a diminished capacity for tissue repair.

4.4 Paper 4: NOTUM from Apc-mutant cells biases clonal competition to initiate cancer

Here, we focused on elucidating the mechanisms by which *Apc*-mutant ISCs acquire a clonal advantage over wild-type ISCs within intestinal crypts, ultimately resulting in the clonal fixation within the crypt and in adenoma formation.

4.4.1 *Apc*-mutant cells secrete Notum that reduces growth of WT cells

To find modulators of *Apc*-mediated tumorigenesis, we performed transcriptomic analysis on tumors that emerged in VillinCre^{ER}; Apc$^{fl/+}$, after the sporadic loss of the remaining WT copy of *Apc* allele, closely resembling human CRC (Moser, Pitot et al. 1990). This analysis was compared to normal intestinal epithelial tissue. The results unveiled an upregulation of the *Notum* gene, which is recognized for its role in inhibiting Wnt signaling by preventing the binding of WNT ligands to the Frizzled receptors (Kakugawa, Langton et al. 2015).

To assess the impact of Notum, secreted by *Apc*-mutant cells, conditioned media was extracted from organoid cultures derived from the VillinCre^{ER}; Apc$^{fl/+}$ mice, and transferred to WT organoid cultures. We evaluated organoid formation capacity after 5 days. The growth and formation capacity of WT organoids exposed to Notum-conditioned media were significantly reduced. These WT organoids also exhibited downregulation of Wnt target genes and stem cell markers.

4.4.2 Notum from Apc$^{-/-}$ cells promotes tumorigenesis by reducing the clonal competitiveness of WT stem cells

To test if the suppressive effect of Notum on WT ISCs carried over to an *in vivo* setting, we analyzed the ability of *Apc*-mutant ISCs to fix crypts in tamoxifen-treated Lgr5Cre^{ER}; *Apc*$^{fl/fl}$ and *Lgr5Cre*^{ER}; *Apc*$^{fl/fl}$; *Notum*$^{fl/fl}$ mice. Deleting Notum resulted in a drastic reduction in clonal outgrowth and impaired clonal fixation of the mutant clones.

Building upon our *in vitro* data, which indicated the inhibitory effect of Notum on stemness, we sought to investigate if this trait would carry over to an *in vivo* setting. Our findings revealed that WT cells located in the same crypt as *Apc*-mutant clones exhibited reduced proliferation compared to WT cells located in crypts distant from mutant clones. However, when we deleted Notum in *Apc*-mutant clones, the

proliferation rates of WT cells in the same crypt as these mutants became similar to those in non-mutant inhabited crypts located further away.

Furthermore, we quantified the levels of the Wnt-regulator Sox9 in ISCs, PCs, and TA cells within crypts harboring *Apc*-mutant clones and in surrounding crypts. Our analysis indicated a significant reduction in Sox9 expression in WT ISCs cohabiting crypts with *Apc*-mutant clones, nearly matching the Sox9 levels observed in TA cells. Interestingly, this suppressive effect of Notum extended to adjacent crypts solely inhabited by WT cells. Even in these WT crypts, stemness and proliferation were reduced, while differentiation increased.

Inhibiting Notum in *Apc*-mutant clones resulted in the restoration of Sox9 expression to normal levels in ISCs, leading to a return of proliferation rates to normal. This intervention reduced the heightened differentiation of cells both in the crypts harboring Apc-mutant clones and in adjacent WT crypts.

Finally, we assessed the impact of Notum inhibition on tumor multiplicity. Notum inhibition led to an increased survival rate in mice developing *Apc*-mutant tumors, decreased tumor burden, reduced clonal fixation of *Apc*-mutant cells, and decreased the number of β-catenin expressing lesions.

Cumulatively, the findings presented in **Paper 4** underscore the pivotal role of *Apc*-mutant ISCs, which leverage Notum secretion to gain a clonal advantage. This mechanism suppresses stemness and enhances differentiation not only within the same intestinal crypt but also in adjacent WT crypts. Inhibiting Notum reduces mutant clonal fixation of crypts, mitigates tumor burden, and ultimately enhances survival.

5 Discussion and future perspectives

5.1 Functionally effective stem cells

Previous studies have delved into clonal cell dynamics in the intestine, particularly focusing on short-term behavior using short-term intravital microscopy (Ritsma, Ellenbroek, et al. 2014). In these investigations, researchers demonstrated that Lgr5+ cells positioned at the central crypt bottom exhibited enhanced survival rates compared to their counterparts at the GFP+ border within the crypt. Additionally, central Lgr5+ cells had a three-fold higher probability of colonizing a crypt during homeostasis, in contrast to border Lgr5+ cells. The variation was ascribed to the crypt's structure, characterized by its "blind-ended" configuration and the positioning of central cells "after/behind" other stem cells. This positioning rendered them the most probable candidates for long-term clones in situations of otherwise neutral competition. However, it remained a challenge to determine the number of ISCs capable of effectively functioning over extended periods and their precise locations within the crypt.

In **Paper 1**, we employed long-term intravital imaging to track the same clone for several weeks and assess clonal retention capabilities in both the small intestine (SI) and the large intestine (LI). Our findings revealed a significantly higher clonal retention in the SI compared to the LI. Intriguingly, in the SI, clones also at the very border of the stem cell niche were found to be effective ISCs. In contrast, in the LI, only clones situated at the central crypt displayed this potential (with a 25% likelihood for central cells to function as a long-term ISC). However, even though effective ISCs in the SI were identified both in the crypt center and the niche border, the percentage of remaining clones at eight weeks was 40% versus 15%, respectively. This suggests that there is indeed an advantage for Lgr5+ cells located at the center compared to the border in the SI. Whether this advantage is due to simple positional advantage as discussed by previous work (Ritsma, Ellenbroek et al. 2014), or if for example increased niche signals from the stroma (Kedinger, Duluc et al. 1998, Yen and Wright 2006, Powell, Pinchuk et al. 2011), or from multilateral contacts with paneth cells (PCs) (rather than TA cells on one side) (Sato, van Es et al. 2011), supply additional niche factors, is not clear. Moreover, the maximal geometric curvature found at the bottom of crypts can enhance the neighbor cell interactions (Pentinmikko, Lozano et al. 2022) and promote stem cell function at the crypt bottom in comparison to cells found at the edge of the niche where the 3D curvature becomes 2D. However, as described below, we discovered that ISCs possess the ability to move in a retrograde manner towards the crypt center, and as the lack of retrograde motility in the colon correlated with the more center-favoring clonal dynamics, it is likely that a complex array of cellular interactions and positional advantages jointly determine the number of effective stem cells in every crypt.

What distinguishes our study is the extended tracking duration of up to eight weeks, a notable improvement over previous short-term intravital microscopy studies that allowed only a few days of tracking (Ritsma, Ellenbroek et al. 2014, Choi, Rakhilin et al. 2018, Rakhilin, Garrett et al. 2019). This extended tracking was made possible by several factors, including the capture of high-resolution XYZ images that incorporated GFP expression. This, combined with the distinctive GFP expression pattern (a consequence of the *Lgr5* knock-in allele) and identifying marks such as blood vessels, enabled us to consistently pinpoint the original location in the intestine over the course of several weeks.

5.2 Active migration in the small intestine

5.2.1 Stem cells

To address the substantial disparities in effective ISC numbers between the SI and LI, as discussed in **Paper 1**, we employed quantitative mathematical modeling. Our model predicted a decline in the probability of long-term clone retention as ISCs are situated farther from the crypt pit. This modeling also suggests that differences in the fate of border ISCs between the SI and LI may arise from mechanisms associated with the downward directional motility of ISCs within the crypt. To validate this prediction, we conducted intravital imaging and confirmed that border ISCs exhibit a capacity to migrate toward the crypt pit in the SI, but this behavior is nearly absent in the LI. This contrast is intriguing considering the shared similarities between SI and LI, such as crypt architecture and the presence of Lgr5+ ISCs, which possess the ability to function as effective ISCs from a molecular standpoint. In **Paper 1,** we turned our attention to Wnt signaling as a potential explanation for this difference. Our reasoning was grounded in the recent discovery that Wnt signaling plays a pivotal role in inducing stem cell migration within the drosophila intestine (Hu, Yun et al. 2021). Additionally, both PCs and pericryptal stromal cells are known to express Wnt ligands. (Gregorieff, Pinto et al. 2005). It is important to highlight that, in contrast, the LI lacks PCs. To functionally test whether Wnt signaling indeed drives the downwards directional motility of ISCs, we developed a live-cell *ex vivo* imaging assay. By decellularizing the SI (dECM) of mice as detailed in **Paper 3**, we could seed single isolated Lgr5+ ISCs onto it and assess their motility in the crypt-villus axis of the SI.

While intravital imaging is an invaluable tool for studying the *in vivo* behavior of ISCs, it has limitations, particularly the influence of neighboring cells, which can exert "pushing" forces on ISCs. This influence prevents the assessment of whether ISCs intrinsically sense cues embedded in the ECM that guide them to their *in vivo* locations. Our *ex vivo* live-cell imaging assay overcame these limitations by allowing us to study the motility of ISCs without the confounding effects of neighboring cells. Notably, we found that inhibiting Wnt secretion in the dECM resulted in the loss of observed directional motility

of ISCs. The combination of the live-cell imaging assay, which enabled precise ISC tracking every 2 minutes over the course of a few hours, along with the long-term intravital imaging spanning several weeks, led us to conclude that ISCs indeed exhibit downwards directional motility and this phenomenon is primarily driven by Wnt.

It was surprising to observe that ISCs in the LI did not display downward directional motility. Despite the absence of Wnt-producing PCs within the LI crypts, the surrounding supporting mesenchymal niche supplies Wnt ligands that support ISC function in both the LI and SI (Degirmenci, Valenta et al. 2018, Karpus, Westendorp et al. 2019, Brügger, Valenta et al. 2020). Consequently, it seems unlikely that the lack of Wnt signaling supplied by PCs is the sole reason for the absence of active downward motility of LI ISCs. A deeper examination of the mesenchymal composition surrounding the colonic crypt structures could shed light on potential factors acting as inhibitors of active migration.

It would be intriguing to investigate ISC movement on LI decellularized intestinal ECM, however, it presents significant challenges. Regrettably, the ECM that separates crypts from each other in LI dECM is narrow, frequently causing crypts to collapse inwards. This obstruction hinders seeded cells from entering the crypt opening. Due to these limitations, conducting live imaging in z of single cells on the LI dECM would not be feasible. It is unfortunate because this approach could have been employed to target specific factors within the ECM, such as various laminin isoforms or growth factors. This would have allowed us to assess their potential effects on ISCs, and potentially promote directional motility toward the crypt bottom.

In addition to the absence of directional downward motility and reduced number of effective ISCs in the LI, ISCs displayed a notable lack of passive rearrangements. Consequently, crypt monoclonality, where a single ISC gives rise to all cells within a crypt, occurred at a significantly higher rate in the LI than in the SI. Given the higher frequency of cancer in the LI compared to the SI in humans, it raises the question of whether the absence of downwards directional motility in the LI contributes to this outcome. While the increased cell rearrangement within SI crypts facilitates a greater number of WT ISCs to compete with mutant ISCs, the reduced number of effective ISCs and decreased cell rearrangement in the LI may partially explain the higher incidence of tumors in this region compared to the SI. However, comprehensive investigations into active downward directional motility are essential before drawing definitive conclusions.

5.2.2 Paneth cells and enterocytes

After discovering the active downward movement of ISCs in the intestinal crypts in **Paper 1**, we employed our *ex vivo* live-cell imaging approach to study the motility of other cell types in relation to ISCs in **Paper 2**. It became evident that ISCs are highly mobile, moving at double the speed of PCs and roughly triple the speed of ECs. This is

perhaps not surprising considering the frequent shuffling of ISCs in the SI, as reported in **Paper 1**. Furthermore, In the *ex vivo* system, ISCs are not embedded within a full epithelial cell layer as in intravital imaging, which suggests that their movement might not be as restricted due to the absence of neighboring cells.

In addition to demonstrating the downward directional motility of ISCs, we discovered that PCs exhibit the same behavior when starting from various regions: the crypt, the villus, or the intermediate region just above the crypt opening. This indicates that, like ISCs, PCs can sense cues within the ECM that guide PCs to their native localization at the crypt bottom. In **Paper 1,** we established that the downward directional motility of ISCs is driven by Wnt. Therefore, it is reasonable to hypothesize that Wnt could serve as a positional cue for PCs. However, unlike ISCs, PCs are Wnt ligand producers. Interestingly, *Apc*-mutant ISCs exhibit constitutively active Wnt signaling (Barker, Ridgway et al. 2009) and do not migrate to the crypt bottom, as demonstrated in **Paper 2**. This suggests that the mechanism guiding the downward directional motility of PCs might not be Wnt-driven but rather responsive to other positional cues in the ECM. Given that the ECM acts as a reservoir for a wide range of molecules, from cytokines to growth factors (Tschumperlin 2015), the possibilities are numerous. On the other hand, Wnt-ligands are produced in modest quantities even by actively secreting cells, and it is unlikely that an isolated PC that is introduced to the naked decellularized ECM could within minutes or hours produce an amount of Wnt-ligands capable of masking the Wnt-ligand gradients deposited by a multitude of stromal and epithelial cells during homeostasis. As we did not probe the motility of PCs on the ECM from porcupine inhibitor-treated mice, it is possible the downward motility of PCs is guided by Wnt-ligands similarly to ISCs.

Deciphering the composition of the ECM has been a challenge, primarily due to limitations in performing mass spectrometry on the ECM (Krasny and Huang 2021, Naba 2023). Particularly, detection of less abundant proteins, in contrast to highly expressed ones like collagen, remains a challenge. However, significant advancements have been made. Notably, the differential distribution of laminin isoforms in the crypt versus villus regions serves as an example. Laminin $\alpha 2$ is predominantly expressed in the crypt, while laminin $\alpha 5$ is prevalent in the villus (Orian-Rousseau, Aberdam et al. 1996, Kedinger, Duluc et al. 1998, Glentis, Gurchenkov et al. 2014). Therefore, in addition to exploring whether Wnt-ligands indeed drive PC downward motility, investigating the role of various laminin isoforms provides an interesting avenue for further research.

When we employed the live-cell imaging approach, we observed that ECs exhibited no directional motility in the absence of neighboring cells, as detailed in **Paper 2**. This finding served as a valuable control, allowing us to investigate potential influences such as gravity that might contribute to the downward directional motility observed in ISCs and PCs.

The absence of directional motility of ECs was initially surprising, especially considering a study published in 2019 Krndija *et al*. demonstrated that ECs could migrate towards the villus tip and increase speed in the process. However, it is important to emphasize that these researchers observed groups of EC units rather than individual cells. They conducted live imaging of gut explants over a 6-hour period, during which EC units indeed exhibited increased speed as they approached the villus tip. Their motility ranged from 3 um/h (0.05 um/min) at the lower part of the villus to 7-8 um/h (0.12 um/min- 0.13 um/min) near the villus tip. In contrast, our live imaging on dECMs extended approximately 4 hours. Towards the end of our imaging, we observed a slight upward trend in ECs within the villus domain, which might be within the lower range motility detected in the lower villus region by Krndija et al. This suggests that single ECs may indeed respond to cues in the villus, but in the absence of neighboring ECs, single ECs might not be able to move as effectively as seen during collective movement. To delve deeper into this phenomenon, it would be intriguing to conduct long-term observations of ECs using *the ex vivo* live-cell imaging approach, shedding light on whether the observed upward trend in the villus region persists over time.

5.3 Could *ex vivo* live-cell imaging be used in other tissues?

Given the discovery of active directional migration in the SI, it raises intriguing questions about the potential for downwards directional motility of stem cells in other tissues. It would therefore be worth exploring this behavior during homeostasis in the lung or even in hair follicles. Adapting and optimizing the *ex vivo* live-cell imaging approach for various tissues could yield valuable insights into tissue-specific stem cell migratory behavior. Additionally, there is a potential advantage in optimizing this live-cell imaging assay for use on human intestinal tissue to investigate whether the downwards directional motility of ISCs is conserved in humans. However, it is important to acknowledge that human intestinal tissue is considerably thicker, which may pose challenges in terms of imaging.

5.4 EphB-ephrin signaling at play?

In vivo, epithelial cells express varying levels of EphB or ephrin, which provide a repulsive migratory signal when cells with opposing expression pattern interact, and contributes to the cellular organization within the intestinal crypt-villus axis (Batlle, Henderson et al. 2002). In **Paper 2**, we co-seeded the EphB-expressing ISCs and ephrin-expressing ECs on the same dECM and assessed their motility. First, ISCs exhibited increased speed compared to when they were seeded alone. Second, the directional motility of ISCs toward the crypt pit was lost. These findings suggest that within our *ex vivo* experimental setting, the EphB-ephrin signaling is at play, inducing repulsion between these two cell types.

Interestingly, ISCs co-seeded with ECs not only failed to move toward the crypt bottom, but ISCs even appeared to start migrating toward the villus. This observation implies that any interactions occurring between ISCs and ECs prior to imaging exert substantial influence to disrupt the intrinsic ability of ISCs to move to the crypt bottom. We wished to delve deeper into investigating the motility between the specific interacting ISC-EC pairs, before, during, and after interaction. As shown in the **supplementary data,** I found that within ISC-EC pairs, xy-motility was enhanced during interaction for both cell types compared to the pre-interaction state.

However, surprisingly ISCs exhibited a more substantial increase in speed than ECs during their interaction. Upon closer examination of the imaging videos, it became evident that ISCs were "nudging" ECs from slightly varying angles while maintaining contact, resulting in an increased speed in xy. In contrast, ECs displayed slightly less movement in xy and tended to move predominantly in one direction. Furthermore, post-interaction, ECs maintained elevated xy-motility, whereas ISCs reduced their speed to levels below that observed before interaction. This underscores the significant influence of EphB-Ephrin signaling resulting from a single interaction.

Unfortunately, we could only analyze a limited number of interacting ISC-EC couples (5), making it challenging to draw conclusions from such a small sample size. Expanding the number of pairs studied would enable us to investigate how directional motility is affected within the pairs. Additionally, it would provide insights into whether directional motility differs depending on whether the pair is located within the crypt or villus domain, representing ISC or EC *in vivo* habitat, respectively.

5.5 Decellularization of the mouse intestine

Chronologically, **Paper 3** serves as the foundation for **Papers 1** and **2**, as it presents the optimized protocol for intestinal decellularization that underpins the *ex vivo* live-cell imaging approach employed in **Paper 1** and **Paper 2**. Accordingly, this discussion will primarily focus on the method detailed in **Paper 3**.

Decellularization of tissues has a long history (Hjelle, Carlson et al. 1979, Rojkind, Gatmaitan et al. 1980, Fu, Wang et al. 2014), but preserving both tissue architecture and ECM components has posed significant challenges. In **Paper 3,** we successfully decellularized mouse intestines (dECM), retaining the villus's large structures and crypt's small invaginations. Importantly, we conducted proteomic analysis with mass-spectrometry to identify the dECM's composition, revealing the removal of most intracellular proteins during the decellularization process while retaining ECM-associated proteins, such as collagens and laminins. Given the challenges of performing mass-spectrometry on the ECM (Krasny and Huang 2021, Naba 2023), the extensive protein identification we achieved was surprising. The ECM's intricate composition, including proteins, proteoglycans, glycoproteins, and other macromolecules, presents

difficulties in accurate separation and identification. The high abundance of collagen in the intestinal ECM can overshadow less abundant ECM proteins, such as growth factors. Moreover, post-translational modifications, such as glycosylation and phosphorylation can further complicate protein analysis, by influencing protein size. The identification of numerous proteins, including Asporin, in the dECM was unexpected and led to the discovery of its role in enhancing Tgfβ-signaling during fetal-like regeneration. Despite our efforts, mass spectrometry analysis did not detect the presence of Wnt in the ECM. However, in **Paper 1** we identified that Wnt-ligands embedded in the ECM have a role in driving ISC downward directional motility. Therefore, it is important to note that the negative data on detectable Wnt-ligands in the dECM by mass spectrometry does not indicate that retention of positional cues such as Wnt is lost during decellularization.

Furthermore, the decellularization protocol outlined in **Paper 3** was not optimized for live-cell tracking as performed in **Papers 1 and 2**. However, the ability of single-seeded ISCs to regenerate a full epithelial cell layer on the dECM in culture implies its viability for tracking cell movement over several hours, provided non-harmful imaging conditions are maintained to minimize cytotoxicity.

5.5.1 Potential caveats

Even though the mice used in the dECMs experiments shared the same C57BL/6 background, were subjected to identical environmental conditions including housing, and were fed the same type of food, inherent variability is always present to some extent, underscoring the importance of biological replicates. However, the factor that likely introduces the most variability is the individual preparation of the dECMs. As the protocol was optimized for supporting long-term culture of epithelium, the chosen protocol may not be optimal for retaining spatial cues for short-term motility assays.

First, considering the influence of increased SDC concentrations during the decellularization process on re-epithelialization, it is crucial to meticulously wash the dECM pieces. Consequently, during experiments comparing different treatments using the dECM model, bias may arise if one set of dECM pieces are not as thoroughly washed as its counterpart. Secondly, when handling the fragile pieces of dECM, it is vital to avoid causing any damage, such as accidental puncturing or tearing with tweezers. Fortunately, such mishaps can be readily identified under a microscope, and any dECM found to be damaged should be discarded. Thirdly, due to the thin nature of the decellularized tissue, it is susceptible to drying out if not handled promptly during the steps leading up to cell seeding. These potential pitfalls emphasize the importance of carefully preparing and handling the dECMs and underscore the value of including technical replicates in the experimental design.

5.6 Loss of crypt-homing capacity of ISCs during aging

During aging, significant alterations occur in the ECM, resulting in increased stiffness and changes in ECM composition (Segel, Neumann et al. 2019, Levi, Papismadov et al. 2020). Additionally, Wnt signaling and stemness in the intestine are downregulated with age (Nalapareddy, Nattamai et al. 2017, Pentinmikko, Iqbal et al. 2019). Given our previous discovery that Wnt drives ISC downwards directional motility in **Paper 1**, we employed our *ex vivo* live-cell imaging approach to investigate whether ISC motility was affected when seeded on aged dECM.

Remarkably, we observed a significant reduction in the overall motility of ISCs, with a complete loss of downwards directional motility of ISCs when seeded on aged dECM. This loss of directional motility could be attributed to the increased production of the Wnt antagonist, Notum (Pentinmikko, Iqbal et al. 2019). This previous research has demonstrated that PCs secrete Notum in the aged SI, which diminishes ISC stemness. Since Wnt ligands are produced not only by PCs, however, also by the surrounding mesenchyme (Gregorieff, Pinto et al. 2005), it suggests that Notum serves as a potent inhibitor of Wnt signaling. Consequently, it is reasonable to speculate that the Wnt positional cues embedded in the young dECM could be lost due to the potent Notum inhibitors in the aged dECM. To explore this further, it would be intriguing to treat aged mice *in vivo* with the Notum inhibitor ABC99 (Suciu, Cognetta et al. 2018), followed by decellularization of the intestine, and perform *ex vivo* live-cell imaging. This could help determine whether the loss of ISC downward directional motility can be reversed.

However, it is important to note that our observation of the loss of ISC downwards directional motility involved young ISCs seeded on aged dECM. To confirm whether this loss of crypt-homing is solely attributed to the ECM and not reflecting some unknown "mismatch" resulting from the different age of ISC and ECM, experiments involving aged ISCs seeded on aged dECM, and aged ISCs on young dECM, should be conducted. It is plausible that aged ISCs respond differently to cues in an aged intestine, possibly as an adaption to the reduced Wnt present in the aged ECM. Additionally, studying the behavior of aged ISCs when seeded on a young ECM could determine if the loss of crypt-homing can be rescued when Wnt-positional cues are present.

Considering the reduced general motility of ISCs when seeded on the aged dECM, it raises the possibility that the dynamic movement of ISCs, observed in a young intestine (as described in **Paper 1)**, might be diminished in an aged intestine. The combination of reduced general motility and a lack of downwards directional motility on an aged dECM underscores the importance of investigating ISC motility in an aged intestine using long-term intravital imaging. This research could reveal whether the loss of downwards directional motility, as observed in **Paper 2**, results in reduced or absent ability for "border" ISCs to migrate to the crypt bottom, potentially affecting their role as effective

ISCs. If aging is indeed found to reduce the number of effective ISCs by limiting downwards directional movement of ISCs, an *in vivo* intervention involving the Notum inhibitor ABC99 (Suciu, Cognetta et al. 2018) becomes an attractive prospect. Notum inhibition may restore the Wnt-positional cues in the ECM, consequently, reinstating the number of long-term efficient ISCs, and promote the clonal competitiveness of WT ISCs against the cells gaining cancer-initiating mutations during aging.

5.7 Initiation of intestinal cancer results in loss of crypt-homing capacity of ISCs

Most intestinal cancers are initiated by mutations in the tumor suppressor gene *Apc* (Brannon, Vakiani et al. 2014). These mutations lead to continuous activation of Wnt signaling, resulting in *Apc*-mutant ISCs losing their niche dependency (Barker, Ridgway et al. 2009). In **Paper 2**, we employed an *ex vivo* live-cell imaging assay to investigate *Apc*-mutant ISCs. We discovered that these cells not only lost their ability for downwards directional motility to the crypt bottom, as observed in WT ISCs in **Papers 1** and **2**, but they also exhibited upward directional motility, escaping the crypt. In **Paper 4**, together with an accompanying publication in Nature (van Neerven, de Groot et al. 2021), we further demonstrated that *Apc*-mutant ISCs secrete Notum, a factor that reduces stemness and increases the differentiation of WT ISCs in the same crypt. Surprisingly, Notum secreted by the incipient adenoma can influence cells even many crypts away from the adenoma. This provides them with a clonal advantage, or competitive edge, effectively making them "supercompetitors" not only in their own crypt, but also in a wider field of crypts and epithelium.

My observation of *Apc*-mutant ISCs escaping the crypt in **Paper 2** raises intriguing possibilities, suggesting that these cells may initiate adenoma formation not only at the crypt bottom but also outside the crypt region. Considering our earlier discovery in **Paper 1**, where we found that ISC downwards directional is driven by Wnt, it raises the hypothesis that *Apc*-mutant ISCs' ability to leave the crypt could be related to their production of Notum (as described in **Paper 4**), which might function to inhibit Wnt positional cues in the ECM. However, given that our live imaging was limited to a few hours, it is unlikely to explain this phenomenon. Additionally, we observed a loss in crypt-homing capacity when pharmacologically activating Wnt signaling. This implies other mechanisms. One possibility is that cells with intrinsic activation of the Wnt-pathway are no longer able to sense lateral differences in Wnt-ligand availability due to their internal Wnt-signaling being "maxed out". Investigating the impact of treating seeded *Apc*-mutant ISCs with the Notum inhibitor ABC99 (Suciu, Cognetta et al. 2018) could shed more light on this complex interplay.

5.8 Inhibition of Notum reduces tumor burden

In **Paper 4** we approach the inhibition of Notum as a potential therapy treatment, by specifically targeting Notum in *Apc*-mutant ISCs. The outcome was reduced tumor burden and increased survival. In one of the accompanying publications by van Neerven *et.al* 2021, researchers approach treatment by boosting Wnt signaling through the Wnt agonist lithium chloride in the WT ISCs. Interestingly, this treatment reduced clonal fixation of crypt-harboring *Apc*-mutant ISCs, as well as reduced tumor burden. However, when investigating later stages of colorectal cancer, by *Kras* deletion, the effect was not observed. This suggests that re-establishing Wnt-signaling in the crypt could offer a treatment option for the onset of intestinal cancer, for instance in individuals who carry APC germline mutations pre-disposing them with FAP, as discussed in **Paper 4**. Even though Notum appears to be a promising target for re-establishing homeostasis of Wnt-signaling in intestinal crypts, further studies are needed to evaluate possible off-target effects, and long-term repercussions of enhancing Wnt signaling.

5.9 Could inhibition of Notum be the answer to age-related intestinal cancer?

Notum is upregulated in *Apc*-mutant ISCs (detailed in **Paper 4** and (Ramadan, van Driel et al. 2022)) and by old Paneth cells in an aged intestine (Pentinmikko, Iqbal et al. 2019). Inhibiting Notum reduced tumor formation by *Apc*-mutant ISCs and enhanced the regeneration of the aged intestine. This suggests that inhibition of Notum might offer avenues to target age-related intestinal cancer. However, there could be repercussions when inhibiting Notum, such as compensation by other Wnt antagonists, like Dkk2 and Wif1 (van Neerven, de Groot et al. 2021), both of which were upregulated in *Apc*-mutant ISCs. One might, therefore, suggest treatment by a Wnt agonist, as performed in the same paper. However, there is a risk that this could, in an aged intestine, instead induce a tumor risk (Pentinmikko, Iqbal et al. 2019).

Given the findings in **Paper 2 and 4,** where the crypt-homing capacity of ISCs was compromised in an aged intestine and *Apc*-mutant ISCs gained a clonal advantage through Notum secretion, it is intriguing to investigate the potential impact of Notum inhibition on the early stages of intestinal adenomas in an aged intestine. Let us first consider the aging aspect. Wnt-signaling decreases in an aged intestine, due to Notum secretion (Pentinmikko, Iqbal et al. 2019). Moreover, as per the insights from **Paper 2**, ISCs lose their ability to home to the crypt bottom in aged intestines. This suggests that the Wnt embedded in the ECM guiding crypt-homing of ISCs, as discovered in **Paper 1**, are lost in an aged ECM. Therefore, inhibiting Notum might reintroduce these Wnt positional cues within the ECM, potentially enabling ISCs to move to the crypt bottom. Drawing from the intravital imaging data in **Paper 1**, which showed a higher percentage of long-term efficient ISCs in a young SI due to their ability for downward directional

motility, it is conceivable that in an aged intestine, the restoration of crypt-homing ability could result in a higher percentage of efficient ISC. This could ultimately counteract the observed decline in stemness associated with aging.

However, the situation becomes more complex in aged intestines harboring *Apc*-mutant ISCs. Since, *Apc*-mutant ISCs are intrinsically activated for Wnt-signaling (Barker, Ridgway et al. 2009), inhibition of Notum would not influence crypt-homing capacity of *Apc*-mutant ISCs. The literature indicates that *Apc*-mutant ISCs typically form adenomas at the crypt bottom (Barker, Ridgway et al. 2009). Nevertheless, as demonstrated in **Paper 2** *Apc*-mutant ISCs can also migrate out of the crypt. Whether such crypt-escaping *Apc*-mutant cells are invariably lost by shedding from the villi, or whether they pose a risk for adenoma formation is unclear. However, Notum inhibition in the old intestine would not only promote the competitiveness of WT ISCs by targeting the Notum produced by *Apc*-mutant cells, but also by promoting their homing towards the crypt bottom and the number of effective ISCs. In this light, Notum inhibition may provide a multipronged advantage for WT cells, particularly in the old intestine. To evaluate the outcomes of Notum inhibition in aged intestines harboring *Apc* mutations, long-term mouse studies would be essential to evaluate if this hypothesis holds true.

In conclusion, Notum inhibition holds promise as a potential therapeutic approach for elderly patients in the early stages of intestinal cancer. By restoring Wnt-positional cues in the ECM, it could bolster the population of long-term ISCS, reducing the competitive edge of *Apc*-mutant ISCs.

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My Ph.D. journey has been a lot like climbing a mountain, filled with both challenges and excitement. It all started at the bottom (as most journeys do), with me gazing up at the summit in the distance. It felt like a long and daunting path ahead, but it was also incredibly beautiful. As I began the ascent, everything seemed to be going smoothly, and I felt optimistic. However, as the day progressed, the sun began to set, casting darkness over the trail. The path became less clear, but I kept moving forward, remembering to turn on my flashlight, which illuminated the way. Time passed, and I continued to focus on reaching the mountaintop. But then, my focus shifted; a storm was approaching, and I had no shelter. Up ahead, I spotted another hiker with their family, waving to me. I quickly approached and was invited into their tent. They offered me a chance to rest and have some food, and suddenly, things were looking up. The storm soon passed, and I continued my journey up the mountain with the family by my side. The path ahead was rough, but it was incredibly beautiful. I found myself learning about the surrounding flowers and trees as I trekked through the challenging terrain. Finally, I reached the summit, gazing down at the side of the mountain. I reflected on the entire journey, with all its challenges and joys, and couldn't help but smile. As I stand here at the summit, reflecting on this challenging yet incredibly rewarding journey, I'd like to express my heartfelt gratitude to those who have been with me every step of the way.

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