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Alterations of niche to stem cell communication in the aging intestine

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ACADEMIC DISSERTATION

to be presented for public discussion with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in Auditorium 2041, Biocenter 2 (Viikinkaari 5D, Helsinki) on the 25th of September, 2020, at 2 pm.

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Cover: Paneth cells (red) support stem cell function in organoids of small intestine
Back cover: Epithelial repair in progress

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'Eteenpäin on menty'
-Antti Muurinen

Abstract

Aging is a biological process where tissue functions deteriorate leading to morbidity. Gastrointestinal tract is a key system for organismal health converting external energy sources to a useful form for the body. With age, intestinal function declines in humans as the absorptive capacity decreases and recovery from damage slows down. Absorptive epithelium is constantly renewed by actively proliferating intestinal stem cells (ISC) that are intermingled between specialized secretory cells named Paneth cells. These cells are located at the bottom of intestinal crypts that forms a specialized microenvironment, the stem cell niche, participating to the maintenance of ISCs and their function. What is the role of the niche in age related changes of ISC function remains poorly characterized.

In this dissertation age-associated changes in the communication between ISCs and the neighboring Paneth cells are analyzed and the outcome for regenerative capacity and homeostatic maintenance of the epithelium is studied. Under homeostasis, old epithelium is functional, but ISCs are biased to differentiate towards the secretory lineage. Using *in vitro* organoid culture, we show that reduced regenerative function of old epithelium is due to defects in both ISCs and the supporting Paneth cells. Aged Paneth cells produce excess amount of Notum, secreted Wnt-inhibitor, that reduces the canonical Wnt-activity in the neighboring ISCs. Moreover, old crypts have enlarged morphology reducing the physical curvature of the niche, and potentially increasing the size of ISCs *in vivo*. Alterations in size and shape of old ISCs can contribute to decreased capacity to receive signals from the niche.

Supplementation of Wnt-ligands, genetic ablation of Notum or culture on bioengineered topologically young scaffolds improved the regenerative growth of old epithelium in vitro. Moreover, inhibition of Notum *in vivo* with a small molecule ABC99 enhanced recovery of old intestine from 5-Fluorouracil induced chemotherapeutic insult. These findings demonstrate that Notum inhibition could be used as a prophylactic treatment to reduce harmful side-effects in elderly patients undergoing chemotherapy. In addition, cellular shape as a facilitator of intercellular communication in the ISC niche suggest that modulation of tissue topology could result in enhanced stem cell function.

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Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AMOT	Angiomotin
APC	Adenomatous Polyposis Coli
CBC	Crypt base columnar
CR	Caloric restriction
DAB	3,3'-Diaminobenzidine
DAG	Diacylglycerol
Dkk	Dickkopf
Dll	Delta like canonical Notch ligand
Dvl	Dishevelled
EDTA	Ethylenediaminetetraacetic acid
Egf	Epidermal growth factor
Eph	Ephrin receptor
F-actin	Filamentous actin
FACS	Fluorescent activated cell sorting
FAO	Fatty acid oxidation
FSC	Forward scatter
Fzd	Frizzled
G-actin	Globular actin
GH	Growth hormone
GI	Gastrointestinal
GSEA	Gene Set Enrichment Analysis
GSK3β	Glycogen Synthase Kinase 3 beta
ISC	Intestinal Stem Cell
Lef	Lymphoid enhancer-binding factor
Lgr	Leucine-rich repeat-containing G-protein coupled receptor
Lrp	Low-density lipoprotein receptor-related protein
mTOR	mammalian Target of rapamycin
NM II	Non-muscle myosin
Mylk	Myosin light chain kinase
Porcn	Porcupine
Ppar	Peroxisome proliferator-activated receptor
qPCR	quantitative polymerase chain reaction
S6	40S ribosomal protein S6
S6K1	p70/p85 S6 kinase 1
SASP	Senescence associated secretory phenotype
SCD1	Stearoyl-CoA desaturase 1
Sfrp	Secreted frizzled-related protein
SSC	Side scatter
TSC	Tuberous sclerosis complex
Wnt	Wingless-related integration site
YAP	Yes-associated protein
Tcf	T-cell factor
Wls	Wnt ligand secretion mediator
Wif1	Wnt inhibitory factor 1

List of original publications

This dissertation is based on the following original publication (I) and a manuscript (II), referred in the text by their Roman numerals:

I. **Pentinmikko N**., Iqbal S.*, Mana M.*, Andersson S., Cognetta III A., Suciu R., Roper R., Luopajärvi K., Markelin E., Gopalakrishnan S., Smolander O-P., Naranjo S., Saarinen T., Juuti A., Pietiläinen K., Auvinen P., Ristimäki A., Gupta N., Tammela T., Jacks T., Sabatini DM., Cravatt BF., Yilmaz Ö., and Katajisto P. Notum produced by Paneth cells attenuates regeneration of aged intestinal epithelium. (2019)

Nature Jul;571(7765):398-402. doi: 10.1038/s41586-019-1383-0.
* Equal contribution

II. **Pentinmikko N**., Lozano R., Scharaw S., Andersson S., Broberg M., Song KY., Albritton N., Teixeira A, Katajisto P. Cellular shape reinforces niche to stem cell signaling in the small intestine.[#]

[#] Manuscript

In addition, some unpublished Supplemental data is presented and referred as Data Figure 1-6 in the text.

Review of the literature

1. Aging

Aging can be defined as progressive tissue deterioration and functional decline that eventually leads to mortality (Lopez-Otin, Blasco et al. 2013). It is conserved among the vertebrate phyla and broadly in animal kingdom. However, some animals, such as hydra and planaria (genus *Hydra* and *Schmidtea* respectively), many plants and single cell prokaryotes can be considered immortal, as they do not die of aging (Petralia, Mattson et al. 2014). This indication that biological aging is not an inevitable feature of biological beings has posed a great interest to understand aging. In humans, increasing age is associated with phenotypic changes, such as graying or losing hair, wrinkling of the skin and reduced gait (Hamilton 1951, Boss and Seegmiller 1981). However aging is also the biggest risk factor for many life-threatening diseases (Harman 1991), and the global increase in the average lifespan (WHO 2019) imposes growing healthcare costs for the society. Therefore, targeting the aging process, would decrease the economic burden and increase the quality and possibly time of human life.

To alleviate the challenges aging poses for the society and individuals, focus should be in preventing or delaying the onset of aging related diseases. This would increase the proportion of time spent in good health in individual life, known as healthspan. In order to target the tissues where age-related functional problems are detected, process of aging must be understood on a cellular level.

1.1 Molecular drivers of aging

Molecular biology entered to the field of aging research in the late 20th century when the first long lived genetically modified model organisms were identified (Klass 1983). Klass and colleagues used a common roundworm *Caenorhabditis elegans* with a lifespan of few weeks. Remarkably, animals possessing mutation in a single gene, later named *age-1*, had longer lifespan than the wild type animals (Klass 1983, Johnson 2013). This was the first hard evidence for scientist that the functional decline due to aging is driven by individual molecular pathways. Moreover, it implied that by tinkering these pathways, aging could be slowed down or even stopped.

Today, it is understood that cells composing the tissues acquire various types of damage during the lifetime (Lopez-Otin, Blasco et al. 2013). Though many aspects and pathways, such as *age-1*, involved in the aging process are conserved, the unpredictable nature and location of cellular damage causes individuals to age differently. Moreover, environmental factors have significant effect for individual's lifespan. Therefore, targeting a single molecular pathway will unlikely extend life in general. However, knowledge on the mechanisms behind the age-associated cellular damage and functional decline enables researchers to target specific aging related diseases and thus extend the healthspan.

DNA damage leads to loss of information

Cellular damage destroying function can appear in several cellular compartments (Lopez-Otin, Blasco et al. 2013). Proliferating cells, such as tissue resident stem or progenitor cells, accumulate mutations to their DNA over time (Rossi, Bryder et al. 2007, Jones and Rando 2011). Moreover, upon each proliferation cycle, telomeres, repeat sequences that protect the integrity of chromosomal DNA, are shortened. Worn out telomeres result to deterioration of cellular function. Telomerase (Tert), enzyme that repairs shortened telomeres, is not expressed widely in somatic cells limiting their replicative lifespan (Flores and Blasco 2010). Therefore, reduced telomere length likely drives some loss of function in old tissues, fueled by stem cells. Remarkably, Jaskelioff and colleagues were able to restore the function of tissue resident progenitor cells by reactivating Tert in mouse model of premature aging, suggesting that Tert activity is critical for lifelong stem cell function (Jaskelioff, Muller et al. 2011). On that line, tissue resident stem

cells often have longer telomeres and higher Tert activity as seen in the intestine (Flores and Blasco 2010, Schepers, Vries et al. 2011). Schepers and colleagues observed that telomere length remained constant in the later life of mice, suggesting that Tert activity does not limit the function of aged stem cells of the intestine (Schepers, Vries et al. 2011).

Epigenetic noise severs cellular identity

On top of the damage on DNA molecule itself, aging has been associated with increased noise in transcription, which results to reduced cellular function (Bahar, Hartmann et al. 2006, Enge, Arda et al. 2017). The source for this uncontrolled gene expression in old cells remains to be elucidated, but likely involves changes in the epigenetic marks of DNA (Kane and Sinclair 2019). Modulation of enzymes capable of editing these marks, such as histone deacetylases, acetyl- and methyltransferases, can either promote or slow down aging in model organisms (Lopez-Otin, Blasco et al. 2013, Kane and Sinclair 2019). The druggable nature of these enzymes, makes them interesting candidates for translational research while some molecules have already reached the market (Dai, Sinclair et al. 2018). The gradual loss of genetic information with age, due to increased transcriptional noise and mutational load of the DNA (see above), postulated by 'the information theory of aging' (Sinclair and Laplante 2019), impairs organismal function by affecting cellular performance.

Proteostasis is required for recycling the cellular building blocks

On another level, inadequate quality control of proteins, collectively termed proteostasis, has been observed in aged tissues (Lopez-Otin, Blasco et al. 2013). Failure to rebuild or to clear misfolded proteins results to formation of aggregates which cause proteotoxic effects (Hipp, Park et al. 2014). Protein aggregates found in Alzheimer's patient brains have been suggested to contribute to the progression of this age-related disease (Ross and Poirier 2004). Moreover, enhancing the clearance of misfolded proteins in the liver of old mice has been shown to increase liver function (Zhang and Cuervo 2008). Taken together, maintaining correct cellular proteostasis can lengthen the functional period of tissues.

1.2 Energy sensors as modulators of aging

When knowledge of the genes that extended lifespan increased, it became obvious that many of them belong to one of the energy sensing pathways of cells. Mutant gene *age-1* in the long lived *C.elegans* was followed by *daf-2*, *daf-16* and others, which were eventually identified to code proteins in the conserved insulin/insulin like growth factor-1 (IGF-1) signaling (IIS) pathway. IIS pathway promotes growth of these animals and mutant versions in *age-1* and *daf-2* hampered the activity of it (Uno and Nishida 2016). Moreover, the first long lived mammalian model organism, Ames dwarf mouse, lacks functional growth hormone (GH), prolactin and thyrotropin (BrownBorg, Borg et al. 1996). Together these findings suggest that anabolism, building of biological material, promotes aging process. Furthermore, experiments done decades earlier, showed that reduced caloric intake without introducing malnutrition, increased lifespan of laboratory rats (McCay, Crowell et al. 1989). This initial study has been complemented by multiple laboratories using various animal models, including non-human primates (Piper, Partridge et al. 2011, Mattison, Colman et al. 2017). Moreover, caloric restricted old animals show improved results in multiple health parameters (Trepanowski, Canale et al. 2011). Taken together, these experiments highlight that decreasing the anabolic growth can extend both life- and healthspan, often taken as supportive for 'the rate of living theory of aging' (Redman, Smith et al. 2018). However, to mechanistically understand why limiting growth improves health in aged animals, knowledge from the molecular pathways involved is needed. Moreover, the behavior of individual cell types upon such systemic modulation holds the answer why the function of given tissue changes.

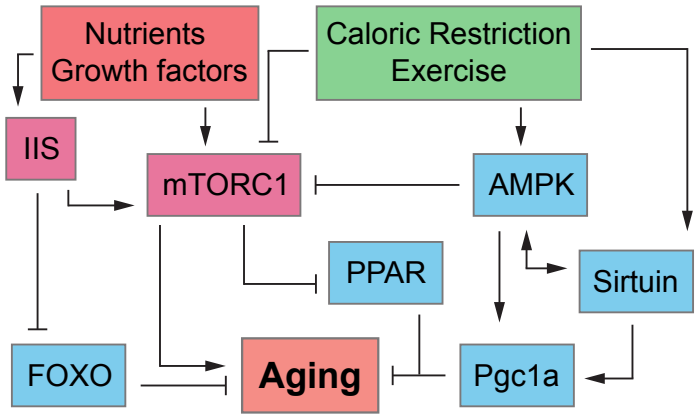


Figure 1 I Energy sensor pathways regulating aging

Crosstalk between energy sensing pathways are depicted. Key factors from each nodule are represented. Aging promoting pathways are highlighted red and inhibiting pathways light blue. Arrows indicate activation and blind-ended arrows inhibition

mTOR pathway, master regulator of cellular growth

Metabolic pathways in cells can either build or break down biomolecules. In order to decide whether cell is in need for energy or building blocks, intertwined pathways to sense the energy status have evolved (Efeyan, Comb et al. 2015)(Figure 1). When energy in the form of nutrients is abundant, sensors driving cellular growth are activated. Mammalian Target of Rapamycin (mTOR) is in the center of the cellular metabolism converging signals from multiple sources such as glucose, amino acids and cholesterol (Castellano, Thelen et al. 2017, Saxton and Sabatini 2017). When these nutrients are present in combination with growth factors from the environment, cellular mTOR-pathway gets activated (Saxton and Sabatini 2017).

mTOR activity is reduced in long lived GH deficient mice and under caloric restriction (Sharp and Bartke 2005, Sun, Akha et al. 2009). Additionally, age dependent increase in mTOR activity is detected in various tissues of model organism, including liver and muscle, where it correlates with decreased function (Sengupta, Peterson et al. 2010, Yang, Tien et al. 2012, Tang, Inoki et al. 2019). Therefore, mTOR pathway has been an appealing target for prolongevity treatments. Correspondingly, treatment of animals with mTOR inhibitor rapamycin produces one of the most robust extension in lifespan (Harrison, Strong et al. 2009). Remarkably, treatment showed effects even if started very late in life suggesting that mTOR inhibition specifically in old organism is sufficient to extend lifespan (Harrison, Strong et al. 2009, Miller, Harrison et al. 2011). This intervention affects multiple aspects of organismal aging. For example, rapamycin treatment enhanced liver and myocardial function of old animals as well as biomechanical properties of muscle tendons (Wilkinson, Burmeister et al. 2012, Johnson, Rabinovitch et al. 2013). Yet, rapamycin is an immunosuppressant and treatment of old mice increased risks for cataracts and testicular degeneration (Wilkinson, Burmeister et al. 2012). Moreover, mTOR activity shows gender specificity in some tissues (Baar, Carbajal et al. 2016). These findings highlight that mTOR inhibition leads to robust lifespan extension, but tissue and gender specific effects limits the applicability of systemic mTOR inhibition. The specific role of mTOR in aging of different cell populations, such as tissue fueling stem cells, requires more investigation.

mTOR acts in two different complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2) (Saxton and Sabatini 2017). mTORC1 regulates anabolic pathways by phosphorylating Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and p70/p85 S6 kinase 1 (S6K1) (Saxton and Sabatini 2017). Phosphorylation of 4E-BP1 prevents its inhibitory action on translation initiation factor, while phosphorylated S6K1 (pS6K1) can further phosphorylate 40S ribosomal protein S6 (S6) (Saxton and Sabatini 2017). S6 protein is phosphorylated on multiple sites by S6K1 and therefore the level of phosphorylated S6 (pS6) protein is a reliable marker for mTORC1 activity. Whether this phosphorylation directly enhances translation is still under a debate (Ruvinsky, Sharon et al. 2005). On the other hand, mTORC2 modu-

lates mainly proliferation and survival, by activating protein kinase Akt (Sarbassov, Guertin et al. 2005). Rapamycin is a potent inhibitor of mTORC1, but prolonged treatment decreases also mTORC2 activity (Lamming, Ye et al. 2012). However, lifespan extension via reduction of mTORC1 activity is uncoupled from mTORC2, proposing that specific inhibitors for mTORC1 would yield even better extension to lifespan (Lamming, Ye et al. 2012). While in proliferating cells, such as tissue resident stem cells, constant growth is a necessity, more quiescent cells of the tissue likely have different metabolic requirements. Cell type specific roles of the mTOR pathway in the context of aging are not well studied, though they might drive the actual life extending phenotype of mTORC1 inhibition.

AMPK and Sirtuins, the low energy sensors

mTORC1 activity interconnects with other energy sensor pathways (Figure 1). AMP-activated protein kinase (AMPK) is activated when cellular Adenosine Monophosphate (AMP) and Adenosine Diphosphate (ADP) levels increase in comparison to Adenosine Triphosphate (ATP), indicating low energy state in the cell (Herzig and Shaw 2018). Upon activation AMPK can mitigate mTORC1 mediated anabolic reactions (Alers, Loffler et al. 2012, Saxton and Sabatini 2017). Low energy is also sensed by Sirtuins, protein and DNA modifiers, that detect high NAD^+ levels (Bonkowski and Sinclair 2016). Sirtuin pathway reinforces AMPK activity therefore also inhibiting anabolic reactions driven by mTORC1 (Price, Gomes et al. 2012).

In low energy state, both AMPK and Sirtuin pathways activate Peroxisome proliferator activated receptor gamma coactivator 1 alpha (Pgc1a) (Bonkowski and Sinclair 2016, Herzig and Shaw 2018). Pgc1a promotes mitochondrial ATP production that is often reduced with aging (Lopez-Otin, Blasco et al. 2013). Remarkably, genetic overactivation of Pgc1a in the intestine of *D.melanogaster* or muscle tissue of laboratory mice prolonged lifespan of these animals (Rera, Bahadorani et al. 2011, Garcia, Nissanka et al. 2018). Together these data highlight, that improving the function of a single tissue can extend the total lifespan.

AMPK and Sirtuin pathways are readily activated upon exercise, possibly contributing to health benefits among exercised elderly (Fiatarone, O'Neill et al. 1994, Bonkowski and Sinclair 2016, Herzig and Shaw 2018). Moreover, AMPK activator Metformin has been shown to increase lifespan in some animal models (Anisimov, Berstein et al. 2011). However, like rapamycin, metformin can cause unwanted effects in elderly, such as blunting the exercise driven muscle gain (Glossmann and Lutz 2019). Therefore, mechanistic understanding of AMPK activity on cellular level is needed to evaluate in which context safe treatments with metformin could be considered.

Ppar pathway, sensor of fatty acids

Transcription factor family Peroxisome proliferator-activated receptors (Ppar) responds to multiple ligands composed of fatty acids and together with the coactivator Pgc1a they increase the expression of many key enzymes in the fatty acid β-oxidation (FAO) pathway (Grabacka, Pierzchalska et al. 2016). Enhancing the capacity of FAO has been shown to improve the function of tissue resident stem cells and restore age-associated defects in them (Ito, Carracedo et al. 2012, Mihaylova, Cheng et al. 2018, Cheng, Biton et al. 2019). Interestingly, Ppar protein levels and FAO are increased in the livers of caloric restricted and long-lived dwarf mice lacking functional GH (Masternak and Bartke 2007). Moreover, Ppar family member alpha (Ppara) knockout animals are viable but develop liver function compromising lesions and have reduced lifespan, indicating important role for Ppar-FAO pathway in metabolism of aged animals (Howroyd, Swanson et al. 2004). FAO produces Acetyl-CoA for tricarboxylic acid cycle (TCA) but also other intermediates such as β-hydroxybutyrate (βOHB) (Grabacka, Pierzchalska et al. 2016). β-OHB and other ketone bodies have been shown to improve tissue function and could explain health benefits of intermitted fasting, caloric restriction (CR) and exercise, lifestyle factors activating Ppar-pathway (Sleiman, Henry et al. 2016).Therefore, Ppara activity likely prevents aging related pathological states. Moreover, mTORC1 activity can result in reduction of Ppara mediated reponses, connecting these opposing pathways (Figure 1) (Sengupta, Peterson et al. 2010). Further studies are required to disclose what proportion

of the life prolonging effects of Ppara are due to improved whole body metabolism versus functional increase locally in tissues or tissue resident stem cells.

Taken together, energy sensory pathways affect many aspects of aging. Moreover, mTOR pathway regulates also proteostasis by inducing autophagy machinery (Saxton and Sabatini 2017). Yet, mTOR and Pgc1a regulate mitochondrial biogenesis, both implicated to be impaired in aging (Lopez-Otin, Blasco et al. 2013). However, age induced damage and alterations in the energy metabolism eventually manifest as a decline of tissue function. In order to understand the underlining mechanisms behind tissue deterioration, the regulatory role of these pathways for the tissues resident stem cells needs to be studied.

1.3. Tissue function and renewal in aging

Maintenance of tissue function throughout the life requires replacement of dysfunctional cells with fresh counterparts. This is done by tissue specific adult stem cells, undifferentiated cells with the ability to create new stem cells, self-renew, and to produce differentiating progenitor cells (Biteau, Hochmuth et al. 2011). Careful balance between the self-renewal and differentiation is required for long term homeostasis. Renewal of many tissues are compromised with age due to reduced stem cell function (Lopez-Otin, Blasco et al. 2013). Therefore, restoring the renewal capacity of aged stem cells could rejuvenate tissue function.

Cellular senescence

In order to protect tissue function, damaged cells can terminally exit the cell cycle in a process called cellular senescence (Campisi 2013). While this protects cells against transformation to a neoplastic phenotype, inefficient clearing of senescent cells can be harmful for tissue function. Evidently, if tissue resident stem cells senesce, maintenance of tissue homeostasis and reparative capacity is compromised. Moreover, accumulation of senescent cells in the proximity of stem cell compartment can be detrimental for their function. Senescence is coupled with a behavior named senescence associated secretory phenotype (SASP) (Campisi 2013). Cells with SASP produce inflammatory cytokines to promote infiltration and activation of immune cells. While ideally this immune reaction leads to clearance of senescent cells, secreted factors affect healthy cells of the tissue. Therefore, SASP has also potential to influence the stem cell function. Many tissues show increased amount of cellular senescence with age (Campisi 2013). Remarkably, clearing of senescent cells in animal models can delay many aging related pathologies and positively affect the healthspan (Baker, Wijshake et al. 2011, Baker, Childs et al. 2016). Whether these effects are due to improved stem cell function, remains under investigation.

Tissue resident stem cells

Tissue function relies on the ability of adult stem cells. Worn out cells need to be replaced in order to maintain functionality. Moreover, upon injury tissues need to be rebuilt, demanding increased capacity from stem cells. All of the aforementioned aging associated damage types have been detected in one or more tissue resident stem cells, likely contributing to functional decline of stem cells (Lopez-Otin, Blasco et al. 2013).

In some cases, decline in the tissue function can be credited to imbalanced differentiation of the old stem cells. For example, adaptive immunity is less competent in old individuals due to differentiation bias towards the myeloid lineage in hematopoietic stem cells (de Haan and Lazare 2018). Moreover, old bone marrow progenitor cells are more prone to make adipocytes than young cells resulting to compromised bone production, possibly contributing to age-associated frailty (Infante and Rodriguez 2018).

Decline in the tissue renewal can result from either exhaustion of the stem cell pool or by reduced potency of individual stem cells. For example, too high proliferation can deplete stem cells, as seen in animal

models lacking cell cycle inhibitors (Cheng, Rodrigues et al. 2000, Kippin, Martens et al. 2005). On the other hand, reduced capability to self-renew contributes to loss of muscle stem cells with age (Bernet, Doles et al. 2014). Yet, reduced functionality can contribute to attenuated renewal capacity, as seen in the aging hematopoietic stem cells (de Haan and Lazare 2018). As proliferative and long-lived stem cells likely collect incremental amount of cellular damage. While mechanisms to pass on damaged particles have evolved (Higuchi-Sanabria, Pernice et al. 2014, Katajisto, Dohla et al. 2015), maintenance of function throughout the life poses a challenge for stem cells. Moreover, alterations in the metabolic state in aged stem cells have been reported, indicating that aging modulating pathways discussed above, likely affect stem cell function (Mihaylova, Cheng et al. 2018).

Stem cells are often found in locations that provide protection from harmful substances of the environment. Whether the composition of these locations change with age is less understood. However, when researchers connected the circulation of young and old animals, in experiments called parabiosis, they learned that exposure to factors circulating in the young blood can rejuvenate the function of muscle stem cells and improve the cognitive functions of old animals (Villeda, Luo et al. 2011, Conboy and Rando 2012). Therefore, the systemic environment around stem cells changes with age and old environment is capable of affecting stem cell function.

Stem cell niche

The undifferentiated state of stem cells is dependent on external factors. Significance of the surrounding microenvironment called 'stem cell niche', was first recognized by Ray Scofield in 1978 (Schofield 1978). His initial observations, that bone marrow is the location for long term maintenance of hematopoietic stem cells were followed by researchers pinpointing other locations where tissue resident stem cells are maintained (Lane, Williams et al. 2014). Now, it is established that the niche is not just a passive location for stem cells to exist, but more dynamic in nature. On top of forming a protected place from environmental damage, niche can fine tune the number of stem cells, affect lineage commitment and the regenerative capacity (Lane, Williams et al. 2014). As the niche can regulate tissue resident stem cells in such many ways, the molecular nature of the crosstalk between stem cells and their niche is under active research.

Niches have been shown to affect stem cell function in numerous ways (Lane, Williams et al. 2014). Systemic signals can regulate stem cell behavior and contribute to the aging of stem cells in quiescent tissues such as muscle and brain (Brack, Conboy et al. 2007, Villeda, Luo et al. 2011, Rodgers, King et al. 2014). Moreover, components of the extracellular matrix (ECM) can partake in the stem cell maintenance (Ahmed and Ffrench-Constant 2016). Attachment to ECM facilitates cell polarization (O'Brien, Jou et al. 2001), feature that is important for stem cell function (Sakamori, Das et al. 2012). Interestingly, loss of polarity is linked to aging of hematopoietic stem cells (Florian, Dorr et al. 2012). The physical properties of the ECM, such as rigidity, can modulate stem cell behavior via YAP signaling pathway, as discussed later in chapter 4.2 (Totaro, Panciera et al. 2018). Recently, it was shown that increased stiffness of the ECM reduced stem cell maintenance in the aging brain (Segel, Neumann et al. 2019).

On the other hand, the cellular milieu, in other words the non-stem cells within the niche, plays an important role in all niches studied (Lane, Williams et al. 2014). For example, cells of the niche in bone marrow, produce chemokines and growth factors to maintain sufficient number of hematopoietic stem cells (Crane, Jeffery et al. 2017). Interestingly, while aged hematopoietic stem cells show bias towards the myeloid lineage (de Haan and Lazare 2018), Ho and colleagues found that part of this bias was driven by the niche (Ho, Del Toro et al. 2019). This highlights the role of the niche in balanced production of differentiated cells. However, how much the cellular niche contributes to age-related reduction in the regenerative capacity of stem cells is not well understood.

Interestingly, morphology of some tissue compartments containing stem cells change with age (Snyder, Piazza et al. 1993, Martin, Kirkwood et al. 1998, Giangreco, Goldie et al. 2010). For example, undulations in the skin epidermis, called rete ridges, define the topography of the environment for basal stem cells

(Solanas and Benitah 2013). Hight of these ridges is decreased in old skin that also harbors less stem cells (Giangreco, Goldie et al. 2010). The concurrent alteration in tissue shape and stem cell number imply connection between these two. Cell morphology has been shown to affect stem cell function in vitro experiments (McBeath, Pirone et al. 2004), but whether tissue topology and cell shape could affect renewal of adult tissues is not known.

Given that modulation of the energy sensory pathways and stem cell activity have improved functionality of aged tissues the interaction of these should be carefully studied. Intestine is an organ that translates the ingested food to energy facilitating functions in the organism. Moreover, its function relies on fast renewal fueled by stem cells in a specific microenvironment. Therefore, it provides an excellent platform to investigate the effect of age for niche regulated stem cell function in a context where energy sensory pathways are heavily involved.

2. Intestine

Gastrointestinal (GI) tract is essential part of the body comprised by continuous hollow open-ended tube where food is first digested, later absorbed and finally unnecessary remains excreted (Figure 2a). Small intestine is the part of GI-tract between stomach and large intestine (Figure 2b). Food digested by enzymes in stomach continues to be broken down by additional enzymatic activity as it mixes with products from pancreas and bile (Reed and Wickham 2009). Food is pushes onwards by peristalsis, involuntarily movement generated by enteric nervous system (Myenteric plexus) and two muscle layers arranged in perpendicular fashion (Muscularis externa). Small intestine can be divided functionally to three parts. Duodenum, the most proximal part is where ducts from pancreas and gallbladder join into the GI-tract. Acidic, partly digested food from stomach is neutralized by alkaline bicarbonate rich pancreatic juice and mucus produced by the Brunner's gland under the duodenal mucosa (Reed and Wickham 2009). Digestive enzymes continue breaking down the proteins in food down to small peptides and individual amino acids. Gallbladder derived bile facilitates lipase function by emulsifying dietary fat. Most of the nutrients are absorbed in jejunum, the midsection of small intestine, while iron and folate are taken up already in duodenum and vitamin B12 and bile salts are absorbed in ileum, the most distal part of small intestine (Kiela and Ghishan 2016).

2.1. Intestinal epithelium

The innermost layer of cells in the mucosa, is called intestinal epithelium and forms a critical barrier between the body and the content of lumen (Figure 2b,c). This interface is one of the largest surface between the host and external environment (Helander and Fandriks 2014). Passing food, digestive enzymes, varying pH, commensal microbes and possible pathogens create a harsh environment in the lumen that abrades the epithelium. To maintain such large barrier, continuously renewing single cell layer epithelium has evolved. For this reason, intestinal epithelium possesses remarkable renewal capacity. The epithelium turns over in just 5 days (Clevers 2013). New cells are constantly produced in intestinal glands, pits or cavities in the epithelium called crypts of Lieberkühn or shortly crypts (Lieberkühn 1745). Cells emerge out from crypts as the pressure generated by formation of new cells pushes them upwards and start actively migrating towards the villus tip (Krndija, El Marjou et al. 2019). At the tip of the villi, cells are shed to the lumen where they die (Clevers 2013).

Cellular subtypes of the villi

The villous-epithelium is mainly composed of three differentiated cell types, enterocytes, goblet cells, enteroendocrine cells (Figure 2c). Enterocytes are the most abundant comprising almost 90 % of the epithelium (Noah, Donahue et al. 2011) Their function is to absorb nutrients from the digested food passing through the intestinal lumen and to transport it to the underlying vasculature (Kiela and Ghishan

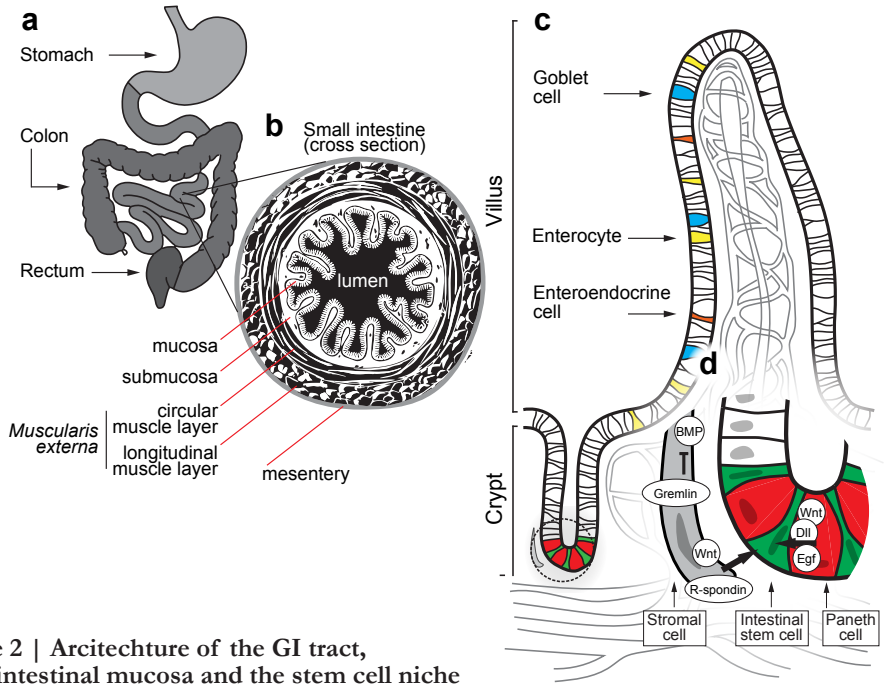


Figure 2 | Arcitechture of the GI tract, small intestinal mucosa and the stem cell niche

a, Organization of the GI tract. **b**, Cross section of the small intestine. Mucosal epithelium is facing the gut lumen and surrounded by underlying cell layers. **c**, Schematic drawing of intestinal epithelium. Epithelium is functionally divided to crypts and villi. Goblet cell (light blue), Enterocyte (yellow), Enteroendocrine cell (orange), Paneth cell (red), Intestinal stem cell (green). **d**, Close-up of intestinal crypt. Paneth cells and underlying stromal cells (gray) support stem cell function by secreting multiple factors.

2016). The second most abundant cell type is mucus producing Goblet cells. The prevalence of these cells increases from the proximal to distal axis of the small intestine (10-15%) and is highest in the colonic epithelium (up to 50%) (Noah, Donahue et al. 2011). Goblet cells produce proteoglycan rich mucus layer protecting epithelium from mechanical abrasion. Moreover, the mucus layer enforces the barrier function of the epithelium by preventing pathogens from facing the epithelium (Johansson and Hansson 2016). On the other hand, at least in rodents, the mucus layer harbors a different composition of microbiota than the luminal content of intestine, suggesting that mucus can compartmentalize microbiome (Schroeder 2019). In addition, the composition of mucus and its microbiome responds to diet (Meng, Li et al. 2019, Schroeder 2019). Enteroendocrine cells are the least common among the major cell types in villus epithelium, accounting only 1% of the cells (Noah, Donahue et al. 2011). They are hormone producing cells, that respond to dietary input and feedback to the systemic circulation. For example, incretins produced by the enteroendocrine cells after meal promote insulin release from the pancreas (Gribble and Reimann 2019). Several subclasses of enteroendocrine cells exist which regulate different aspects of digestion (Gribble and Reimann 2019). Other cell types found from the differentiated epithelium are Tuft cells, rare cell type that participates in innate immunity be recognizing intestinal pathogens and microfold cells (M-cell), specialized antigen presenting cells located at the top of lymphoid tissue called Peyer's patches (Clevers 2013).

Lgr5-positive intestinal stem cells

Constant production of new cells in the intestinal epithelium is driven by actively cycling stem cells located at the base of the crypts (Clevers 2013). The exact nature and location of these cells remained enigmatic until faithful marker gene was identified in 2007 (Barker, van Es et al. 2007). This seminal finding by the laboratory of Hans Clever showed that under homeostasis, bona fide stem cells are intermingled

between specialized secretory cells at the crypt base, called Paneth cells (Barker, van Es et al. 2007). This resolved a long debate, whether stem cells exist at the very bottom of crypts or in the supra-Paneth, often called +4, position (Potten, Hume et al. 1978, Clevers 2013). Later, it has been shown that a distinct population of slow cycling 'reserve' stem cells occupy the +4 position (Tian, Biehs et al. 2011, van Es, Sato et al. 2012, Buczacki, Zecchini et al. 2013, Barriga, Montagni et al. 2017). Reserve stem cells contribute little to tissue renewal at homeostasis, but are more resistant to damage compared to actively cycling stem cells (Buczacki, Zecchini et al. 2013, Metcalfe, Kljavin et al. 2014), providing a backup-mechanism for restoring actively cycling stem cells if they are acutely lost.

Each crypt contains multiple stem cells, effectively ranging from 5-7 in mice and 5-10 in humans (Kozar, Morrissey et al. 2013, Nicholson, Olpe et al. 2018). These stem cells, sometimes named crypt base columnar cells (CBCs) due to their distinctive morphology (slender columnar shape when compared to Paneth cells), express Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) (Barker, van Es et al. 2007). Lgr5 is a receptor tyrosine kinase of which respective ligands are R-spondins (de Lau, Peng et al. 2014). In this dissertation, Lgr5-positive stem cells are referred as intestinal stem cells (ISCs).

ISCs divide daily to produced two daughter cells that either differentiate or maintain stem cell identity (Schepers, Vries et al. 2011, Clevers 2013). The divisions are symmetric and the faith of the progeny is decided in competition from the space (Snippert, van der Flier et al. 2010). Daughter cells pushed out from the crypt bottom lose contact with Paneth cells and begin to differentiate (Clevers 2013). Due to this random outcome of daughter cell fate, with time each crypt drifts towards clonality (Snippert, van der Flier et al. 2010). Although this 'clonal competition' is random, ISCs located at the very bottom have highest chance to outcompete others due to smallest chance of relocation from the crypt bottom (Ritsma, Ellenbroek et al. 2014). Progeny pushed out from the crypt base enters so-called transit-amplifying (TA) zone where progenitor cells divide two-to-three times before exiting the crypt and terminally differentiating (Clevers 2013).

ISC identity is maintained by conserved signaling pathways Wnt, Notch and BMP and perturbing the normal function of one of them results to a rapid loss of stem cell pool (Figure 2d) (Spit, Koo et al. 2018). Wnt pathway modulates the stem cells capability to proliferate by suppressing p21 mediated cell cycle arrest (Krausova and Korinek 2014). Moreover, Wnt dependent expression of multiple Wnt pathway regulators, such as Axin2, Rnf43, and Lgr5, participate in the maintenance of correct Wnt levels in the ISCs. (Clevers 2013, Krausova and Korinek 2014). Yet, Wnt pathway suppresses differentiation in the ISCs, as excessive amount of activity increases ISC pool (Kim, Kakitani et al. 2005). Notch pathway also partakes in maintenance of stemness and proliferative capacity of ISCs. Notch activity is high in ISCs as seen by the expression of Notch target gene Olfm4 (VanDussen, Carulli et al. 2012). Moreover, deletion of Notch receptor leads to a rapid loss of stem cells, indicating its crucial role in maintenance of stemness (VanDussen, Carulli et al. 2012). Additionally, Notch pathway regulates cell fate decision and patterning of the niche in the differentiating progeny, as discussed later in chapter 4.1. BMP pathway negatively regulates ISC fate, by inducing differentiation and suppressing Wnt pathway (Spit, Koo et al. 2018). Moreover, proliferative capacity in ISCs is maintained by Epidermal growth factor (Egf) signaling, as disruption in Egf signaling leads to quiescence of ISCs (Basak, Beumer et al. 2017).

Ligands that modulate these pathways originate from the surrounding microenvironment, the stem cell niche, of ISCs. Stromal cells in the mucosa that underline the epithelium, sometimes referred as Telocytes, are important source of Wnt ligands and R-spondins, agonist of the Wnt pathway (Farin, Van Es et al. 2012, Shoshkes-Carmel, Wang et al. 2018). Moreover, the stroma produces Gremlin and noggin, antagonist of BMP-pathway to prevent differentiation at the crypt base (Spit, Koo et al. 2018, McCarthy, Manieri et al. 2020). Notch ligands, Delta like canonical Notch ligands (Dll 1 and 4) are provided by the neighboring Paneth cells, which also produce canonical Wnt ligand, Wnt3 and Egf (Sato, van Es et al. 2011). Together Wnt, Notch and BMP signaling nodes allow the maintenance of stem cell identity (Spit, Koo et al. 2018). (Figure 2d)

Paneth cells

In small intestine, base of the crypt is occupied by not only ISCs but also large granulated secretory cells. These were named after Joseph Paneth, a 19th century physicists who initially characterized them (Paneth 1887). Paneth cells bear distinctive looks due to the large secretory granules that fill up their cytoplasm. These granules are filled with antimicrobial compounds such as defensins, cryptdins and lysozyme (Porter, Bevins et al. 2002). By secreting antimicrobials to the crypt and gut lumen, Paneth cells can control the microbiota (Ganz 2000). Under inflammatory conditions, such as inflammatory bowels disease, increased number of Paneth cells are observed reflecting body's effort to overcome the source of inflammation, microbes in the gut lumen (Shi 2007).

Given the localization of Paneth cells at the crypt base, a region where new cells are produced, and their immune function suggested immunoprotective role for crypt resident cells. However, visualization of $Lgr5^+$ cells showed, that ISCs are intermingled between Paneth cells (Barker, van Es et al. 2007)(Figure 2d). Interestingly, already 1887 Joseph Paneth reported that very slender cells ('schmale Zellen') were sandwiched between Paneth cells (Paneth 1887). The intimate contact between ISCs and Paneth cells hinted that these cells might have also direct functional interactions.

To investigate the relationship between Paneth cells and ISCs, researchers at the Clevers laboratory isolated and profiled both cell types (Sato, van Es et al. 2011). Results indicated that Paneth cells were producing multiple ligands for receptors that were present on the ISC surface. Wnt, Notch and Egf -ligands made by Paneth cells were likely provided for the neighboring ISC (Sato, van Es et al. 2011)(Figure 2d). Elegantly, by isolating cell-doublets, researchers were able to show that doublet containing one ISC and one Paneth cell was forming an *ex vivo* organoid (see below) more efficiently than ISCs alone, or doublets containing two ISCs. Paneth cells did not form organoids as they are post mitotic (Sato, van Es et al. 2011). Moreover, *in vivo* deletion of Sox9, a transcription factor required for Paneth cell maturation resulted to concomitant decrease in ISC numbers. This indicated that Paneth cells, separately demonstrated to be derived from $Lgr5^+$ ISCs, partake in the formation of the niche.

Interestingly, Paneth cells were found to be highly responsive to nutrient status, as their mTORC1 activity is tightly coupled to food availability and could be swiftly promoted by insulin (Yilmaz, Katajisto et al. 2012). When the life prolonging treatment, caloric restriction (CR), was applied on laboratory animals, mTORC1 activity was reduced in Paneth cells. Surprisingly, this led to an increased pool of ISCs. Under CR Paneth cells produced Bst-1, enzyme capable converting NAD^+ to cADPR in the extracellular space. cADPR is a second messenger taken up by ISCs and promoting self-renewal (Yilmaz, Katajisto et al. 2012). This mechanism reduces production of differentiated cells and shortens the villi. It likely evolved to reduce the replacement of differentiated cells when food is scarce, and facilitates quick tissue remodeling and villus lengthening upon nutrient availability. Recently, Paneth cells were shown to rely more on glycolysis as source of energy while ISCs are utilizing oxidative phosphorylation (Rodriguez-Colman, Schewe et al. 2017). Moreover, Rodríguez-Colman and colleagues showed that lactate formed as a by-product of Paneth cell glycolysis facilitated the growth of neighboring ISCs. Together, these studies show that Paneth cells are not just a steady source of niche factors, but a dynamic sensory unit, able to integrate the metabolic status of an organism and guide the output from ISCs.

Regardless their importance for intestinal function, genetic ablation studies have shown that mice lacking Paneth cells are surprisingly viable and contain renewal capable ISCs (Shroyer, Wallis et al. 2005, Durand, Donahue et al. 2012, Kim, Escudero et al. 2012). While complete loss of Paneth cells in some of these mouse models is controversial (Sato, van Es et al. 2011), other cells in the niche are clearly able to compensate the reduction in Paneth produced niche factors. Under such circumstances, ISCs rely more on the Wnt-agonists and BMP inhibitors made by the underlining stroma (Farin, Van Es et al. 2012, Shoshkes-Carmel, Wang et al. 2018). Fascinatingly, if Paneth cells are acutely removed and their formations prevented, enteroendocrine and Tuft cells occupy opened slots at the crypt base. These cells are able to produce Delta ligands required for the ISC function (van Es, Wiebrands et al. 2019). On the other hand, Paneth cells antimicrobial activity is clearly required for efficient clearance of pathogens (Bevins

and Salzman 2011). Analysis of the bactericidal peptides discharged by Paneth cells, indicated that the crypt lumen remains sterile even if exposed to microbes (Ayabe, Satchell et al. 2000). Moreover, when Zhang and colleagues introduced pathogenic quantity of Klebsiella pneumoniae bacteria to young mice where Paneth cell were selectively destroyed, they observed a severe epithelial damage (Zhang, Sherman et al. 2012). This suggested, that reduced number of Paneth cells sensitizes the whole intestine for microbial infection, which might underline the high incidence of necrotizing enterocolitis in prematurely born infants (Heida, Beyduz et al. 2016). In humans, Paneth cells emerge around the second trimester of gestation and their numbers increase till birth, suggesting immature crypt immunity in prematurely born infants (Heida, Beyduz et al. 2016). However, often pathogen triggered inflammation leads to a local damage which requires epithelial repair (Zhang, Sherman et al. 2012). Therefore, unhealthier outcomes from experiments where Paneth cell ablated intestines are exposed to pathogens might also reflect impaired reparative function of the neighboring ISCs. On that note, when Zou and colleagues blocked Wnt production by genetically removing Wnt ligand secretion mediator (Wls) from the intestinal epithelium including Paneth cells, epithelial regeneration after rotavirus infection was impaired (Zou, Blutt et al. 2018). Taken together, Paneth cells form an important part of the ISC niche as guardians of the microbial content of crypts and by supporting the identity and reparative ability of ISCs.

2.2 Regeneration of intestinal epithelium

Normal turnover of the epithelium requires continuous production of new cells by ISCs. Upon injury, boost of proliferation is needed from ISCs and TA cells to cope with the acute loss of tissue. Yet, in the worst case when the injury ablates most of the ISCs, intestinal epithelium shows remarkable plasticity. (Clevers 2013)

Genetically modified mice, carrying Cre-recombinase activity under specific promoters, such as Lgr5 for ISCs or Dll1 for secretory precursors (Barker, van Es et al. 2007, van Es, Sato et al. 2012), and ubiquitous Cre inducible reporter genes, allow tracing of the progeny of distinctive cell populations in crypts. Experimental removal of actively cycling ISCs in these mouse models have shown that stem cell pool is quickly rescued by dedifferentiation of more mature progenitor cells (Tian, Biehs et al. 2011). Virtually all progenitors and even more mature cells residing in the crypt have shown ability to restore the ISC pool by dedifferentiation. These include $Alpi^+$ enterocyte and $Dll1^+$ secretory precursor cells as well as Paneth cells (Roth, Franken et al. 2012, van Es, Sato et al. 2012, Tetteh, Basak et al. 2016). The level of plasticity could be due to more open chromatin state of crypt resident cells allowing swift respond to dramatic changes in transcription factor activity (Kim, Li et al. 2014). Moreover, as progenitor cells now occupy the crypt base, signals from the stromal and cellular niche could further promote acquisition of the stem cell fate. Together the remarkable plasticity of reserve stem cells and crypt progenitors ensures that the critical absorptive and barrier function of the intestinal epithelium is retained even upon severe damage.

Regenerating intestine requires Wnt activating signals from both stromal and epithelial niche (Degirmenci, Valenta et al. 2018, Shoshkes-Carmel, Wang et al. 2018, Zou, Blutt et al. 2018). Intriguingly, some stimuli can reprogram ISCs and possibly other cells at the site of injury to more fetal state (Nusse, Savage et al. 2018, Yui, Azzolin et al. 2018, Wang, Chiang et al. 2019).This transient fetal conversion promotes recovery and indicates that tissues may utilize developmental programs for efficient repair.

Organoid models for stem cell biology

The lack of methods to maintain ISCs *in vitro* was a barrier for cost- and time-efficient research of the intestinal epithelium. This hurdle was removed in 2009 when methods to culture intestinal epithelium as organotypic cultures were developed (Ootani, Li et al. 2009, Sato, Vries et al. 2009). Thereafter, knowledge from the biology of tissue renewal has increased greatly. These cultures, collectively named organoids, are self-renewing when growth factors activating Wnt- and MAPK- pathways and inhibiting BMP-pathway are provided in the form of R-spondin, Egf and noggin respectively (Sato, Vries et al. 2009). Organoids

contain ISCs and Paneth cells in crypt resembling crypt domains, and other differentiated cell types in the inter-crypt region, called villus domain (Sato, Vries et al. 2009). Organoids allow researchers to investigate stem cell behavior in more controlled fashion. The continuously growing organoids provide and *ex vivo* regeneration model for the intestinal epithelium. Moreover, it allows researchers to dissect the responsible cell type for observed changes in regenerative capacity, as shown by elegant co-cultures of Paneth cells and ISCs (Yilmaz, Katajisto et al. 2012). Yilmaz and Katajisto showed that organoid formation capacity after CR was improved only if Paneth cells were derived from CR mice. Moreover, patient derived organoids permit, for example, personalized testing of cancer derived organoids for their responsiveness to first line chemotherapeutics. This improves targeting of tumor sub-classes that often show resistance against some treatments (Tuveson and Clevers 2019). Moreover, developing functional assays facilitates testing of drugs against genetically uncharacterized variants of hereditary disease (Dekkers, Wiegerinck et al. 2013). Therefore, organoid technology shows great promise for advances in basic science and personalized medicine.

2.3 Problems of the aging intestine

Intestine is in the interphase between digested food and rest of the body (Kiela and Ghishan 2016). In addition, pathogens, and toxic compounds consumed together with the food, pass through the gut, can harm epithelial cells (Bintsis 2017). Moreover, the lumen contains high quantities of commensal microbes that can serve organismal health, need to be kept in place (Johansson and Hansson 2016). Functionality of the body relies on the capacity to absorb nutrients while keeping external environment in place. Therefore, complications resulting decreased function of the intestine affect the health of aging individuals in many way (Drozdowski and Thomson 2006).

Absorptive capacity of the aged epithelium

Loss of proper absorptive function by intestinal epithelium leads quickly to malnutrition and cachexia (Costa 1977). This is life threatening particularly for elderly with frailty and age-induced decline in tissue function (Drozdowski and Thomson 2006). Clinical studies have suggested that the capacity to absorb nutrients, especially carbohydrates is decreased among older individual (Feibusch and Holt 1982). One possible reason being decreased epithelial surface area on old individuals (Warren, Pepperman et al. 1978). Moreover, Chen and colleagues observed age-dependent decline in glucose and amino acid transport in old laboratory mice(Chen, Currier et al. 1990). These observations indicate that reduced absorptive capacity in old age is conserved and could be studied using laboratory animals. However, reduction in absorptive function has been disputed in later studies. Weight loss and cachexia might result from changed ability to mechanically process food (Berkhout, Cools et al. 1998). Moreover, social interactions reducing feeding behavior or reduced appetite can alter intake of calories (Landi, Calvani et al. 2016).

Alterations in the gut microbiome

Gut lumen has sizable amount of microbial activity (Johansson and Hansson 2016). Commensal microbes, naturally present in the gut can even promote the health and digestion of the intestinal epithelium (Martin, Miquel et al. 2013). However, if pathogenic microbes overwhelm the commensal microbes, intestinal health decreases due to inflammation and associated problems (Bintsis 2017). Microbial content has been shown to change with age in animal models and in human populations (Odamaki, Kato et al. 2016, Smith, Willemsen et al. 2017). Moreover, researchers have observed increased amount of pathogenic *Helicobater pylori* in gut biopsies of old humans (Feldman, Cryer et al. 1996). This was accompanied with increased inflammation pointing out the connection between microbiome and health of the tissue. Furthermore, recently identified associations between certain taxa of microbes and Parkinson's disease suggest that functional link between gut health and aging related diseases might exist (Boertien, Pereira et al. 2019).

Compromised barrier function

To control luminal content, epithelial lining forms a continuous sheet of tightly connected cells (Parrish 2017). Tight junctions maintain integrity in the epithelial layer that prevents pathogens and unwanted compounds from infiltrating the body. Moreover, intact epithelium is critical for efficient absorption as transport of nutrients, glucose and amino acids through the enterocyte layer requires coordinated localization of Na^+ dependent symporters in the apical side while keeping the Na^+K^+ -ATPase and nutrient uniporters in the basal side (Lodish H 2000). Barrier function has been shown to reduce with age in animal models including non-human primates (Parrish 2017). Remarkably, if leakiness in the gut of aged *D.melanogaster* flies was repaired, lifespan was extended (Akagi, Wilson et al. 2018). This proposes, that intestinal health is lifespan limiting factor in some animals. In humans, *ex vivo* experiments have shown similar reduction on barrier function (Man, Bertelli et al. 2015). Reduced barrier function can cause chronic inflammation, condition that is a risk factor for many aging-related diseases (Chung, Cesari et al. 2009). Moreover, it could partly explain reduced absorptive capacity among aged individuals (Feibusch and Holt 1982, Feldman, Cryer et al. 1996). In order to maintain effective barrier function throughout the life, functional cells need to be produced by ISCs to replace worn out enterocytes.

Ulceration and regeneration

Intestinal epithelium faces harsh environment of the gut lumen that is filled with acidic gastric juices originating from the stomach and basic content derived from the pancreas (Greenwood-Van Meerveld, Johnson et al. 2017). Under some conditions, gastric juices are produced in too high quantities and can lead to ulceration of the epithelium (Barreras and Donaldson 1967). Moreover, pathologies such as Celiac disease or exposure to rotavirus, norovirus or salmonella family of pathogens can cause gastroenteritis, inflammatory condition of the GI tract causing severe diarrhea (Majowicz, Musto et al. 2010, Marshall and Bruggink 2011, Gujral, Freeman et al. 2012, Tate, Burton et al. 2012). Recovery from such insults requires efficient and coordinated regeneration of the intestinal epithelium. Throughout the lifetime of humans, intestinal epithelium has typically faced multiple events that require regenerative capacity. The increasing incidence of inflammation in the gut of elderly individuals underlines the importance of regenerative capacity by healthy and efficient ISCs. Moreover, the compromised regenerative capacity of the aged intestine decreases tolerance towards the harmful side-effects of cancer therapies, which is a major clinical problem and cause of collateral morbidity for old individuals (Chang, Goldstein et al. 2017). Interestingly, in some cases signals from the stem cell surroundings seem to facilitate regeneration. For instance, Zou and colleagues found, that epithelial derived Wnt ligands are required for efficient recovery from rotavirus induced damage (Zou, Blutt et al. 2018).

3. Wnt signaling pathway

As multicellular organisms developed, tasks between individual cells of the organism could be divided. In order to establish communication between cells of the organism, new signaling pathways emerged (Schenkelaars, Pratlong et al. 2017). One of these pathways, involved in mammalian development, disease and in particular maintenance of tissue resident stem cells is known as Wingless-related integration site (Wnt) -pathway (Holstein 2012, Nusse and Clevers 2017).

3.1 Ligands and receptors

The Wnt-pathway was discovered in the latter half of the 20th century by scientists working with tumor viruses. Roel Nusse and Harold Varmus identified and characterized one of the genes activated by tumor viruses and named it Integration 1 (Int-1) (Nusse and Varmus 1982). The discovery that Int-1 drives proliferative and undifferentiated state of the cells suggested its role in tumor formation and growth in mammals. Further studies revealed high conservation of the Int-1 gene within the metazoan phyla and

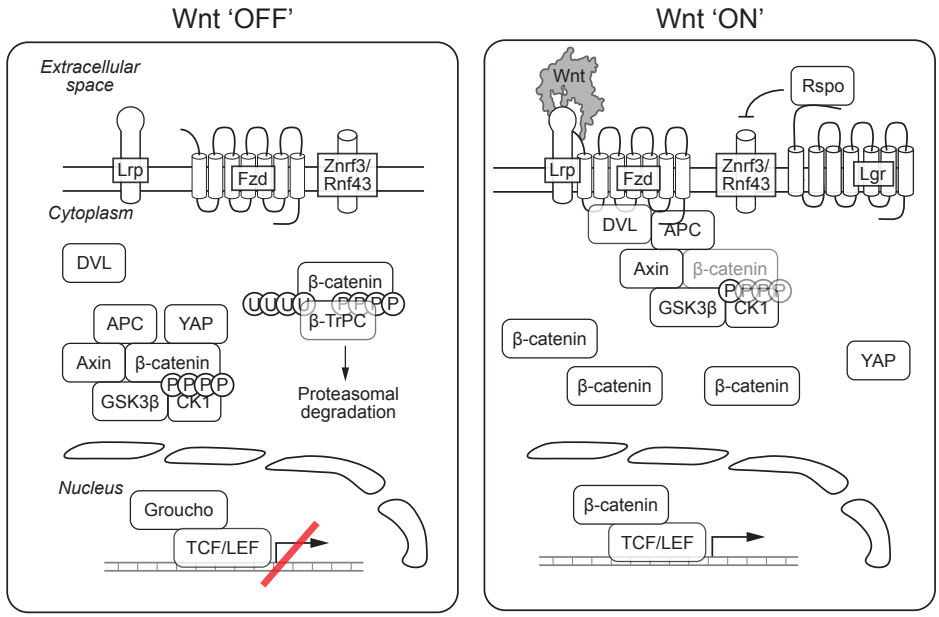


Figure 3 | Canonical Wnt signaling cascade

Schematics of the canonical Wnt signaling cascade. In the absence of Wnt ligands 'Wnt OFF', β-catenin is bound to the destruction complex. Glycogen synthase kinase 3β (GSK3β) and Casein Kinase 1 (CK1) phosphorylate (P) β-catenin targeting it to β-TrPC mediated ubiquitinylation (U) and degradation. Simultaneously, destruction complex sequesters cytoplasmic Yes-associated protein (YAP). When Wnt ligands are present 'Wnt ON', Dishevelled (DVL) is reqruited to the plasmamembrane followed by relocalization of β-catenin destruction complex. GSK3β activity is inhibited and β-catenin is released from proteasomal targeting. Accumulation of cytoplasmic β-catenin leads to nuclear translocation, where β-catenin can bind to TCF/LEF transctription factors superceding the inhibitory factor Groucho and activating transcription. Binding of R-Spondin (Rspo) to the Leucine-rich repeat-containing G-protein coupled receptor (Lgr) prevents Znrf3/Rnf43 mediated internalization and deactivation of ligand bound Fzd, amplifying Wnt activity. Moreover, YAP is released from sequestration by the destruction complex enabling downstream signaling.

that a homologous gene was identified previously in *D.melanogaster* where it was called *wingless* (*wg*), due to the absence of wings in mutant flies (Rijsewijk, Schuermann et al. 1987, Holstein 2012). This indicated that Int-1 would likely participate in embryonic development in mammals as well, fact that was later proven my multiple studies (Nusse and Clevers 2017). Mammalian Int-1 gene was later known as Wingless-related integration site 1 (Wnt1).

Subsequently, it was evident that multiple Wnt-genes, coding for secreted proteins, are present in the genomes of animal cells. Today, in humans and mice, total of 19 Wnt-genes are identified (The Wnt Homepage). Significant progress has been made in understanding the role of Wnts in development and disease states. However, the large number of ligands and receptors expressed in various tissue compartments and crosstalk with other major signaling pathways enables cells to respond stimuli in context dependent manner.(Nusse and Clevers 2017)

3.2 Canonical Wnt pathway

Protein named Beta-catenin (β-catenin), is in the heart of the conventional, canonical Wnt-signaling pathway. β-catenin is as multifunctional protein participating to cellular adhesion as well as transcriptional co-activation (Valenta, Hausmann et al. 2012). Under steady state, β-catenin has fast turnover, half-life

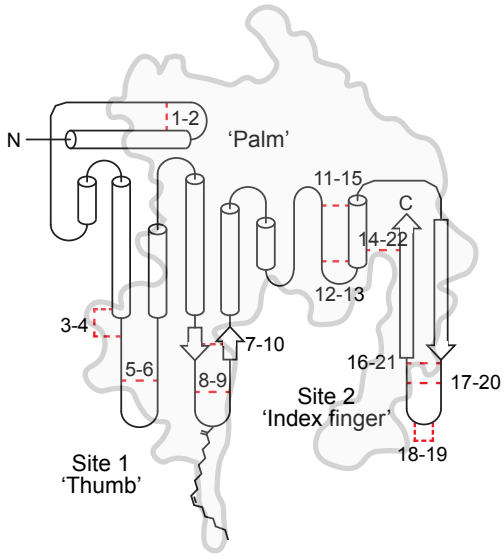


Figure 4 | Structure of Wnt proteins

Schematic sturcture of Wnt8 protein. Folded protein resembles a hand with three distinct domains, 'palm' contains functionally relevant N-glycosylations, 'index finger' forms critical interaction site with Fzd receptors and 'thumb' contains lipid modification critical for secretion and function of Wnt proteins. Alpha-helices (barrels), beta-sheets (arrows) and conserved disulphide bonds between Cystein residues (dashed red line, numbers correspond the order of conserved Cysteins in the sequence) are depicted. Lipid modification on Site 1 presented as cis-16:1n-7 palmitoylation. Modified from (Janda et al 2012, Willert and Nusse 2012)

being approximately an hour (Salic, Lee et al. 2000). This is achieved by a destruction complex that phosphorylates and ubiquitinates β-catenin leading to proteasomal degradation (Figure 3).

Wnt-ligands bind to their respective receptors called Frizzled (Fzd). Binding recruits a co-receptor Low-density lipoprotein receptor-related protein 5 or 6 (Lrp5/6). The formation of Wnt-Fzd-Lrp complex induces translocation of the β-catenin destruction complex, composed of Axin, Glycogen Synthase Kinase 3 beta (GSK3β), APC and Casein Kinase 1 alpha (CK1α) to plasma membrane via adaptor protein Dishevelled (DVL) (Figure 3). Membrane localization reduces the phosphorylation and destruction capacity of the complex and stabilizes β-catenin. Stabilized β-catenin can translocate to the nucleus where it interacts with family members of transcriptional regulators named T-cell factor/ lymphocyte enhancer factor (Tcf/Lef) (Valenta, Hausmann et al. 2012). This interaction dislocates the Tcf/Lef bound transcriptional repressor Groucho to activate transcription of target genes (Valenta, Hausmann et al. 2012)(Figure 3).

3.3 Non-canonical Wnt pathways

Wnt-proteins can modify cellular behavior independent of β-catenin, to mediate protein stability, cell motility, polarity and patterning of tissues (Green, Nusse et al. 2014, Acebron and Niehrs 2016, Humphries and Mlodzik 2018). Collectively these are described as non-canonical Wnt-pathways. Non-canonical pathways can utilize different set of Fzd co-receptors. For example, Receptor tyrosine kinase-like orphan receptor (Ror) family receptors Ror1 and Ror2 can bind both Wnt ligands and Fzd receptors (Green, Nusse et al. 2014). Signaling through Fzd-Ror receptor complex integrates Wnt signaling to cellular morphology and motility as discussed in 3.7.(Schlessinger, Hall et al. 2009).

3.4 Structure

All 19 Wnt proteins are approximately 350 to 400 amino acid long peptides and share 27 to 83% similarity in amino acid sequence. The universal features of Wnt proteins are signal peptide required for secretion and conserved 22 Cystein-residues forming disulfide bonds that participate to the maintenance of the protein structure (Miller 2002) (Figure 4).

Wnts are also post-translationally modified. Two conserved glycosylation sites affect Wnt-function (Komekado, Yamamoto et al. 2007). Another characteristic modification is addition of palmitate or pal-

mitoleic acid by an enzyme called Porcupine (Porcn) (Nile and Hannoush 2016). Porcupine (Porcn) is an ER resident acyltransferase which adds an acyl group to conserved Serine-residue on Wnt. This modification is required for the secretion of all Wnt molecules as inhibition of Porcn leads to accumulation of Wnt to ER (Nile and Hannoush 2016).

First high-resolution structure of Wnt bound to its receptor Fzd revealed additional functional outcomes for the acyl group (Janda, Waghray et al. 2012). Wnt proteins form unique hand-like structure, where two distinct domains, called sites 1 and 2, interact with the Fzd -receptor. Acylated Serine is located at the site 1, also known as the "thumb", forming a protruding structure where acyl-group points outwards (Figure 4). When Wnt is bound to Fzd-receptor, site 1 has a large interaction surface with Fzd and the acyl-group is buried in the groove of the receptors CRD-domain (Janda, Waghray et al. 2012). This suggests that acylation of Wnt is required for proper interaction with its receptors. Moreover, from the structure it is clear that hydrophobic acyl is not buried within the Wnt-proteins making them less soluble in aqueous solutions (Janda, Waghray et al. 2012). This highlights the fact, that although secreted protein, Wnt is likely to interact with lipid membranes in close proximity of the producer cells. Therefore, Wnt ligands likely act very locally in cell-to-cell communication.

The carboxy-terminal end of Wnts contains another conserved domain, site 2, often named as 'index finger' (Janda, Waghray et al. 2012)(Figure 4). Index finger forms additional interaction surface with Fzd (Janda 2012). As site 1 interaction with Fzd is dominated by the lipid, site 2 might mediate selectivity over Wnt-Fzd interactions. Indeed, in vitro experiments with modified Wnt proteins have shown that site 2 has some selectivity over different Fzd receptors (Janda, Waghray et al. 2012). However, the discrimination between the canonical and non-canonical pathways is unlikely due to specificity of Wnt-Fzd interactions, as interaction sites 1 and 2 are conserved (Janda, Waghray et al. 2012). More likely explanation would be the abundance of co-receptors on the surface or receiving cell (Willert and Nusse 2012).

3.5 Extracellular modulation of Wnt pathway

Wnt signaling activity is controlled by plethora of agonists and antagonists (Nusse and Clevers 2017). Many of the components of Wnt-pathway inactivation are direct Wnt targets, generating efficient negative feedback loop. For example, key components of the Wnt-destruction complex, Axin2 and β-TrCP, are upregulated upon canonical Wnt activation (Spiegelman, Slaga et al. 2000, Yan, Wiesmann et al. 2001). Another way to control Wnt pathway is by altering the extracellular components of the pathway.

Cell membrane associated Wnt inhibitors

An interesting type of Wnt activity modulation happens on the cell surface via Wnt target genes Rnf43 and Znrf3 (de Lau, Peng et al. 2014). These proteins are E3 ligases that recognize Wnt-bound Fzd on the cell surface and cause internalization and destruction of the receptor complex (de Lau, Peng et al. 2014, Nusse and Clevers 2017). This feedback inhibition makes sure that Wnt activity is quickly shut down upon receptor binding (Figure 3).

Inhibition of Rnf43/Znrf3 allows an elegant way to amplify Wnt signals in a subset of cells. This is accomplished by a protein family called R-spondins and their corresponding receptors Lgrs (de Lau, Peng et al. 2014). As described above, Lgr5, marks ISCs (Barker, van Es et al. 2007). Upon binding, R-spondin/Lgr5 complex inhibits the Rnf43/Znrf3 ligases on the cell surface, preserving active Wnt-Fzd complex on the surface (Figure 3). This enables amplification of Wnt only in cells expressing Lgr -family receptors.

Additional cell surface proteins interfere with Wnt pathway. Tiki is a metalloprotease that can proteolytically cleave off 8 residues long peptide from the N-terminus of Wnt (Zhang, Abreu et al. 2012). This cleavage produces oligomerized Wnt aggregates that are unable to activate Wnt-signalling pathway. Apcdd1 is a cell surface glycoprotein, that is mutated in hereditary disorder causing hair loss (Shimomura,

Agalliu et al. 2010). It can prevent Wnt signaling, likely by sequestering Wnt activators away from Fzd (Shimomura, Agalliu et al. 2010).

Secreted Wnt inhibitors

Wnt pathway activity is also inhibited by a group of secreted proteins (Langton, Kakugawa et al. 2016). A family of proteins called Secreted frizzled-related proteins (Sfrp) bind Wnt-ligands in the extracellular space with their CRD-domain, homologous to Fzd (Leyns, Bouwmeester et al. 1997, Wang, Krinks et al. 1997). Dickkopf (Dkk) proteins are capable of sequestering the co-receptor Lrps on the cell surface (Langton, Kakugawa et al. 2016). This interaction acts much like the Rnf43/Znrf3, as it eventually leads to internalization of Lrps (Mao, Wu et al. 2002). Wnt inhibitory factor 1 (Wif1) is secreted protein that prevents Wnt activity likely by altering their interactions with the cell surface Glypicans (Avanesov, Honeyager et al. 2012).

Notum

An interesting mechanism to deactivate Wnts was described by two laboratories simultaneously (Kakugawa, Langton et al. 2015, Zhang, Cheong et al. 2015). Wnt signaling inhibitor identified previously in *D.melanogaster*, named *notum* (also known as *wingful*) (Gerlitz and Basler 2002, Giraldez, Copley et al. 2002), was shown to inhibit Wnt signaling by removing the lipid moiety from Wnt. Kakugawa and colleagues resolved the structure of human version of Notum that indicated a hydrophobic cleft fitting 16-carbon long fatty acid (Kakugawa, Langton et al. 2015). This site contained a catalytic triad able to hydrolyze lipid esters. Delipidated Wnt loses signaling capacity, possibly due to decreased affinity to Fzd receptors (Janda, Waghray et al. 2012, Kakugawa, Langton et al. 2015). Moreover, Zhang and colleagues showed that Wnts have the propensity to form inactive oxidized oligomers when the lipid group is removed (Zhang, Cheong et al. 2015). As an enzyme, Notum can repeatedly inactivate Wnt ligands in the extracellular space. Such powerful inhibition is likely kept in guard by sequestering Notum on the surface of cells expressing Glypicans (Kakugawa, Langton et al. 2015). However, glypicans can also bind Wnt proteins (Langton, Kakugawa et al. 2016), which facilitates the proximity of Notum and Wnt.

Laboratory mice lacking Notum have developmental defects. Gerhardt and colleagues showed that lack of Notum alters dorsal to ventral patterning of the tracheal mesenchyme leading to premature death of mutant animals (Gerhardt, Leesman et al. 2018). Another mouse model shows less lethal phenotype, but animals lacking Notum suffer from kidney analgesia and hyperplastic growth in teeth (Vogel, Read et al. 2016). Development of these tissues involves Wnt signaling (Jernvall and Thesleff 2012, Little and McMahon 2012). Moreover, these animals have higher bone density and mass indicating that Notum modulates bone growth (Brommage, Liu et al. 2019). These results indicate that Notum is finetuning Wnt activity during the development and possibly regulating bone growth in adult tissues as well. Interestingly, Seldin et al found liver produced Notum from the peripheral circulation of animals (Seldin, Koplev et al. 2018). When the amount of Notum was experimentally increased, white adipose tissue began to express markers of brown adipose tissue. The browning effect suggests, that Wnt activity in the adipose tissue regulates metabolism of adipocytes and circulatory Notum could modulate Wnt ligands in peripheral tissues. When Notum is conditionally deleted from the liver, no obvious changes are seen in the liver function (Canal, Charawi et al. 2016). However, male mice accumulated more fat with old age and had higher insulin levels than control animals indicating glucose intolerance. Together these findings suggest, that liver produced Notum can modulate adipocyte fate and alter whole body metabolism. Whether these effects were Wnt-dependent was not directly studied. Yet, Wnt signaling is known to affect cellular metabolism in osteoblast suggesting a metabolism regulating role for Wnt in other tissues as well (Frey, Li et al. 2015). While the role of Notum in vertebrate tissue development and patterning is established, whether it participates in regulation of stem cells is not known.

3.6 Wnt signaling in the intestine

Wnt activity is vital for the maintenance of multiple adult stem cell populations (Nusse and Clevers 2017). This is exemplified in the intestine, where absence of canonical Wnt-effector, Tcf4 prevents formation of adult ISCs (Korinek, Barker et al. 1998). Moreover, if Tcf4 is removed from the adult epithelium, rapid halt in renewal of the epithelium is observed due to loss of stem cells (van Es, Haegebarth et al. 2012). Many of the marker genes for ISCs are direct Wnt targets, and some functionally critical for stem cell identity (The Wnt Homepage , Barker, van Es et al. 2007). The best of them, Lgr5, codes for receptor enabling amplification of the Wnt cascade by R-spondins (de Lau, Peng et al. 2014). Removal of Lgr5 together with a sister protein Lgr4 from adult intestine leads to disappearance of ISCs, stressing the importance of R-spondins (de Lau, Peng et al. 2014). Moreover, when Pinto and colleagues overexpressed Wnt-ligand inhibitor Dkk1 in the intestine, proliferative stem cells were lost and tissue compromised (Pinto, Gregorieff et al. 2003).

As discussed above, ISCs rely on Wnts and R-spondins, which are not made by themselves. The main source of Wnt ligands for ISCs has been extensively studied. Gregorieff et al characterized the expression pattern of multiple Wnt ligands and receptors in the mouse intestine (Gregorieff, Pinto et al. 2005). Many ligands were detected in the underlying stroma while only few were made by the epithelial cells, mainly at the crypt bottom. Later, it was shown that Paneth cell produced Wnt3, was the vital Wnt ligand for *ex vivo* organoid cultures (Farin, Van Es et al. 2012). Moreover, overexpression of mesenchyme produced Wnt2b and epithelial Wnt6 and Wnt9b were able to compensate the loss of Paneth cell produced Wnt3 (Farin, Van Es et al. 2012). These results indicated, that canonical Wnt-activity in ISCs is maintained by Wnt3 made by the Paneth cells and Wnt2b originating from the underlining stroma. Interestingly, Gregorieff and colleagues studied also the expression pattern of secreted Wnt inhibitors, identifying that under normal homeostasis they are only made by the stroma (Gregorieff, Pinto et al. 2005). However, what regulates the expression of these inhibitors and how much they affect the function of intestinal epithelium was not studied.

While exogenously administration of R-spondin enables *ex vivo* culture of intestinal organoids (Sato, van Es et al. 2011) and stromal Wnt-ligands were able to compensate the Paneth cells loss (Farin, Van Es et al. 2012) the exact source of these Wnt agonists *in vivo* was not clear. Recently, the work done in multiple laboratories has identified markers for subepithelial cells that contribute critically to Wnt and R-spondin production. In the small intestine, Wnts made by the $Pdgfra^{+}$ stromals cells are needed for recovery after injury (Greicius, Kabiri et al. 2018). Moreover, Shoshkes-Carmel and colleagues showed, that Wnt production from $Foxl1^{+}$ stromal cells, known as Telocytes, are critical for intestinal homeostasis (Shoshkes-Carmel, Wang et al. 2018). Telocytes underline intestinal epithelium and were shown to compartmentalize canonical Wnt2b to the crypt base and non-canonical Wnt5a towards the crypts neck and villous stroma. Furthermore, $Gli1^{+}$ stromal cells that are critical source of R-spondin3 in the colon, are required in the small intestine when epithelial Wnt-production is impaired (Degirmenci, Valenta et al. 2018). How much the described markers for stromal niche cells overlap requires further research. Evidently some level heterogeneity exists allowing localized production of niche factors.

Interestingly, if epithelial Wnt production by Paneth cells is halted by preventing secretory cell maturation (Durand, Donahue et al. 2012), epithelial renewal is maintained. However, when regenerative capacity is needed, Wnts made by the epithelium are necessary for proper repair (Zou, Blutt et al. 2018). Together, multiple sources for Wnt agonist provides safe maintenance of canonical Wnt activity in the ISCs. Upon injury, both stromal and epithelial Wnt agonists are needed for efficient regeneration.

The key role of Wnt signaling in maintenance of stemness and undifferentiated cellular state underlines the risk that uncontrolled Wnt activity brings. The fact that Wnt signaling was identified during a quest to understand pathways driving cancerous growth was not a coincidence (Nusse and Varmus 1982). Aberrant Wnt activity is driving multiple cancer types originating from various tissues (Nusse and Clevers 2017). In the light of this, the existence of multifaceted machinery that keeps Wnt pathway in place or shuts it down upon high activation (see above) is not surprising. Furthermore, multiple genes involved in

the control of Wnt pathway are recognized as tumor suppressors today (Polakis 2012). GI tract is a good example, where vast majority of tumors originate from aberrant activation of Wnt pathway due to loss-of-function mutations in the APC gene (Muzny, Bainbridge et al. 2012).

3.7 Wnt and cytoskeleton

The importance of proper Wnt activity during development is highlighted by the fact that mutant animals for Wnt-pathway genes are often embryonically lethal (van Amerongen and Nusse 2009). As described above, Wnt activity is important for maintenance of a pool of undifferentiated stem cells that can regenerate or form tissues (Nusse and Clevers 2017). However, Wnt pathway is also modulating cellular behavior that is not directly linked to cell fate (Schlessinger, Hall et al. 2009). As tissues form during the development, cellular morphology and movement has to change a lot. Epithelial sheets are invaginating to form more complex tissues. Wnt signaling can play a role in this, as changes required in specific developmental steps in cell shape and cytoskeleton are often missing in mutants where Wnt-pathway is impaired. (Schlessinger, Hall et al. 2009)

Molecularly, Wnt pathway modulates cell cytoskeleton and movement primarily via the non-canonical signaling arm. Ligand binding activates small Rho GTPases that modulate actin cytoskeleton (Schlessinger, Hall et al. 2009). Particularly RhoA and Rac1 are of interest, as they drive somewhat opposing effects in cellular morphology. Rac1 promotes actin filament assembly on cell periphery, promoting protrusions required for motility (Sit and Manser 2011). Conversely, RhoA activation leads to phosphorylation of Rho-associated protein kinase (ROCK) that has multiple downstream substrates (Riento and Ridley 2003). A major downstream effect of ROCK activation is increased stress fiber assembly and contraction. This is achieved by phosphorylation of Myosin light chain kinase (Mylk), which increases its capacity to promote actomyosin contractility (Amano, Ito et al. 1996). Moreover, ROCK can inactivate Myosin light chain phosphatase, an inhibitor of myosin activity, further reinforcing contractility (Kimura, Ito et al. 1996). Furthermore, ROCK stabilizes F-actin by inhibiting actin severing Cofilin via LIMK (Riento and Ridley 2003). Rho-ROCK driven actomyosin contractility is crucial for invagination of epithelial sheets, as it is the driving force of an event called apical constriction (Vicente-Manzanares, Ma et al. 2009). For example, Chauhan and colleagues showed that balanced activity between Rac1 and RhoA was required for proper cellular shape in the developing eye (Chauhan, Lou et al. 2011). When they genetically deleted RhoA from the epithelial layer, eye cup failed to invaginate. On the other hand, if Rac1 was removed, formed eye cup had higher curvature, suggesting that Rac and Rho -pathways balance each other's activity by trans-inhibition. In summary, Wnt signaling can contribute to morphogenesis of tissues by altering cell motility and shape. Whether this feeds back to the signaling capacity of cells or is independent phenomena requires further research.

4. Physical shape of tissues and niches

Tissue architecture has evolved to facilitate function of a given tissue (Hagios, Lochter et al. 1998). Continuous layer made by epithelial cells can generate enough force to partake in formation of tissues morphology as seen in the developing eye and intestine (Chauhan, Lou et al. 2011, Heisenberg and Bellaiche 2013, Sumigray, Terwilliger et al. 2018). Moreover, morphology of the epithelial layer can compartmentalize distinct units within the tissue, serving distinct function. For example, hair follicle contains distinct compartments for hair shaft and sebum production, bulge and sebaceous gland respectively (Solanas and Benitah 2013).

Interestingly, many adult stem cell niches are found from such curved pits. For example, multipotent stem cell populations exist in the curved environments of the hair follicle (Solanas and Benitah 2013). Similarly, cell population responsible of corneal renewal and repair exists in the curved regions of the edges of corneal limbus (Nowell and Radtke 2017). Moreover, throughout the GI tract, stem cell responsible for the renewal of epithelium exist inside curved crypts (Clevers 2013). While stem cell niches likely benefit

from the protective nature of invaginated tissue morphology, the topology might offer other gains such as facilitating organization and hierarchy in the renewing tissue.

4.1 Self-organization requires coordinated intercellular communication

Complex architecture of tissues arises during the development. However, in high turnover tissues, the architecture has to be maintained while cells move. In the case of severe injury, lost tissue needs to be rebuilt. This ability highlights the capacity of cells to communicate with each other in order to maintain or restore homeostasis. Investigation of this phenomena has been relying on snapshots of collected tissue at different timepoints of regeneration, but organoid technology has allowed investigators to follow these events live on cellular level (see above).

As a prime example, intestinal epithelium, grown as organoids in hydrogel with uniform composition and without any spatial cues for the epithelium, self-organizes to units that resemble *in vivo* like architecture of the epithelium (Sato, Vries et al. 2009). In this organoid system, Paneth cells, as the main source of Wnt, dictate where high Wnt-activity exist (Sato, van Es et al. 2011, Farin, Van Es et al. 2012). Gradient in activity is formed, as membrane bound Wnt ligands dilute during the ISC and progenitor divisions (Farin, Jordens et al. 2016). Near Paneth cells, Wnt target genes Ephrin receptors (Eph) are produced, whereas low Wnt induced differentiation leads to production of Eph receptor interacting proteins (Ephrin) (Solanas and Batlle 2011). The interaction of these proteins on cellular interface results in bi-directional signaling that promotes physical separation of both cells. This signaling cascade prevents Eph producing ISCs and Paneth cells mixing with differentiated cells, eventually leading to formation of distinct crypt domains in organoids (Clevers 2013).

Cellular organization and fate determination in intestinal epithelium are largely driven by Notch-Delta lateral inhibition (Sancho, Cremona et al. 2015). At the crypt base trans-activation of Notch receptors by the Dlls made in Paneth cells, drives non-secretory cell fate in ISCs (VanDussen, Carulli et al. 2012). This results in production of more Notch receptor and suppression of ligand production (Sancho, Cremona et al. 2015). Vice versa, Paneth cells producing Notch ligands are surrounded by receptor producing ISCs that cannot trans-activate Notch in Paneth cells. Low Notch activity leads to promotion of secretory cell fate and increases production of Notch ligands (Sancho, Cremona et al. 2015). Lateral inhibition also drives the differentiation of TA cells. Small differences in the Notch-Delta expression between undifferentiated progenitors can stochastically initiate a cascade resulting to either enterocyte (Notch high) or secretory (Notch low) cell fate. Moreover, it promotes the salt-and-pepper pattern of Paneth and ISCs at the crypt base (Clevers 2013). Similar mechanism likely establishes crypts from patches of homogenous ISCs at the inter-villus region of developing intestine and organize crypt domains in growing organoids (Sato, Vries et al. 2009, Chin, Hill et al. 2017).

Experiments with single cell derived organoids have indicated, that also YAP pathway is crucial in establishment of the crypt units. Serra and colleagues noticed, that organoids formed exclusively from ISCs, require uneven YAP activity in order to create the first Paneth cell and establish asymmetry (Serra, Mayr et al. 2019). This is required for the organoid growth and later formation of crypt domains (Gregorieff, Liu et al. 2015). Therefore, it seems that normal growth of small intestinal organoids requires Paneth cells and crypt like morphology. Paneth cells provide Wnt and Notch ligands to fuel ISC function but how the morphology contributes to better growth is not known.

4.2 Cellular shape

Intestinal epithelial cells are stereotypically cuboidal in shape, jointly forming a continuous lattice of often hexagonal or pentagonal array. The side facing external environment, the gut lumen, is called the apical surface and is enriched with specialized structures such as microvilli. Basal side faces the stromal side of the tissue, and contains adhesions to the underlining basement membrane. To maintain barrier function

of epithelium, cells are connected with various adhesions with their neighbors and the basal lamina (Parrish 2017). Tight junctions are strong adhesions between adjacent epithelial cells that intracellularly connect with the cytoskeleton rich in actomyosin cables (Vicente-Manzanares, Ma et al. 2009, Parrish 2017). This interconnection of contractile structures on the apical side of epithelium allows formation of invaginating structures as individual cells contract apically (Heer and Martin 2017). Apical constriction in epithelial cells requires activity from NM II which is regulated mainly by the Rho-ROCK pathway (discussed in 3.7). This ability is required at the development already during the gastrulation of the embryo and when various tissues form (Chauhan, Lou et al. 2011, Sumigray, Terwilliger et al. 2018).

The importance of apical constriction and cell shape for development are discussed above, but whether they regulate other aspects of cell behavior is less understood. Recently, changes in the cellular shape has been connected to a differentiation of intestinal epithelium (Shyer, Huycke et al. 2015, Sumigray, Terwilliger et al. 2018). Remarkably, forcing developing and still smooth intestine to adopt undulating shape, induced Sonic Hedgehog (Shh) mediated differentiation of the epithelium (Shyer, Huycke et al. 2015). Yet, when not fully differentiated cells exit the intestinal crypt, their morphology changes dramatically inducing compartmentalization of intestinal epithelium to crypts and villi (Sumigray, Terwilliger et al. 2018).

To link cytoskeletal changes and cellular fate, main research focus has been around the YAP pathway (Totaro, Panciera et al. 2018). Curvature, stiff substrate or sharp contours, increase polymerization of globular actin (G-actin) monomers to form filamentous actin (F-actin) promoting cellular stiffness (Aragona, Panciera et al. 2013, Totaro, Panciera et al. 2018). Aragona and colleagues showed, that by artificially promoting F-acting assembly, YAP localization to nucleus was induced even on substrates that normally did not trigger YAP activity (Aragona, Panciera et al. 2013). Mechanistically, increased F-actin concentrations have been proposed to increase nuclear translocation of YAP by sequestering Angiomotin (AMOT), negative regulator of YAP activity (Totaro, Panciera et al. 2018). Another mechanism how cellular stretching can promote YAP activity is opening of nuclear pores when tension is applied on cells (Elosegui-Artola, Andreu et al. 2017).

Besides, YAP has been shown to promote proliferative and undifferentiated state in cells of many tissues (Mo, Park et al. 2014). Mechanical tension via YAP has been shown to dictate differentiation of mesenchymal progenitors to osteoblast or adipocytic lineages (McBeath, Pirone et al. 2004, Engler, Sen et al. 2006, Dupont, Morsut et al. 2011). As a transcriptional co-activator, YAP alone unlikely mediates these effects alone. The intimate connection with other fate determining pathways such as Notch and Wnt likely play a significant role (Totaro, Panciera et al. 2018)(Figure 3). Interestingly, low cellular volume can induce nuclear localization of YAP, but also contributes to increased concentration of cellular mRNA (Bao, Xie et al. 2017). High concentration of biomolecules could contribute to effectiveness of intracellular signaling. Moreover, low volume allows better protein translation in yeast and mammalian cells (Neurohr, Terry et al. 2019). Consequently, as shape and volume modulate the signaling capacity, they are likely to play an important role in cell-to-cell communication such as heterotypic interaction between the niche and stem cells.

Knowledge from the biology behind the aging process has increased significantly during the last decade. Altered metabolism has been proposed to drive many aging related pathologies and functional decline. While reduced capacity of tissue resident stem cells in this process has been acknowledged, the role of the stem cell supporting niche has not been extensively studied. Investigation of niche derived signals regulating stem cell behavior might open possibilities to alleviate some aspects of aging associated health problems.

Aims of the study

Beforehand, little was known about age-related changes in the intestinal epithelium. Reduced capacity to recover from injuries of the intestine among elderly was reported, but careful analysis of the regenerative ability of aged intestine was lacking. Moreover, whether physical architecture of intestinal epithelium affects signaling in the stem cell niche had not been studied. Therefore, this dissertation aims to answer the following points:

i) Whether cellular composition and regenerative capacity of intestinal epithelium change with age?

ii) What is the role of the stem cell niche in regeneration of aged intestine?

iii) Can crypt architecture facilitate signaling between the niche and ISCs?

Materials and methods

Materials and methods used in the unpublished data figures are described here. Full list of methods used in I and II are included in the corresponding articles.

Isolation of small intestinal crypts from a mouse

Mice were sacrifice under CO2 narcosis by cervical dislocation. Abdomen was opened and small intestine from stomach to cecum removed and placed in ice cold PBS. Working on ice intestine was flushed with PBS to remove partially digested food and cleaned from residual fat and mesentery. It was opened longitudinally and further washed with fresh PBS to remove excess mucus. Preparation was cut to approximately 3 mm long segments and place into a Falcon tube. Tube was filled with 30 ml of cold 10 mM EDTA – PBS and incubated on ice for 15 min. Tube was shaken by hand to detach villus epithelium. Supernatant was removed and both incubation and shaking were repeated twice. After removing most of the villus epithelium, preparation was incubated in 10 mM EDTA – PBS for 1 h. Incubation was followed by vigorous shaking of the tube to detach crypts. Supernatant was filtered through 70-um nylon mesh (FisherBrand) to enrich for crypts and centrifuged 300g +4 C 5 min. Optionally second and third yield was collected by repeating the incubation step for additional 15 min. Pelleted crypts were washed once with fresh PBS without EDTA.

Isolation of single cells from mouse epithelium

For single cells experiments, crypts isolated as above were dissociated for 90 seconds at +32 C in 1 ml of TrypLE Express (Life Technologies) containing 1U/ul of DNase I (Roche). Cell suspension was washed once with 12 ml of isolation media (SMEM (Sigma) or DMEM containing 10 mM Hepes and 0,2 % BSA) and centrifuged 300 g +4 C 5 min. Pellet was resuspended to 500 ul of isolation media containing desired combination of fluorescent conjugated antibodies (all 1:500) and incubated 15-30min on ice. Sample was washed once with 10 ml of isolation media and resuspended to 2-4ml of isolation media containing 7-AAD or SytoxBlue (1:500). Sample was filtered through 40um mesh prior analysis and sorting with BD FACSAria II or FACSAria Fusion.

Culture of isolated single cells

Isolated Lgr5^{hi} ISCs were resuspended to ENR media (Advanced DMEM/F12 (Life Technologies), 10 mM Hepes, 1x Glutamax (Life Technologies), 100 U/ml of Penicillin and 100 ug/ml Streptomycin, 1x B27 supplement (Life Technologies), 1x N2 supplement (Life Technologies), 1uM N-acetylcysteine (Sigma), 50 ng/ml EGF (RnD), 100 ng/ml noggin (Peprotech), 500 ng/ml Rspondin-1 (RnD) and mixed to growth factor reduced Matrigel® (Corning) in 2 : 3 ratio. 20 ul drops containing 1000 cells were placed on 48-well tissue culture plate (CellStar) and let to solidify for 30 min +37 C. Matrigel-cell mixture was overlaid with 300 ul of warm ENR supplemented with 3uM of Chir99021 (Sigma) and 1mM Valproic Acid (Cayman chemicals), hereafter ENRCV. 10 uM of ROCK-inhibitor Y-27632 (Sigma) was used in the culture for the first 2 days. Media was changed every 2-3 days.

Stem cell pure organoids were expanded in ENRCV followed by mechanical dissociation with p200 pipette tip. Subcultured organoids were grown for additional 4 days in ENRCV (stem cells) or in plain ENR (organoids), or ENR supplemented with either 10uM of DAPT (Sigma) and 3uM of Chir99021 (Paneth cells), 1mM Valproic acid and 2uM IWP-2 (Sigma) (Enterocytes) or 10uM DAPT and 2uM IWP-2 (Goblet cells).

Immunoblotting

Protein from sorted Paneth cells was isolated in RIPA buffer containing 1x Halt protein inhibitor, 1x phosphoSTOP phosphatase inhibitor and 5 mM EDTA and homogenized in bath sonicator. Equal number of sorted cells were loaded per lane. Protein samples were denaturated by boiling in 1x LDS -sample buffer (Thermo) under reducing conditions. Samples were run on NuPAGE 4-12% Bis-Tris protein gels in Bolt Transfer buffer (both Thermo) and transferred to Protran nitrocellulose membrane (Perkin-Elmer) using Novex Mini Gel Tank system (Thermo).

Nitrocellulose membrane was blocked with 3% Milk in 0,05% Tween20-TBS (TBST) over 1 h RT or overnight +4 C followed by incubation overnight in primary antibody +4 C (rbanti-pS6 S240/244 CST, mo anti-S6 CST, rb anti-H3 Abcam). The following day membrane was washed 3 times with TBST and incubated in HRP-conjugated secondary antibody 1 h RT. Subsequently membrane was washed 3 times with TBST and incubated in chemiluminescent substrate, ECL (Thermo) or Supersignal West femto ECL (Life Technologies). Chemiluminescence was detected by exposing X-Ray film (FUJI) on membranes. Primary and secondary antibodies were diluted in 3% Milk-TBST.

RNA isolation and qPCR

RNA from cell isolates was purified with Trizol reagent (Life Technologies) according to manufacturer's protocol. Briefly, cells were lysed in Trizol reagent by vortexing and a freeze-thaw cycle. 200 ul of chloroform per 1 ml of lysate was used to separate aqueous phase containing RNA by centrifugation. RNA was precipitated by adding equal volume of isopropanol supplemented with 15 ug/ml of Glycoblue coprecipitant (Life Technologies). RNA was washed twice and resuspended to DNase/RNase-free water. Concentration of RNA was measured with NanoDrop. 100 ng to 3 ug of RNA was DNase I treated and transcribed to cDNA by RevertAid first strand synthesis kit (Life Technologies).

qPCR was performed from diluted cDNA by Power SYBR green PCR master mix with 0,5 uM mRNA specific primers. Technical triplicates were run. Gene-expression was normalized to a housekeeping gene and represented as Log2 Fold change with deltaCt method. Reactions from RNA samples without reverse transcriptase ('-RT') and without template (non-template control 'NTC') were used to control primer specificity.

Primer:	**Sequence 5' - 3':**
Beta-Actin-F	CCTCTATGCCAACACAGTGC
Beta-Actin-R	CCTGCTTGCTGATCCACATC
Alpi-F	GGCTACACACTTAGGGGGACCTCCA
Alpi-R	AGCTTCGGTGACATTGGGCCGGTT
Dll4-F	TTCCAGGCAACCTTCTCCGA
Dll4-R	ACTGCCGCTATTCTTGTCCC
Neurogenin3-F	AGTGCTCAGTTCCAATTCCAC
Neugorenin3-R	CGGCTTCTTCGCTTTTTGCTG
Atoh1-F	GAGTGGGCTGAGGTAAAAGAGT
Atoh1-R	GGTCGGTGCTATCCAGGAG
Gfi1-F	GAGCAACACAAGGCAGTG
Gfi1-R	TCTTGCCACAGATCTTACAGTC

Spdef-F	AAGGCAGCATCAGGAGCAATG
Spdef-R	CTGTCAATGACGGGACACTG
Muc2-F	AGGGCTCGGAACTCCAGAAA
Muc2-R	CCAGGGAATCGGTAGACATCG
Lysozyme-F	GCCAAGGTCTACAATCGTTGTGAGTTG
Lysozyme-R	CAGTCAGCCAGCTTGACACCAGC
Lgr5-F	ACCCGCCAGTCTCCTACATC
Lgr5-R	GCATCTAGGCGCAGGGATTG
ChgA-F	CAGGCTACAAAGCGATCCAG
ChgA-R	GCCTCTGTCTTTCCATCTCC
Notum-F	CTGCGTGGTACACTCAAGGA
Notum-R	CCGTCCAATAGCTCCGTATG
Wnt3-F	TGGAACTGTACCACCATAGATGAC
Wnt3-R	ACACCAGCCGAGGCGATG
Egf-F	AGAGCATCTCTCGGATTGACC
Egf-R	CCCGTTAAGGAAAACTCTTAGCA
Axin2-F	AGTGCAAACTCTCACCCACC
Axin2-R	TCGCTGGATAACTCGCTGTC
Hmgcs2-F	ATACCACCAACGCCTGTTATGG
Hmgcs2-R	CAATGTCACCACAGACCACCAG

Histological and immuno-staining of tissue sections

Tissues were fixed in 4% Paraformaldehyde (PFA) 16 to 32 hours. Fixed tissues were stored in 70% EtOH until embedded to paraffin. 5 um paraffin sections were deparaffinized and stained with routine histological protocols (H&E, Alcian Blue). Samples to be immuno-stained were boiled in antigen retrieval solution, pH 6 Citrate buffer (Sigma) or Target retrieval solution (DAKO), followed by blocking and incubation with desired antibodies. For immunohistochemical staining, samples were pretreated in 0,3% H2O2 (Sigma) in methanol before blocking and probing with primary antibodies (mo anti-pS6 240/244 CST, rb anti-Lysozyme DAKO, rb anti-ChgA Abcam). HRP-based detection kit, VectaStain ABC kit (Vector laboratories), was used in conjunction with ImmPACT DAB peroxidase substrate kit (Vector laboratories). Hematoxylin was used to counterstain the nuclei, followed by dehydration and mounting with xylene-based media, Pertex.

Animal experiments

Overnight fasted animals were refed for 4 hours and tissues collected for analysis. Refed and control (continuously fasted) mice were weighed before fasting, after overnight fast and after 4 hour refed period. For stem cell damage, 5-Fluorouracil was used as single 100 – 200 mg/kg dose as described in the corresponding figure legends and was reconstituted to DMSO in concentration of 150 mg/ml. All animals were housed according to the institutional laws of the host university. Mouse lines used in I and II are listed below.

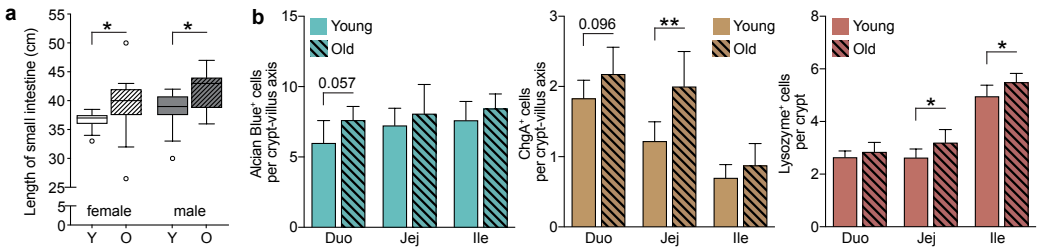
Mouse line:	Used in:	Reference:
C57BL/6JRccHsd (wild type)	I,II	Envigo
Lgr5-EGFP-IRES-CreERT2	I,II	(Barker, van Es et al. 2007)
Rosa26-mTmG	I,II	JAX 007576
Rosa26-LSL-Cas9-EGFP	I	JAX 024857
Rosa26-LSL-TdTomato	I	JAX 007909
Rosa26-LSL-ZsGreen	I	JAX 007906
TSC1(fl/fl)	I	(Kwiatkowski, Zhang et al. 2002) Tsc1tm1Djk/J, JAX 005680
Villin-CreERT2	I	(el Marjou, Janssen et al. 2004)
Rag2(-/-)	I	B6(Cg)-Rag2tm1.1Cgn/J, JAX 008

Results and discussion

1. Changes in the aging intestine (I and Supplemental data)

Age-related reduction in intestinal function has been reported in model organisms and humans (Drozdowski and Thomson 2006). However, to what extent stem cell function changes with age has not been studied carefully. To address changes in the aging intestine, transgenic mouse model where EGFP-fluorescent protein has been knocked in to the Lgr5 locus allowing visualization and isolation of Lgr5 expressing intestinal stem cells, Lgr5-EGFP-IRES-CreERT2, (Barker, van Es et al. 2007), was utilized. Cohorts of these mice were aged until they reached the age of 20 to 24 months, approximately corresponding to humans age of 60 to 70 years (Flurkey 2007).

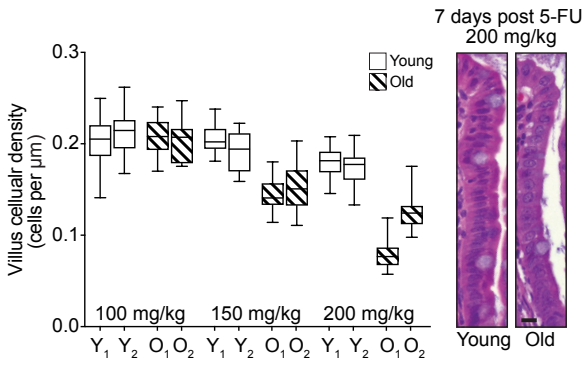
Old animals were larger and less active than young when examined (data not shown). The larger body size was reflected in increased body mass and length of the small intestine (I, Extended Data Fig. 4c, Data Figure 1). These observations propose, that the core metabolism in aged mice is altered with age, like in humans (Auro, Joensuu et al. 2014). Apart from the length, intestines from old animals were macroscopically similar with young counterparts (I, Extended Data Fig. 1f). This was not surprising, given the important role of intestinal function for the viability of an organism. The function of the small intestine relies on the differentiated cells covering the fingerlike protrusions called villi (Figure 2c). To address whether the cellular composition of the villus remains unchanged with age, tissue sections of small intestine from young and old animals were stained with histological stain Alcian Blue to detect mucin rich granules of Goblet cells and immuno-stained against Enteroendocrine cell marker Chromogranin A (ChgA). Interestingly, in the proximal intestine, composed of duodenum and jejunum, number of Goblet cells was near significantly higher and Enteroendocrine cells showed clear age-dependent increase (Data Figure 1b). This data corroborates previous reports of aged intestine harboring more Goblet and Enteroendocrine cells (Nalapareddy, Nattamai et al. 2017, Igarashi, Miura et al. 2019). Moreover, to cover the main secretory cell spectrum, number of Lysozyme positive Paneth cells was assessed in crypts. While Paneth cells are more abundant in the distal intestine, ileum, old animals had increased number of Paneth cells in both regions (Data Figure 1b). These findings propose more general bias towards the secretory lineage in old homeostatic epithelium. Intriguingly, higher proportion of hormone producing Enteroendocrine cells in old intestine suggests differential response to food and other hormone releasing stimuli (Gribble and Reimann 2019). Further studies are required to investigate whether this could lead to altered whole body metabolism of old animals.



Data Figure 1 | Characterization of the old intestine

a, Quantification of the small intestinal length in young (Y) and old (O) animals. n= 30 animals per age group. Line at the box- and whisker plot represents median, box interquartile range and whiskers range. Extreme cases called out by the Tuckey's method are shown (circles). **b**, Quantification of Alcian Blue^{+} Goblet, Chromogranin A^{+} Enteroendocrine and Lysozyme^{+} Paneth cells along the proximal to distal axis of small intestine. Mean + s.d. n = 6 animals per age group analysed. * $P < 0.05$, ** $P < 0.01$, Student's t-test.

Regeneration of the intestine relies on functional Lgr5-positive stem cells (ISCs)(Metcalfe, Kljavin et al. 2014). Old intestine has been shown to regenerate slower than young from irradiation induced damage (Martin, Kirkwood et al. 1998). Moreover, aged humans show sensitivity to chemotherapeutics that poses a challenge for cancer therapies of old (Chang, Goldstein et al. 2017). To test whether aged mice respond differently to chemotherapy caused side effects, young (3-4 months old) and old (20-22 months old) wild type animals were subjected to acute treatment with various doses of commonly used cancer drug 5-Fluorouracil (5-FU) (Longley, Harkin et al. 2003). Following the body weight of animals indicates how well food and water are absorbed by the intestine (Song, Park et al. 2013). While young animals recover from the treatment in 5 days as shown by the restoration of initial bodyweight, old animals fail to do so (I, Extended Data Fig. 9a). To assess if regenerative output of stem cells is altered in old, cellular density in the villi was assessed 7 days post 5-FU treatment. If cellular output is impaired, lower number of differentiated cells are found from villi as the migration of cells continue even when proliferation is halted (Krndija, El Marjou et al. 2019). Old animals receiving highest dose of 5-FU showed decreased cellular density even after 7 days of recovery (Data Figure 2). These results are in line with previous reports showing reduced tolerance for irradiation in old animals (Martin, Kirkwood et al. 1998, Nalapareddy, Nattamai et al. 2017). Moreover, elderly cancer patients are affected more by cytotoxic agents targeting also healthy dividing ISCs indicating that attenuated regenerative capacity of aged intestine is conserved feature (Chang, Goldstein et al. 2017).



Data Figure 2 | Analysis of cellular density post 5-FU

Quantification of cellular density 7 days post 5-Fluorouracil (5-FU, 100-200 mg/kg) injection in ileal villi of young (Y) and old (O) animals. 15 villi counted per animal. In box plots, the line represents median, box interquartile range and whiskers range. Represetative image of H&E stained ileal villi of young and old animals 7 days post 5-FU (200 mg/kg). Scale bar 10 µm. Young animals 3-4 months, old 20 months.

To analyze how much of these changes are due to epithelium intrinsic aging, organoid cultures (Sato, Vries et al. 2009) were set up from isolated crypts of human and mouse intestine. In these cultures, crypt cells grow organoids that resemble intestinal epithelium *in vivo*. Organoids recapitulate the epithelial stem cell niche (crypt domains) and differentiated epithelium (villus domain) that harbors all the differentiated cells types (Sato, Vries et al. 2009). The capacity of isolated crypt to form an organoid is correlating with the *in vivo* number of ISCs per crypt as well as their functional capability together with the function of neighboring Paneth cell niche (Yilmaz, Katajisto et al. 2012). Organoid forming capacity of both human and mouse crypts declined with age (I, Figure 1a and Extended Data Fig. 1a). Moreover, the ability to grow and form new crypt domains was reduced in mouse small intestinal organoids (I, Figure 1b). These findings indicated that number or function of ISCs is reduced in old tissues. Furthermore, the phenotype was maintained in serially passaged organoids suggesting that at least some of the changes observed were intrinsic to epithelium (I, Extended Data Fig. 1d).

To test if number of ISCs changes with age, as suggested by the observed decline in regenerative capacity *in vitro*, frequency of EGFP expressing cells was analyzed from isolated crypts of young and old Lgr5-EGFP-IRES-CreERT2 mice with flow cytometry. Expression level of cell surface protein CD24 and level of side scatter (SSC) was used to additionally identify Paneth ($CD24^{hi}SSC^{hi}$) and enteroendocrine ($CD24^{hi}SSC^{lo}$) cell populations (Sato, van Es et al. 2011, Yilmaz, Katajisto et al. 2012). The analysis of 30 young animals (3-9 months old) and 26 old animals (24 months or older) revealed a clear drop in the number of ISCs while number of Paneth cells was increased (I, Figure 1c). Number of enteroendocrine cells remained unchanged (I, Extended Data Fig. 1i). These findings were validated with immunostainings

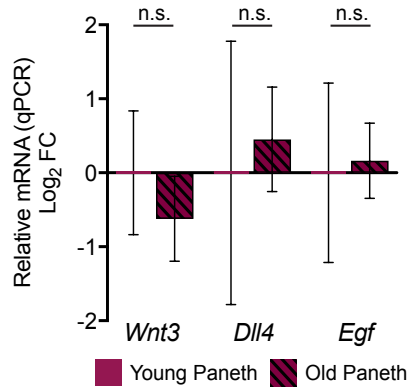
of tissue sections with antibodies detecting stem and progenitor marker Olfm4 and Paneth cells marker Lysozyme (I, Extended Data Fig. 1k, Data Figure 1). The low frequency of differentiated Enteroendocrine cells in crypts might explain why difference seen in the immunostaining of the whole tissue was not captured by the flow analysis (I, Extended Data Fig. 1i, Data Figure 1).

The drop in ISC to Paneth cell ratio in old tissues (I, Extended Data Fig. 1l) suggested that observed decline in the regenerative capacity could lie in altered communication between old stem and Paneth cells. In normal homeostasis Paneth cells maintain ISCs by producing Wnt, Egf and Dlls and any given cell at the bottom of a crypt touching a Paneth cell is expressing stem cell marker Lgr5 (Sato, van Es et al. 2011). Moreover, under caloric restriction ISC and Paneth cell numbers are increased hand in hand leading to boosted self-renewal and regenerative capacity (Yilmaz, Katajisto et al. 2012). To functionally test, whether the crosstalk between these cell types is impaired, ISCs and Paneth cells were isolated from different aged donor mice by fluorescent activated cell sorter (FACS) and mixed in different combinations into co-culture assay as developed by Yilmaz and Katajisto (Yilmaz, Katajisto et al. 2012). In this assay, non-proliferative Paneth cells alone do not form organoids, but are able to support growth of ISCs. Young ISCs co-cultured with young Paneth cells grew significantly more organoids than ISCs alone. However, when young ISCs were supplemented with old Paneth cells, their organoid forming capacity decreased (I, Figure 1d). In addition, the organoid forming capacity of old ISCs was lower even when supplemented with young Paneth cells.

These results indicate that age-dependent decline in function of both cell types (ISCs and Paneth cells) contribute to decreased regenerative capacity of old epithelium. Interestingly, coculture of young ISCs with old Paneth cells decrease ISC function also in prolonged culture, suggesting that Paneth cell made factors can rewire young ISC long term, as fresh Paneth cells made by ISCs are not able to reinstate regenerative growth quickly (I, Extended Data Fig. 1n). Thus, it'll be interesting to evaluate age-dependent changes in expression of secreted factors in Paneth cells.

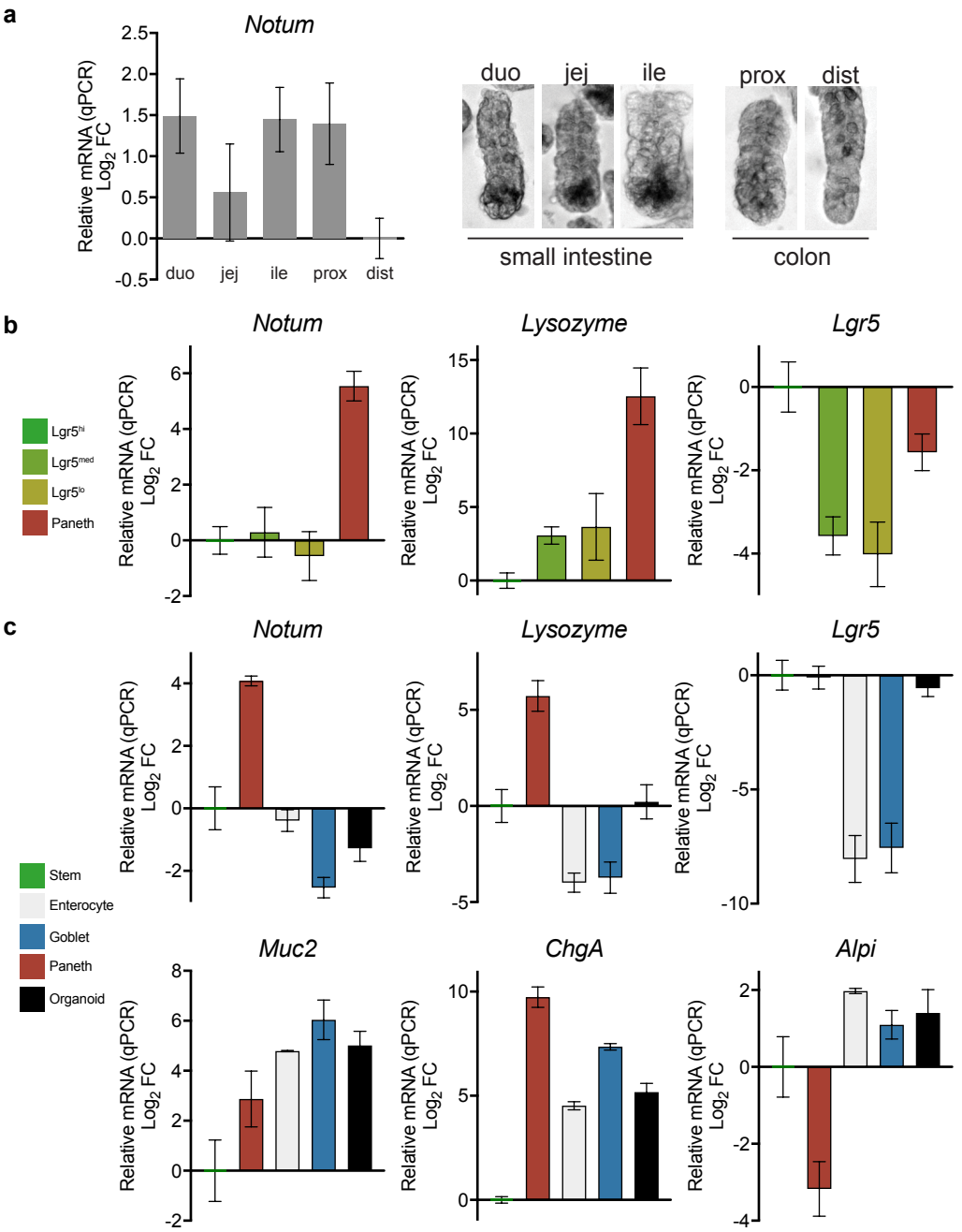
2. Notum, a Wnt inhibitor (I and Supplemental data)

To characterize potential changes in niche-to-stem cell signaling transcriptional profile of Paneth and ISCs from young and old animals were analyzed. To explore factors involved in cell to cell communication, focus was on proteins either secreted or present on plasma membrane (I, Extended Data Fig. 2a). In Paneth cells, no significant change was observed in factors known to regulate stem cell function (I, Extended Data Fig. 2b, Data Figure 3). However, increased expression of secreted Wnt-inhibitor Notum was detected in old Paneth cells (I, Figure 2a). Notum could potentially regulate ISCs function, as Wnt-signaling is crucial for the maintenance of stemness (Krausova and Korinek 2014) and as a potent Wnt-deacylase it can act on multiple ligands produced either by Paneth cells or subepithelial stroma (Kakugawa, Langton et al. 2015). To test if Notum expression was restricted to Paneth cells in the intestine, its expression levels were evaluated from region specific crypt isolates (Data Figure 4a). Expression decreased significantly in the distal colon, where Paneth cells are absent. Interestingly, proximal colon showed comparable expression to ileum (Data Figure 4a), suggesting that Reg4+ deep crypt secretory cells (DCS), colonic counterparts of the Paneth cell niche (Sa-



Data Figure 3 | Expression analysis of niche factors

qPCR analysis of isolated Paneth cells from young and old animals. Three well known niche factors analysed. Data is represented as Log2 Fold Change +/- s.d compared to young Paneth cells. n = 3-5 animals analysed. Student's two-tailed t-test, n.s. = not significant

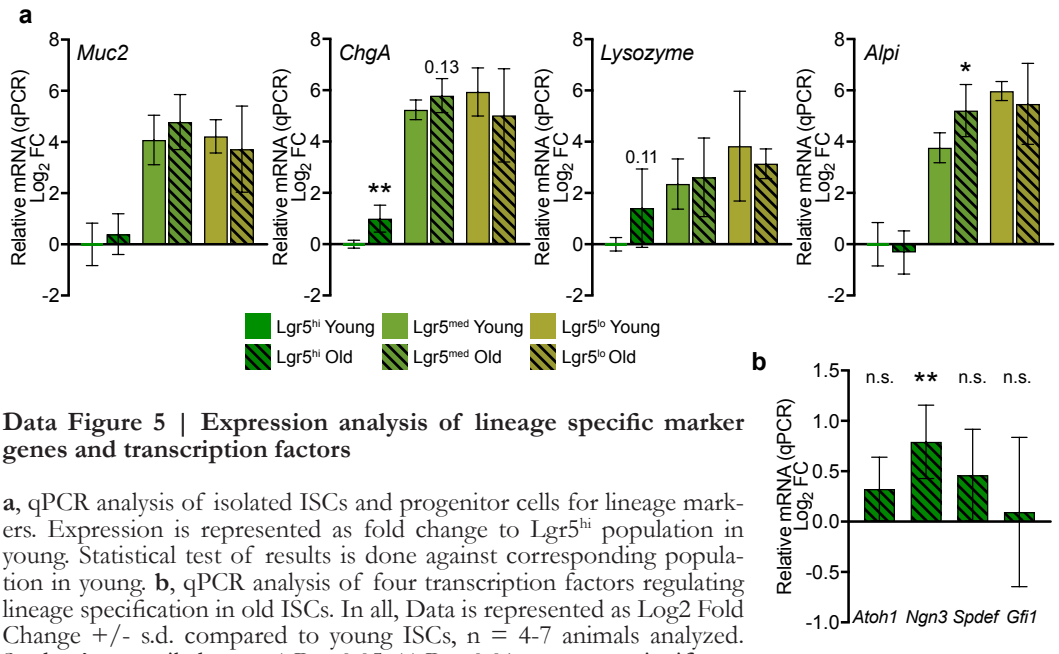


Data Figure 4 | Analysis of Notum expression in the GI tract

a, qPCR analysis of Notum expression and representative images of crypts isolated along the proximal to distal axis of GI tract. Duodenum (duo), jejunum (jej), ileum (ile), proximal colon (prox) and distal colon (dist). Data is represented as Log2 Fold Change +/- s.d compared to crypts from distal colon, n = 3 animals analyzed. **b**, qPCR analysis of Notum expression in isolated ISCs ($Lgr5^{hi}$), progenitors ($Lgr5^{hi}$ and ^{med}) and Paneth cells. Data is represented as Log2 Fold Change compared to ISCs, n = 3-4 animals analyzed. Notum was detected only in 2 out of 3 samples of ISCs. **c**, Expression of Notum and lineage markes from organoids differentiated towards ISC (green), Paneth (red), Enterocyte (gray), Goblet (blue) and mixture (black) faith. n = 3 replicate cultures analysed.

saki, Sachs et al. 2016) could produce Notum as well. To dissect the cell specific expression pattern, different cell populations were isolated and analyzed (Data Figure 4b). The expression was several folds higher in Paneth cells and was barely detected in ISC or TA-cells. Furthermore, organoid cultures enriched for different cell lineages (Yin, Farin et al. 2014) showed strikingly similar outcome (Data Figure 4c). Highest expression was observed in lines enriched for Paneth cells. Finally, RNA *in situ* hybridization revealed that expression was limited to Paneth cells in the epithelium and was not detected in the underlying stroma (I, Figure 2b). These results indicate that in the small intestine, Notum is expressed specifically in Paneth cells and its production increases with age.

Profiling of old ISCs suggested decreased Wnt-activity which was validated by qPCR (I, Figure 2c, Extended Data Fig. 2f). This agrees with previous report where decrease in canonical Wnt signaling was observed in old crypts (Nalapareddy, Nattamai et al. 2017). Interestingly, profiling also revealed that bias towards secretory lineage might exist already at the stem cell state (I, Supplementary table 1). To further validate, whether such bias exists in old ISCs, qPCR analysis for specific lineage markers was performed (Data Figure 5a). While cell purity was not compromised in sorted ISCs (I, Supplementary Figure 2), ISCs showed age dependent increase in the expression of the enteroendocrine marker ChgA. Yet, expression of the Paneth cell marker Lysozyme was increased in some animals (Data Figure 5a). Moreover, old ISCs expressed significantly more transcription factor Neurogenin3 (Ngn3), the fate determining factor of enteroendocrine lineage (Jenny, Uhl et al. 2002)(Data Figure 5b). Interestingly, analysis of the progenitor cells (Lgr5^{med}) revealed significant increase in expression of Alkaline phosphatase (Alpi), marker for differentiated enterocytes, in old animals (Data Figure 5a)(Clevers 2013). This indicates faster differentiation in the old intestinal crypt, possibly reflecting reduction in trophic factors that maintain undifferentiated state. These data are in line with analysis of tissue histology where increased number of ChgA positive enteroendocrine and Lysozyme positive Paneth cells in the old intestine was detected (Data Figure 1). Besides, decreased Wnt combined with reduced Notch activity drives differentiation to Enteroendocrine and Goblet cell lineages further supporting the low Wnt state of old ISCs and Notum as a possible driver of the old phenotype (Basak, Beumer et al. 2017).



Data Figure 5 | Expression analysis of lineage specific marker genes and transcription factors

a, qPCR analysis of isolated ISCs and progenitor cells for lineage markers. Expression is represented as fold change to Lgr5^{hi} population in young. Statistical test of results is done against corresponding population in young. **b**, qPCR analysis of four transcription factors regulating lineage specification in old ISCs. In all, Data is represented as Log2 Fold Change +/- s.d. compared to young ISCs, n = 4-7 animals analyzed. Student's two-tailed t-test, * $P < 0.05$, ** $P < 0.01$, n.s. = not significant

Notum function in mammalian stem cell compartments had not been studied. It was originally shown to inhibit Wnt signaling by genetic analyses in *D.melanogaster* (Gerlitz and Basler 2002, Giraldez, Copley et al. 2002). Genetic ablation of Notum did prevent Wnt-inactivation and correct regeneration in planaria, indicating its role in the regeneration process (Petersen and Reddien 2011). Biologically active Notum can be produced and is available from commercial vendors. To test if Notum produced by Paneth cells could influence ISCs, that are known to rely on Wnt ligands (Pinto, Gregorieff et al. 2003), ISCs were isolated and immediately submerged into media containing recombinant Notum. Their ability to form colonies in Paneth cells and Wnt-ligand free culture was decreased significantly if recombinant Notum was present in the media (I, Figure 2d). Wnt3 produced by the Paneth cells is known to bind ISC surface forming a reservoir of Wnt ligands that eventually are diluted when cells divide and are moved away from the Paneth cell niche (Farin, Jordens et al. 2016). Treatment of isolated ISCs with Notum can deactivate these ligands and hinder their capacity to form colonies in vitro. Interestingly ISCs from old animals showed reduced ability to form colonies and their function was unaffected by addition of Notum (I, Figure 2d). Together these results show that membrane bound Wnt ligands are functional reservoir for stem cells, and that ISCs in old animals are experiencing lower Wnt activity possibly due to increased production of Notum from old Paneth cells (I, Figure 2a).

Interestingly, recent evidence suggests, that not all Wnts depend on the acylation equally for proper signaling capacity (Speer, Sommer et al. 2019). Thus, Notum might deactivate certain Wnt ligands completely while affecting others less. Plethora of Wnt ligands are produced from the stroma of the intestine (Gregorieff, Pinto et al. 2005, Farin, Van Es et al. 2012, Shoshkes-Carmel, Wang et al. 2018). Whether Notum production from the Paneth cells can deactivate all of them is not known. While similar local regulatory mechanisms may exist around the Wnt dependent cells of other tissues, the liver produced Notum could regulate stem cells of distant peripheral tissues (Seldin, Koplev et al. 2018). Yet, Notum produced by the DCS may regulate ISC function in the proximal colon (Data Figure 4a). How much this contributes to the reduced regenerative capacity of the colonic epithelium should be addressed (I, Figure 1a). Local production of Notum provides a powerful regulatory mechanism for Wnt-activity of ISC. Therefore, control over the expression or activity of Notum would enable targeted control on ISC behavior.

3. Metabolic changes in aged Paneth cells (I and Supplemental data)

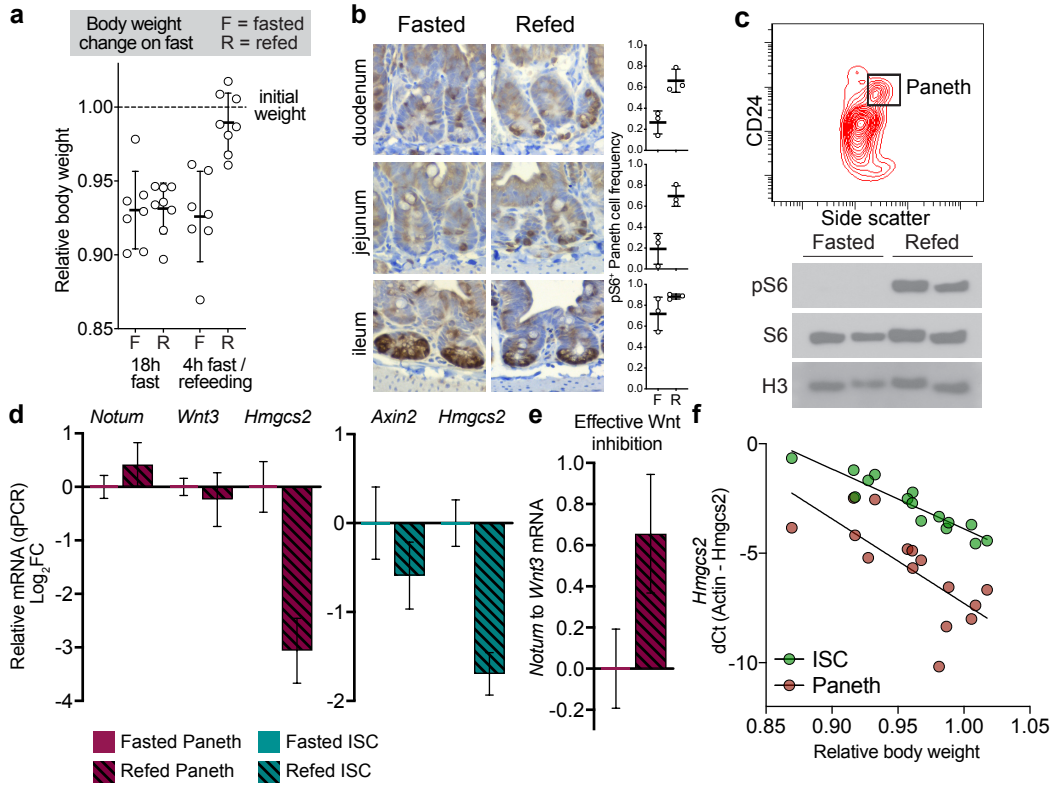
Notum expression increases when canonical Wnt signaling is activated (Kakugawa, Langton et al. 2015, Canal, Charawi et al. 2016). This places it in to the negative feedback loop of Wnt signaling cascade. However, transcriptional profiling showed no clear indication that Paneth cells in old animals would experience higher Wnt-activity than young animals (I, Supplementary Table 1). Gene set enrichment analysis (GSEA) of the Hallmark gene sets suggested increased mTORC1 activity in old Paneth cells (I, Extended Data Fig. 4a). Upon activation of mTORC1 complex, S6K1 is phosphorylated and activated by mTORC1 resulting to enhanced translation (Saxton and Sabatini 2017). Activated pS6K1 subsequently phosphorylates ribosomal S6 protein (Saxton and Sabatini 2017). Using antibodies against pS6K and pS6 mTORC1 activity in tissue sections and in isolated crypts and Paneth cells was studied in old animals. Immunostainings of tissue sections revealed, that mTORC1 activity in the crypt is highest in Paneth cells regardless of the age of animals (I, Extended Data Fig. 4b,c). This supports findings of Paneth cells as nutrient sensors in the intestinal crypt (Yilmaz, Katajisto et al. 2012). Analysis of crypt preparations showed that mTORC1 activity was increased in the old niche (I, Extended Data Fig. 4e). To test if Paneth cells and ISCs both show increase in mTORC1 activity, given cell types were isolated and their mTOR-status studied independently. Old Paneth cells showed increased mTORC1 activity based on the pS6 levels but ISCs from young and old animals seemed to have comparable activity (I, Figure 2e, Extended Data Fig. 4d,f,g). Paneth cell specific mTORC1 hyperactivity in old animals is interesting, as it was recently shown that the stem cell supporting function of Paneth cell is improved under caloric restriction due to reduced mTORC1 activity (Yilmaz, Katajisto et al. 2012). The results here contradict recent report where old animals show reduced mTORC1 activity in old ISCs (Igarashi, Miura et al. 2019). Differences might reflect variability in the animal husbandry between facilities, which may affect diet and microbiome. More-

over, aging kinetics are different between substrains of C57BL/6 mice. The mice used by Igarashi et al, were a mixture from NIA aging colony (C57BL/6N), Jackson Laboratories Lgr5-EGFP-IRES-CreERT2 (C57BL/6J) and Japan SLC Inc. (strain not defined), compared to Jackson Laboratories (C57BL/6J) used in this study. C57BL6/J mice have reported to have longer maximal lifespan than C57BL/6N, suggesting that 24-month-old animals might reflect different timepoints of life (Turturro, Witt et al. 1999, Yuan, Meng et al. 2012). Moreover, antibody for pS6 used to evaluate mTORC1 activity in ISCs detects different phosphorylation site than the antibody used in this study (Ser235/236 and Ser240/244 respectively). While phosphorylation of S6 occurs in ordered manner, Ser235/236 site is phosphorylated before Ser240/244, the latter might represent more stable and persistent mTORC1 activity (Martin-Perez and Thomas 1983, Wettenhall, Erikson et al. 1992). Regardless, transcriptional analysis of old ISCs by Igarashi et al. also showed reduced expression of Wnt target genes Axin2, cMyc and CD44 supporting findings in this dissertation (Igarashi, Miura et al. 2019)(I, Figure 2c).

Under caloric restriction low mTORC1 leads to increased production of Bst-1, ectoenzyme converting NAD^+ to cADPR, influencing ISCs self-renewal capacity (Yilmaz, Katajisto et al. 2012). Modest reduction in Bst-1 was observed in old Paneth cells, but Bst-1 KO did not recapitulate aging phenotype in vitro (I, Extended Data Fig. 2d,e). However, inhibition of mTORC1 activity in old animal with rapamycin improved regenerative function of aged epithelium by improving niche function of Paneth cells and colony forming capacity of old ISCs (I, Extended Data Fig.5b-h). Surprisingly, rapamycin treatment altered expression of multiple stromal Wnt-ligands in old animals, foremost elevating expression of Wnt4 (I, Extended Data Fig.5i). Analysis of crypt preparations revealed increased Notum expression after rapamycin treatment (I, Extended Data Fig.5i,j). These data corroborate previous reports where Wnt4 is shown to be required for Notum expression and that rapamycin treatment increases Paneth cell numbers (Yilmaz, Katajisto et al. 2012, Naillat, Yan et al. 2015). They also reflect, that systemic treatment with rapamycin makes interpretation of response to mTORC1 inhibition in one cell type difficult. To overcome these limitations, mTORC1 activity was induced specifically in the epithelium by inactivating Tuberous Sclerosis Complex 1 (TSC1) with Villin-Cre mediated deletion (Kwiatkowski, Zhang et al. 2002, el Marjou, Janssen et al. 2004). This led to increased mTORC1 activity and Notum production in Paneth cells and reduced regenerative capacity of the epithelium (I, Extended Data Fig. 5k-m).

Together these data indicate, that mTORC1 activity can drive Notum expression in the intestinal epithelium. Moreover, as mTORC1 activity is critical for Paneth, but not enteroendocrine cell function, it likely participates in the separation of cellular identities between the two (Sampson, Davis et al. 2016). Thus, it can explain some of the Paneth cell specificity in the Notum expression (I, Figure 2b, Data Figure 4).

As mTORC1 does not directly regulate transcription, additional downstream pathways were screened. GSEA analysis suggested that Ppar-pathway was downregulated in old Paneth cells (I, Extended Data Fig. 6a). mTORC1 activity can inhibit Ppara via S6K1 and NCoR1 as shown in aging liver (Sengupta, Peterson et al. 2010). To test if similar relationship between mTORC1 and Ppara exists in intestinal epithelium, young mice were fasted overnight to prevent caloric intake and reduce mTORC1 activity in Paneth cells. After fasting, group of mice were refed and analyzed for mTORC1 activity. In four hours, refed mice almost regained the initial body weight (Data Figure 6a). Analysis of the tissue sections showed increased amount of pS6+ Paneth cells in the refed mice. Interestingly, Paneth cells in the ileum retained their pS6 positivity under fasted state (Data Figure 6b), possibly reflecting incomplete emptying of the GI tract (data not shown). For subsequent analysis, Paneth cells were isolated from jejunal region yielding striking difference in mTORC1 activity between the fasted and refed state (Data Figure 6c). Consistently, this coincided with decreased expression of Ppara target gene Hmgcs2 (Data Figure 6d)(Hsu, Savas et al. 2001). Simultaneously, expression of Wnt3, the main Wnt ligand made by Paneth cells (Farin, Van Es et al. 2012) showed modest decrease while expression of Notum slightly increased (Data Figure 6d). The balance between Notum and Wnt3 production seemed to follow mTORC1 activity (Data Figure 6e) while Wnt target gene Axin2 decreased in the neighboring ISCs in some cases (Data Figure 6d). Moreover, Hmgcs2 expression and the endpoint bodyweight correlated well (Data Figure 6f), indicating that within this experimental window, the amount of ingested food translates directly to mTORC1-Ppara activity in intestinal epithelium. This response was strongest in the Paneth cells further validating their status as



Data Figure 6 | Paneth cells respond to food

a, Body weight of animals after 18 h fast and additional 4 h of fasting (F) or refeeding (R). Weight presented in relation to body weight before fasting. n = 7 fasted and 8 refed animals, mean +/- s.d. **b**, Left: Representative images of histological sections of crypts stained with pS6 (S240/244) antibody (brown). Countestained nuclei (blue). Right: quantification of pS6+ Paneth cell frequency from different regions of the small intestine. Mean +/- s.d., n = 3 animals per group. **c**, Representative FACS gating strategy to sort out Paneth cells (CD24hiSSChi) from jejunum. Immunoblot of sorted jejunal Paneth cells. Leveles of phopsorylated S6 (pS6), total S6 (S6) and Histone 3 (H3) analysed, n = 2 animals per group. **d**, qPCR analysis of Notum, Wnt3, Hmgcs2 and Axin2 from sorted jejunal Paneth and ISCs. In all, Data is represented as Log2 Fold Change +/- s.e.m. compared to fasted state, n = 7-8 animals analyzed. **e**, Expression of Notum compared to Wnt3 in the corresponding samples, mean +/- s.e.m. **f**, Correlation of relative body weight after fasting or refeeding to expression of Hmgcs2 in Paneth (red) and ISCs (green). Student's two-tailed t-test, * $P < 0.05$, ** $P < 0.01$, n.s. = not significant

nutrient sensors (Data Figure 6f). These results support the model, where high mTORC1 activity inhibits Ppara in old Paneth cells. Moreover, they show that nutrient status might regulate Wnt on-off switch in the epithelial stem cell niche.

To directly target Ppar-pathway, organoid cultures were exposed to GW6471, an inhibitor of Ppara (Xu, Stanley et al. 2002). Strikingly, inhibition of Ppara in young organoids mimicked the observed aging phenotypes. Formation of *de novo* crypt domains in organoids was reduced, ISC to Paneth cell ratio decreased and Notum expression was elevated (I, Figure 2f,g, Extended Data Fig. 6c). Moreover, addition of excess Wnt-ligands was able to revert seen regeneration defects (I, Figure 2g, Extended Data Fig. 6d). These findings indicate, that high mTORC1 activity can repress Ppara signaling in old Paneth cells leading to increased Notum expression.

Paneth cells respond to nutrient status promptly (Data Figure 6)(Yilmaz, Katajisto et al. 2012). Therefore, elevated mTORC1 activity in old Paneth cells propose persistent activators of mTORC1 in the systemic

circulation. While not experimentally tested, old mice were heavier suggesting increased adiposity and elevated base line for systemic glucose (Khawandanah 2019)(I, Extended Data Fig. 4h). Moreover, attenuated control in glucose metabolism leading to elevated circulatory glucose has been reported among older humans (Chia, Egan et al. 2018). Increased blood glucose and or insulin levels could lead to increased mTORC1 activity in old Paneth cells (Saxton and Sabatini 2017). Consequently, these results underline, that lifestyle choices inhibiting mTORC1 activity or promoting FAO, such as diet and exercise, can increase the regenerative capacity and health of intestinal epithelium (Campisi, Kapahi et al. 2019).

4. Regenerative therapies for aged intestine (I)

Cancer incidence increases with age and with the increasing median life expectancy, the number of aged patients in need for chemotherapeutic treatment are on the rise (WHO 2019). Chemotherapy or focused irradiation destroys proliferative cancer cells, but also targets actively cycling ISCs (Metcalfe, Kljavin et al. 2014). The reduced regenerative capacity of the old epithelium (I, Figure 1a-d, Extended Data Figure 9a) explains the challenge that clinicians face in treatment of older patients (Chang, Goldstein et al. 2017). As an enzyme, Notum is a potential target to increase the regenerative capacity of healthy tissue. Inhibitors for Notum have been developed but little characterized for their *in vivo* efficiency (Han, Pabba et al. 2016, Suciu, Cognetta et al. 2018). To investigate, if one of these inhibitors, named ABC99 (Suciu, Cognetta et al. 2018), would function against Paneth cell produced Notum in vivo, different aged mice were treated daily for one week before analysis (I, Extended Data Fig. 8b). The activity of ISCs was evaluated based on their Wnt-activity and proliferative capacity. ISCs in the old tissue showed reduced amount of nuclear β-catenin, indicating lowered Wnt activity (I, Figure 3d). ABC99 treatment resulted in enhanced Wnt activity in old ISCs. Moreover, ISCs from treated old mice had proportionally increased proliferation and enhanced colony forming capacity (I, Figure 3c,d,e). To examine, whether treatment with ABC99 could protect old intestine from damage similar to side effects from chemotherapy, 5-FU was administered to treated mice (I, Extended Data Fig. 9b). While old mice showed significantly reduced capacity to regain weight after damage, ABC99 treatment was able to alleviate this defect. (I, Figure 3f, Extended Data Fig. 9b). Moreover, the analysis of the tissue revealed enhanced production of new epithelium in ABC99 treated old mice, indicating that quicker recovery was due to enhanced functionality of the epithelium (I, Figure 3g). Taken together, results above demonstrate that pharmacological inhibition of Notum could be used as a chemoprotective treatment.

Wnt signaling promotes cell proliferation and survival, both needed for regenerative response of ISCs (Nusse and Clevers 2017). The declined Wnt in old ISCs (I, Figure 2c, 3d Extended Data Fig. 2f) underlines the reduction in reparative abilities of old intestinal epithelium. Therefore, enhancing Wnt activity in old epithelium could be considered as protective treatment. However, too much of Wnt leads to hyperproliferation and preneoplastic growth of the intestinal epithelium (Kim, Kakitani et al. 2005), increasing the pool of tumor initiating ISCs (Barker, Ridgway et al. 2009). In this context, Wnt inhibitor Notum expressed only in post-mitotic Paneth cells and potentially affecting Wnt activity in a local microenvironment, is suitable target for controlled Wnt activation. Moreover, restoring Wnt activity in the old ISC niche, could reduce tumor incidence, by reducing chances of mutated stem cell clones to persist and initiate a tumor (Huels, Bruens et al. 2018).

5. Cellular shape is required for efficient niche-to-stem cell signaling (II)

Many adult stem cell populations reside in niches that possess curved topology (Clevers 2013, Solanas and Benitah 2013, Nowell and Radtke 2017). Intestinal crypts are a good example of a niche with high curvature where ISCs apical surface is minimal creating a conical shape (II, Figure 1a). Niche curvature in the intestine has been considered to protect ISCs from luminal environment and could be just a passive outcome of the tissue organization (Clevers 2013). Whether the shape plays any significant role in signaling between ISCs and their niche is not known. Interestingly, if epithelium is isolated and cultured in homogenous 3D matrix, forming organoids adopt shapes resembling the native tissue where ISCs retain

their conical shape (II, Figure 1b). This demonstrates, that epithelium has intrinsic capacity to maintain cellular shapes present in the tissue. To investigate the role of crypt cells in this process, activity of NM II was evaluated based on phosphorylation status of the Myosin regulatory light chain (pMRLC). ISCs at the base of both *in vivo* crypts and *in vitro* crypt domains demonstrated pMRLC localization in the apical side, which was lacking from the neighboring Paneth cells (II, Figure 1c), suggesting generation of constrictive force by the ISCs. Furthermore, if organoids are cultured under conditions where only ISCs are present crypt width is significantly lower than organoids enriched for Paneth cells (Yin, Farin et al. 2014) (II, Figure 1d, Data Figure 4b). Together these data imply, that ISCs have intrinsic capacity to acquired conical shape resulting in bending of the surrounding epithelium. Moreover, it explains some aspects of the self-organizing capacity of *ex vivo* organoid cultures where crypt domains containing ISCs form and mimic the native tissue morphology (Sato, van Es et al. 2011).

Actively acquired conical shape proposes that cellular shape might improve function of these cells. Conical shape in epithelial tissues can be achieved by apical constrictions, phenomena where apically located actomyosin bundles are contracting and reducing the apical area of polarized cells (Vicente-Manzanares, Ma et al. 2009)(II, Figure 1c). To prevent curved niche to form, organoids were treated with two inhibitors against actomyosin contractility, Rho-associated kinase inhibitor Y-27632 and NM II inhibitor Blebbistatin. Concentrations that did not affect proliferation of organoids were used (II, Supplementary Figure 1c). Treatment with both inhibitors hindered crypt formation and reduced the curvature of formed crypt domains (II, Figure 1e-g). Immunofluorescent staining with Lysozyme indicated that treated organoids had a large number of Paneth cells suggesting that ISCs were not lost (II, Figure 1e). However, when organoids were analyzed for their cellular composition with flow cytometry, treatments with both inhibitors reduced the ISC to Paneth cell ratio (II, Figure 1h). To test if observed reduction manifests as functional impairment of ISCs, organoids were allowed to form crypt domains before treatment was initiated. After treatment, crypt domains were larger and particularly ISC shape was altered (II, Figure 1i,j, Supplementary Figure 1d). Organoids were mechanically dissociated to single crypt domains and these were re-plated without inhibitors. Treatments reduced regenerative capacity of crypt domains, assessed by quantifying *de novo* crypt domains two days later (II, Figure 1k). Together these data propose that apical constriction in ISCs is required for the maintenance of regenerative output in crypts.

Altering cells capability to contract affects it's mechanosensing properties (Aragona, Panciera et al. 2013, Totaro, Panciera et al. 2018). As mechanosensing YAP pathway has been shown to regulate ISC function by promoting Notch and Wnt-pathway activation it is possible that inhibitors of NM II contractility can affect ISCs YAP status (Totaro, Panciera et al. 2018). Nuclear localization of the YAP protein can be used as a proxy of the pathway activity (Totaro, Panciera et al. 2018). Analysis of control organoids indicated, that YAP localization in nucleus is greater in ISCs than Paneth cells (II, Supplementary Figure 1e) as reported previously (Gregorieff, Liu et al. 2015). Treatments with Y-27632 and Blebbistatin reduced YAP's nuclear localization comparable to treatment with 10 nM Verteporfin, inhibitor of YAP activity (II, Supplementary Figure 1f). Interestingly, ISCs and Paneth cells seem to respond differently to Y-27632, as its effect on YAP localization was significantly milder in ISCs (II, Supplementary Figure 1f). Wnt activity strengthens YAP signaling by alleviating sequestration of YAP by the β-catenin destruction complex (Figure 3)(Totaro, Panciera et al. 2018). Moreover, Y-27632 has been reported to increase canonical Wnt activity in intestinal cancer cells (Rodrigues, Macaya et al. 2014). Furthermore, as inhibition of ROCK promotes actin depolymerizing ability of Cofilin, reduced F-actin pool in Y-27632 treated cells could alleviate AMOT mediated YAP sequestration (Vicente-Manzanares, Ma et al. 2009, Totaro, Panciera et al. 2018). Together these might explain the differences between Y-27632 and Blebbistatin. However, treatment of organoids with 10 nM Verteporfin did not change the shape nor affected the regenerative capacity of crypt domains (II, Supplementary Figure 1g). Jointly these data suggest that observed effects of the Y-27632 and Blebbistatin could not be solely explained by reduction in YAP pathway activity.

To remove possible side-effects of used inhibitors, artificial scaffolds to culture epithelial cells on alternative curvatures were designed (II, Figure 2a). Initial design mimicked the native crypt width (50 μm) and curvature (II, Supplementary Figure 2a). When isolated Lgr5+ ISCs were cultured on these scaffolds under conditions that intrinsically activated Wnt and Notch -pathways, followed by a release period in media

enabling differentiation, ISCs were maintained at the bottom of curved pits (II, Figure 2b,c). When similar experiment was performed on scaffolds with larger crypt diameter, reduction in stemness, as indicated by the decreased Lgr5-EGFP at the crypt bottom and attenuated ability to maintain pits populated, was observed (II, Figure 2d,e). Strikingly, when epithelium grown on scaffolds was treated with the inhibitors of NM II activity, its renewal capacity was not affected, as indicated the resulting area of new epithelium (II, Figure 2f). This data can explain the rather contradicting report of RhoA inhibition increasing tumor formation in the mouse small intestine (Rodrigues, Macaya et al. 2014). The surrounding stroma and ECM scaffold are likely able to maintain ISC shape even in the absence of RhoA activity. Therefore, the number of tumor-initiating ISCs are not reduced and other mechanisms, such as intrinsic activation of Wnt pathway can promote tumor formation (Rodrigues, Macaya et al. 2014). Together these data indicate, that ISCs require curved topology for their function. Moreover, inhibition of NM II reduces the regenerative capacity of intestinal epithelium only if morphology of the crypt domain is altered, suggesting that the cellular shape participates in the maintenance of stemness and regenerative capacity of ISCs.

Conically shaped ISCs are also smallest cells at the cypt base (II, Supplementary Figure 3a). Small size has been reported to be a feature of other adult stem cells as well (Li, Rycaj et al. 2015). Stem cells lacking the specialized duties of differentiated cells logically require less complex cellular machinery and thus are functional with smaller size. On the other hand, small volume could serve signal receiving stem cells, as it increases surface-to-volume ratio. Epithelial cells that rely on signals emanating from their neighbors, such as ISCs depending on Paneth cells, could further increase the receiving surface-to-volume ratio by reducing the area of surfaces with less function (II, Figure 3a). In the ISCs apical surface has no known functions, rendering it dispensable.

To investigate, whether surface-to-volume ratio regulates ISCs capacity to regenerate, different sized subpopulations of ISCs were isolated with FACS (II, Figure 3b,c). These cells have equal Lgr5 expression, based on the EGFP reporter, and comparable capacity to form clonal colonies regardless for enrichment of cell cycle state G1 in the small ISC (S-ISC) (II, Figure 3b,d, Supplementary Figure 3b). Remarkably, when the function was tested in co-cultures with Paneth cells, large ISCs (L-ISC) formed fewer organoids than S-ISCs (II, Figure 3d). This suggests, that small size, and consequently large surface-to-volume ratio, is beneficial in situations where signaling platform between neighboring signal producing cells is formed. In this context, small volume could help to intensify signals from Paneth cell derived Wnt- and Notch-ligands. One example of such behavior is reported in experiments where Notch-signal intensity was studied in the context of surface area (Shaya, Binshtok et al. 2017). Sheya et al. showed that large contact area increased Notch response in receiving cells. Moreover, experiments where cellular volume has been regulated by artificial 3D cultures have indicated that low volume increases transcriptional output of cells (Bao, Xie et al. 2017). These data highlight that cellular size and shape can modulate cell behavior and capacity to communicate with the environment. Features that are critical for stem cells.

Interestingly, the morphology of intestinal crypts is reported to change with age (Martin, Kirkwood et al. 1998). To accurately measure *in vivo* crypt curvature, freshly resected intestinal segments were imaged and the largest diameter measured (II, Figure 4a). Old crypts exhibited larger size throughout the small intestine which is in line with previous reports (Martin, Kirkwood et al. 1998, Nalapareddy, Nattamai et al. 2017). Moreover, analysis of cell size indicated increased volume in old ISCs, while no difference was observed between other cell populations (II, Figure 4b). To understand the mechanisms behind differences in the cell shape and size, transcriptional profiling of young and old ISCs (I) was extended to contain more differentiated TA cells (Lgr5lo) (II, Figure 4c). Two-dimensional principal component suggested that the four populations analyzed (young and old ISCs and TA cells), were best separated by an axis of differentiation (PC1 67%), followed by an axis of age (PC2 11%) (II, Figure 4c). Comparison of young ISCs and TA cells indicated contractility as a one key feature enriched in ISCs (II, Figure 4d). This data further supports the intrinsic tendency of ISCs to acquire conical shape. Moreover, it suggested that evaluation of the gene expression changes between old and young could enlighten the mechanisms behind altered crypt morphology and cell size in old animals. Interestingly, Myosin heavy chain 14 (Myh14), was among the differentially expressed genes between old and young ISCs (II, Supplementary Table 1). Myh14 codes for the heavy chain of NM IIC isoform, capable of forming actomyosin cables with F-ac-

tin and NM IIA, the main NM II isoform in intestinal epithelium (Vicente-Manzanares, Ma et al. 2009, Sumigray, Terwilliger et al. 2018). Expression analysis for the two NM II isoforms in ISCs, indicated that both Myh14 and Myh9 were downregulated in old ISCs (II, Figure 4e). These data imply, that old ISCs reside in a low curvature niche resulting to reduced signaling platform with the neighboring Paneth cells. Moreover, reduced expression of the two major components in actomyosin contractility, might reduce the capacity for apical constriction in old ISCs. Parallel increase in cell size propose a link between cell shape and size.

If the low curvature of old niche is partly behind the reduced ISC function, narrow curvature should augment the growth of old epithelium. To investigate this, epithelium from old animals was cultured on scaffolds with 50 μm pits, resembling young tissue topology. Remarkably, the growth of old epithelium was enhanced, while the young epithelium grew less on scaffolds with large 100 μm pits(II, Figure 4f). The capacity of old epithelium did not fully reach the youthful level, indicating that other epithelium intrinsic factors, such as low Wnt, potentially reduce growth regardless the topology (I). These results imply, that signaling between the ISCs and Paneth cells could be improved by correcting the topology of old tissues.

Together these data demonstrate that tissue and cell shape are critical regulators of stem cell identity in the intestinal epithelium and play a role in tissue maintenance in the physiological context of aging. Interestingly, decreased lateral surface-to-volume ratio can reduce input from any signaling cascade originating from the cell membrane. As low activity in Wnt-, Notch- and MAPK-pathways in ISCs directs them to enteroendocrine lineage (Basak, Beumer et al. 2017), reduced niche morphology can partly explain the increased number of enteroendocrine cells in old tissues (Data Figure 1) and the presence of enteroendocrine precursor markers in ISCs (Data Figure 5). Therefore, the topology of the niche could also affect lineage decisions of differentiating intestinal epithelium.

Whether cellular shape regulates cell-to-cell communication in other stem cell niches remains under investigation. Interestingly, tissue topology has been reported to compartmentalize and regulate the stem cell functions in adult fish gill (Stolper, Ambrosio et al. 2019). These observations from distantly related tissues and taxa among the chordata suggest that topology as a regulator of cellular communication might be a feature utilized over and over again in biology.

Conclusions and future perspectives

Findings of this dissertation highlight the importance of the niche in maintenance of stem cell function. Age-related differentiation bias towards secretory cell fate in the intestinal epithelium indicates changes in the niche derived signals (Data Figure 1,5). Paneth cell produced Notum in combination with altered niche topology in old animals could decrease the signal intensity of Wnt, Notch and MAPK pathways and underline the increased production of Enteroendocrine cells (I,II, Data Figure 1,5)(Basak, Beumer et al. 2017). These findings support other published data on the aging intestine where reduction in Wnt activity and increased number of secretory cells were observed (Nalapareddy, Nattamai et al. 2017, Igarashi, Miura et al. 2019). The consequence of more abundant secretory cells for epithelial function remains to be investigated in the future.

When old epithelium is injured, it fails to regenerate as efficiently as young. This was demonstrated to be due to stem cell intrinsic and extrinsic factors (I,II). The role of Paneth cells and niche topology in this process emphasize the sensitivity in communication between ISCs and their surroundings. Alterations in the mTOR-pathway activity were shown to drive the aged phenotype in Paneth cells (I, Data Figure 6). However, the underlining cause for such activity requires more investigation. Whether systemic factors and stromal cells contribute to altered behavior of differentiated cells in the old intestinal epithelium needs to be carefully dissected. Improving ISCs regenerative function might not rejuvenate old Paneth cells, if non-epithelial trophic factors are driving mTORC1 activity. Interestingly, circulatory glucose levels are increased among older individuals (Chia, Egan et al. 2018), possibly factoring to elevated mTORC1 activity in old Paneth cells. Moreover, the resulting increase in incretin producing enteroendocrine cells could reflect an attempt from the body to increase glucose absorption in peripheral tissues. Further research is needed to identify the driver factors that enable aging process in the intestine.

Furthermore, these results provide proof of principle for niche targeted therapeutics, that could be safe alternatives for treatments that directly modulate stem cell behavior. In the future, harmful side effects on elderly patients undergoing chemotherapy could be alleviated by targeting Paneth cell derived Notum with pharmacological inhibitors such as ABC99 (I)(Suciu, Cognetta et al. 2018). Moreover, as nutrient sensing mTORC1 regulates some aspects of Notum expression, lifestyle interventions could alleviate age related gastrointestinal problems (I, Data Figure 6). To what extent Notum and other Wnt inhibitors partake in age-related decline of other tissue resident stem cells remains under investigation. Recently, Wnt inhibitor Sfrp5 has been suggested to promote quiescence in aged neural stem cells (Kalamakis, Brune et al. 2019), proposing that deregulated Wnt inhibition is more universal effector of compromised tissue renewal in aging.

Finally, the finding that altered ISC shape results to decreased signaling capacity between the niche and stem cells emphasizes the importance of the architecture for proper tissue function (II). This work opens new avenues, as similar mechanisms for effective signaling may also be important in other stem cell niches. Therefore, targeting cytoskeletal contractility and tension might provide novel ways to improve tissue repair. Moreover, the impact of cellular shape highlights that improved cell culture platforms may be generated by mimicking the native tissue topology. Meeting the need of biotechnology for the long-term ex vivo tissue cultures could improve drug development and personalized medicine.

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Pursuing a PhD is like a hike through the wilderness. In the beginning you set a goal and choose a trail. The trail is unfamiliar and contains variable terrain, with easier and more demanding sections. You never know what lies behind the next turn or a hilltop. It can be a beautiful and breath-taking scenery where you decide to stop and have a rest. However, quite often it is just another marsh swarming mosquitoes, or even a tougher ascent. Nice bits are easy, but it is also possible to find a peaceful mindset when scrambling up on the steep rocky face of the fell. Just remember that by moving you're (likely) getting closer to your goal and by doing that you continuously gain something valuable. Remembering this is not always so easy, but easier when you are surrounded by an awesome and versatile bunch of people who I want to acknowledge here. Without them, I would have not found the end of this trail.

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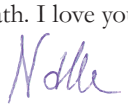

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