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Rôles de l'autophagie dans l'homéostasie des cellules souches intestinales

Coralie Trentesaux

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Rôles de l'autophagie dans l'homéostasie des cellules souches intestinales

Thèse de doctorat de l'Université Paris-Saclay
préparée à L'Université Paris Sud

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Spécialité de doctorat: aspects moléculaires et cellulaires de la biologie

Thèse présentée et soutenue à Paris, le 23 octobre 2018, par

Coralie TRENTESAUX

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Role of Autophagy in Intestinal Stem Cell Homeostasis

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of the Université Paris-Saclay
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Thesis presented and defended in Paris, on the 23rd of october 2018, by

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“A painting is not thought out in advance. While it is being done, it changes as one’s thoughts change. And when it’s finished, it goes on changing, according to the state of mind of whoever is looking at it” –Pablo Picasso on *Guernica*



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2. *Book chapter* : Trentesaux and Romagnolo. (2018). Intestinal stem cells and their defining niche.

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ABBREVIATIONS

+4SC	+4 label-retaining stem cells
α SMA	α smooth muscle actin
β -TrCP	β -transducin repeats-containing proteins
4E-BP1	Inhibitor of the eukaryotic initiations factor 4E binding protein 1
4OHT	4-hydroxy-tamoxifen
5-FU	5-fluorouracil
53BP1	Tumor protein p53 binding protein 1
ALPI	Intestinal alkaline phosphatase
AMP	Antimicrobial peptide
AMPK	AMP-activated protein kinase
AOM	Azoxymethane
APC	Adenomatous polyposis coli
ASCL2	Achaete-scute homologue 2
ATB	Antibiotics
ATF6	Activating transcription factor 6
ATG	Autophagy-related gene
ATM	Ataxia telangiectasia mutated
ATM	Ataxia telangiectasia mutated
ATOH1	Atonal BHLH transcription factor 1, Math1 in mice
BAD	Bcl2 antagonist of cell death
BCL-XL	B cell lymphoma extra large
BER	Base-excision repair
BID	BH3-interacting domain death agonist
BiP	Binding immunoglobulin protein
BMP	bone morphogenic protein
BMPR1	type 1 BMP receptor
BMPR2	type 2 BMP receptor
BNIP3	Bcl2 interacting protein 3
BNIP3L	BCL2 interacting protein 3 like, also called NIX
CaMKII	Calcium calmodulin kinase II
CBC	Crypt basal columnar cells
CHK	Checkpoint kinase
CHOP	c/EBP homologous protein
CIMP	CpG island methylation phenotype
CIN	Chromosome instability
CK1 α	Casein kinase 1 α
CMA	Chaperone-mediated autophagy
CMS	Consensus molecular subtypes
CQ	Chloroquine
CRC	Colorectal cancer
CRCS	Colorectal cancer subtyping consortium
DAMP	Danger-associated molecular pattern
DAPK1	Death-associated protein kinase 1
DCAMKL1	Doublecortin like kinase 1

DCS	Deep crypt secretory
DDB2	DNA damage-binding protein 2
DDR	DNA damage repair
DEPTOR	DEP-domain containing mTOR-interacting protein
DKK	Dickkopf
DLL	Delta-like
dMMR	Deficient MMR
DNA-PK	DNA-dependent protein kinase
DRAM	Damage-regulated autophagy modulator
DSB	Double-stranded break
DSS	Dextran Sulfate Sodiun
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
DUOX2	Dual oxidase 2
ECM	Extra-cellular matrix
EGF	Epidermal growth factor
EGFR	EGF receptor
eIF2 α	Eukaryotic translation initiation factor 2 α
eIF4e	Inhibitor of the eukaryotic intiations factor 4E
ER	endoplasmic reticulum
ERO1 α	ER oxidoreductase 1 α
ESCRT	Endosomal sorting complex required for transport
FAP	Familial adenomatous polyposis
FIP200	Focal adhesion kinase family-interacting protein of 200 kDa
FOXL1	Forkhead box L1, also known as Fkh6
FOXO	Forkhead box family, class O
FZD	Frizzled
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GALT	Gut-associated lymphoid tissue
GAP	GTPase-activating protein
GSEA	Gene set enrichment analysis
GSK-3 β	Glycogen synthase 3 β
HCQ	Hydroxychloroquine
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
HIF1	Hypoxia-inducible factor 1
HMGB1	High mobility group box 1, a chromatin-associated protein
HNPCC	Hereditary non-polyposis colon cancer, or Lynch syndrome
HOPX	Homeodomain-only protein
HP1 α	Heterochromatin protein 1 α
HR	Homologous recombination
HSC	Hematopoietic stem cell
HSC70	Heat shock cognate 70 kDa protein
IBD	Inflammatory bowel disease
IFN	Interferon
IgA	Immunoglobulin A
IHH	Indian Hedgehog

IL	Interleukin
ILC	Innate lymphoid cells
IMM	Inner mitochondrial membrane
IPA	Ingenuity pathway analysis
IR	Ionizing radiation
IRE1 α	Inositol-requiring enzyme 1 α
IRGM	Interferon-inducible immunity-related GTPase family M member 1
ISC	Intestinal stem cells
JNK	c-Jun N-terminal kinases
KAP1	Krüppel associated box associated protein 1
KEAP1	Kelch-like ECH-associated protein 1
LAMP2	Lysosome-associated membrane protein 2
LC3	Microtubule-associated protein 1 light chain 3
LEF	Lymphoid enhancer factor
LGR5	Leucine-rich repeat-containing G-protein-coupled transmembrane receptor 5
LIG4	Ligase 4
LIR	LC3 interacting region
LPR	Lipoprotein-related protein
LPS	Lipopolysaccharide
LRIG1	Leucine rich repeats and immunoglobulin like domains 1
LRRK2	Leucine-rich repeat kinase 2
LTA	Lipoteichoic acid
M cells	Microfold cells
MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen activated protein kinase
MCL1	Myeloid cell leukemia sequence 1
MDP	Muramyl dipeptide
MLH1	MutL homolog 1
mLTS8	Mammalian lethal with SEC13 protein 8
MMR	Mismatch repair
MNV	Murine norovirus
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MSI	Microsatellite instability, MSI-H or -L for High or Low MSI
MSS	Microsatellite stable
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MyD88	Myeloid differentiation primary response gene 88
MYH11	Myosin heavy chain 11
NAC	N-acetyl Cysteine
NBR1	Neighbor of BRCA1 gene 1
NCID	Notch intracellular domain
NDP52	Nuclear dot protein 52
NER	Nucleotide-excision repair
NF κ B	Nuclear factor κ B
NHEJ	Non-homologous end joining
NK	Natural killer

NLR	NOD-like receptor
NOX1	NADPH oxidase 1
NRF2	Nuclear factor erythroid 2
NSC	Neural stem cells
OLFM4	Olfactomedin 4
OMM	Outer mitochondrial membrane
p70-S6K	Ribosomal protein S6 kinase-1
PARP1	Poly (ADP-ribose) polymerase 1
PDGF	Platelet-derived growth factor
PE	Phosphatidylethanolamine
PERK	Protein kinase RNA-like ER kinase
PGC1 α	Peroxisome proliferator-activated receptor α coactivator 1 α
PI(3)P	Phosphatidylinositol 3-phosphate
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3K	Phosphoinositide 3 Kinase
PINK1	PTEN-induced kinase 1
PKR	Protein kinase R
POLD1	DNA polymerase δ
POLE	DNA polymerase ϵ
PolyIC	Polyinosinic:polycytidylic acid
PPAR γ	Peroxisome proliferator-activated receptor γ
PRAS40	Proline-rich Akt substrate of 40 kDa
PRR	Pattern recognition receptor
PTCH	Patched
PTEN	Phosphatase and tensin homolog
PTPN2	Protein tyrosine phosphatase, non-receptor type 2
RAB8a	Ras-related proteins in brain 8a
RAG	Ras-related GTP-binding protein
RAPTOR	Regulatory-associated protein of mTOR
REG3	Regenerating islet-derived protein 3
REG4	Regenerating islet-derived 4
RHEB	Ras homolog enriched in the brain
RNF168	Ring finger protein 168
<i>RNF3</i>	Ring finger 3
RNF43	Ring finger protein 43
RNS	Reactive nitrogen species
ROS	Reactive oxygens species
SCNA	DNA somatic copy number alterations
SHH	Sonic Hedgehog
SLC	Solute carriers
SMO	Smoothed
SMOC2	SPARC related modular calcium binding 2
SNAP	Soluble N-ethylmaleimide-sensitive factor attachment protein
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SOD	Superoxide dismutase
SQSTM1	Sequestosome1, or p62

SSA	Sessile serrated adenomas
STAT3	Signal transduce and activator of transcription 3
TA	Transit amplifying
TAX1BP1	Tax-1 binding protein 1
TCF	T cell factor
TCGA	The cancer genome atlas
TERT	Telomerase reverse transcriptase
TFEB	Transcription factor EB
TGF	Transforming growth factor
TIM	Translocase of the inner mitochondrial membrane
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOM	Translocase of the outer mitochondrial membrane
TRIF	Toll-IL-1 receptor domain-containing adapter-inducing interferon
TRMP5	Transient receptor potential cation channel subfamily M member 5
TSA	Traditional serrated adenomas
TSC	Tuberous sclerosis complex
ULK	Unc51-like autophagy activating kinase
UPR	Unfolded protein response
UVRAG	UV radiation resistance-associated gene protein
VEGFR	Vascular endothelial growth factor
VMP1	Vacuole membrane protein 1
VPS34	Vacuolar protein sorting 34
XBP1	X-box binding protein 1
XPC	Xeroderma pigmentosum, complementation group C
XRCC4	X-ray repair cross complementing 4
ZNRF3	Zinc and Ring Finger 3
ZNRF43	Zinc and ring finger 43

RESUME EN FRANÇAIS

Le renouvellement de l'épithélium intestinal repose sur la prolifération incessante de cellules souches intestinales (CSI). Le maintien de ces dernières est donc essentiel à l'homéostasie de l'épithélium intestinal mais aussi à sa régénération suite à des dommages. Ces CSI sont également considérées comme étant à l'origine de la transformation et l'initiation tumorale. L'étude des mécanismes impliqués dans la protection des CSI face à différents stress est donc essentielle pour mieux comprendre l'homéostasie et les pathologies intestinales. Notre équipe a précédemment pu démontrer, à la fois dans des échantillons de cancers colorectaux humains et dans un modèle murin prédisposé à développer des tumeurs intestinales, une induction de l'autophagie dans le tissu tumoral. L'autophagie est un mécanisme hautement conservé au cours de l'évolution et permettant la dégradation de différentes composantes cytoplasmiques par la voie des lysosomes. Ce mécanisme est impliqué dans de nombreux types de cancers ainsi que dans l'homéostasie de différentes populations de cellules souches adultes, telles que les cellules souches hématopoïétiques, les cellules souches neuronales, ou encore les cellules satellites. Grâce à des modèles murins permettant l'inactivation d'un gène clé du processus autophagique, *Atg7*, spécifiquement dans l'épithélium intestinal, l'équipe a pu démontrer un rôle clé de ce processus dans l'initiation et la croissance des tumeurs intestinales, ainsi que dans l'homéostasie microbienne de l'intestin. Suite à ces travaux, mes travaux de thèse visent à étudier le rôle de ce processus catabolique dans l'homéostasie des CSI. Pour ce faire, nous utilisons des modèles murins génétiquement modifiés et des cultures d'organoïdes afin d'étudier les effets de l'inhibition de l'autophagie dans l'homéostasie intestinale et en particulier dans les CSI.

Nos travaux indiquent que l'inhibition de l'autophagie par l'inactivation du gène *Atg7* conduit à une activation de p53 et à une apoptose spécifique des CSI. De même, les cryptes invalidées pour *Atg7* ont une survie moindre que des cryptes contrôles en culture, ce qui souligne d'avantage un défaut de cellules souches. L'inactivation simultanée du gène *Tp53* empêche la mort des CSI déficientes en autophagie. De plus, au long terme, ces souris doublement invalidées pour *Atg7* et *Tp53* développent des tumeurs, contrairement aux souris invalidées uniquement pour les gènes *Atg7* ou *Tp53*. Nous avons donc émis l'hypothèse que l'inhibition de l'autophagie sensibilisait les CSI à l'apoptose suite à une accumulation de dommages cytotoxiques.

Par une analyse d'expression génique des CSI issues de cryptes contrôles et invalidées pour le gène *Atg7*, nous avons mis en évidence une altération des réponses associées au stress oxydant et à la réparation de l'ADN. Confirmant ces signatures, nous avons observé des dommages de l'ADN dans les cryptes déficientes en autophagie et un défaut de réparation de ces dommages suite à une irradiation. Nous observons également une accumulation d'espèces réactives de l'oxygène dans les CSI déficientes en autophagie associée à une atténuation de la réponse antioxydante médiée par NRF2. Des traitements antioxydants améliorent la survie des CSI invalidées pour *Atg7* autant *ex-vivo* sur cultures d'organoïdes qu'*in vivo*. Ces expériences soutiennent l'implication des espèces réactives de l'oxygène accumulées suite à la perte de l'autophagie dans la mort des CSI.

Par ailleurs, l'inhibition de l'autophagie induit des défauts de défense antimicrobienne, notamment au niveau de la sécrétion de peptides antimicrobiens par les cellules de Paneth, type sécrétoire retrouvé au fond des cryptes intestinales à proximité des CSI. Grâce à des traitements antibiotiques à large-spectre, nous avons observé un rôle important de la flore intestinale sur la survie des CSI déficientes en autophagie *in vivo*. Ce résultat est confirmé par la survie normale d'organoides inhibés pour l'autophagie seulement une fois en culture (et donc isolé du microenvironnement intestinal), mais cette survie est réduite en présence de certains signaux microbiens.

Nos travaux indiquent donc un rôle important de l'autophagie dans la protection et le maintien des CSI, de par son contrôle des espèces réactives de l'oxygène, du microenvironnement bactérien et des voies de réparation de l'ADN. Cependant, malgré la mort en continu des CSI déficientes en autophagie, un pool de CSI est maintenu en continu, ce qui permet le renouvellement normal de l'épithélium et souligne une plasticité importante au sein des cryptes épithéliales. L'ensemble de ces travaux suggèrent que l'autophagie pourrait être une cible thérapeutique prometteuse pour traiter les cancers colorectaux sans pour autant empêcher la fonction homéostatique de l'épithélium.

INTRODUCTION

FOREWORD

The work of my host team and Dr. ROMAGNOLO's group focuses on the physiopathological renewal of the intestinal epithelium. For years, the group has investigated the molecular mechanisms involved in both the homeostatic regeneration of this highly proliferative epithelium and its deregulation in colorectal cancer. Among these, the WNT/ β -catenin signaling pathway is both a crucial driver of proliferation and differentiation in the intestinal crypt and a critical target of oncogenic processes resulting in tumor development. Importantly, this pathway is essential for the maintenance and function of intestinal stem cells, which drive homeostatic proliferation in the epithelium and have also been implicated in tumor initiation and growth. Using both mouse models of intestinal tumorigenesis through WNT/ β -catenin hyperactivation and human colorectal cancer samples, our group identified a crucial role of autophagy, a highly conserved cellular catabolic mechanism, in intestinal tumorigenesis. They further showed that blocking autophagy in the intestinal epithelium dramatically hindered tumor development and tumor growth, in part by affecting the gut's microbiota and immunity. Interestingly, autophagy inhibition also affected the intestinal stem cell compartment of the non-tumoral epithelium. The work of my thesis aimed to understand the physiological functions of autophagy in the homeostatic intestinal epithelium and, more specifically, in intestinal stem cells.

This introduction will focus on the two principal aspects of my thesis work. First, the current knowledge on the intestinal epithelium at homeostasis and the rupture of this homeostasis in colorectal cancer will be reviewed. Particular attention will be paid to intestinal stem cells and the mechanisms regulating their maintenance, self-renewal, and proliferation. Second, we will explore the mechanisms and cellular functions of autophagy and its physiological impact. In both of these fields, major advancements have been made using invertebrate models such as *Drosophila melanogaster* and *Caenorhabditis elegans* or, in the case of autophagy, in unicellular eukaryotes like *Saccharomyces cerevisiae*. Nevertheless, throughout this introduction, we will focus primarily on mammalian systems and, whenever possible, on *in vivo* studies.

CHAPTER 1:
PHYSIOPATHOLOGY OF THE
INTESTINAL EPITHELIUM

I. STRUCTURE & FUNCTION OF THE INTESTINE

1.1 OVERVIEW OF THE ADULT DIGESTIVE SYSTEM & GASTROINTESTINAL TRACT

The adult digestive system consists of the organs along the length of the digestive tube – the mouth, esophagus, stomach, small intestine, and large intestine (or colon) – as well as several accessory organs including the tongue, salivary glands, liver, pancreas and gallbladder [FIGURE 1]. Along the route of ingested food from the mouth to the anus, the digestive tube plays two principal functions: it allows the breakdown of food and absorption of nutrients (digestion), and it acts as a physical and chemical barrier between the outside luminal environment and the organism.

Digestion begins directly upon ingestion of food, in the mouth. The chewing and grinding action of the teeth (mastication) helped by the muscular action of the tongue allow the mechanical breakdown of food into smaller pieces. This increases the surface area accessible to the digestive enzymes in saliva, produced in the salivary glands. In addition to digestive enzymes, saliva also contains antimicrobial proteins, antifungal proteins and immunoglobulins, which act as early defense against microbes arriving in the gastrointestinal tract along with the ingested food. Saliva also softens the food, transforming it into a soft bolus, and facilitating its passage through the pharynx and into the esophagus upon swallowing.

The esophagus is a muscular tube carrying the bolus to the stomach through rhythmic contractions called peristalsis that will continue along the rest of the gastrointestinal tract. Two sphincters at the top and bottom of the esophagus open upon swallowing and prevent backflow from the stomach, respectively. Once in the stomach, the bolus is further broken down by the action of numerous digestive enzymes. Gastric acid (mainly hydrochloric acid) creates an acidic pH that both allows pepsin proteases to work and kills most ingested bacteria. The whole of the gastrointestinal tract, and particularly the stomach, is covered by mucus that lubricates the passage of digested food and protects the underlying, single-layered columnar epithelium. Along with the enzymatic digestion, mechanical churning helps transform the bolus into chyme.

It is at this stage, approximately one to two hours after ingestion, that the partially-digested food enters the small intestine through the pyloric sphincter. The majority of digestion and absorption occurs in the small intestine. The segment directly connected to the stomach is called the duodenum. Bile produced by the liver and stored in the gallbladder is released into the duodenum upon the arrival of the chyme to help break down fats. The pancreas also releases pancreatic juice rich in bicarbonate and digestive enzymes into the duodenum to neutralize the acidic chyme from the stomach and continue the digestion of carbohydrates, proteins and lipids into monosaccharides, amino acids and fatty acids, respectively. As nutrients are taken up by the organism, the muscular walls of the intestine help slowly pass the chyme along the relatively short duodenum and the other two segments of the small intestine: the jejunum and ileum.

The intestinal wall contains many folds, which allow the intestine to stretch and contract as food passes along. To increase the surface area of absorption, the small intestinal epithelium consists

of a single layer of cells folded upon itself creating finger-like protrusions called villi. Moreover, the absorptive cells of the epithelial lining, called enterocytes, have densely packed microvilli on the luminal side to further increase surface area. These cells express additional digestive enzymes at their surface, as well as transporters that take up monosaccharides, amino acids, some vitamins and ions. Fatty acids, other vitamins and water, on the other hand, can diffuse passively across the epithelium and into the bloodstream. Once in the bloodstream, nutrients and other substances absorbed in the intestine are directly sent to be processed by the liver.

The majority of nutrients are thus absorbed in the duodenum and jejunum. Along with the amount of available nutrients, the length of the epithelial villi decrease from the jejunum to the ileum [FIGURE 2]. Remaining water, salts, and undigested materials are passed on to the large intestine, or colon, through the ileocecal valve. The colon is subdivided into several segments; the first pouch is called the cecum, followed by the proximal and distal colon – subdivided in humans into the ascending, transverse, descending, and sigmoid colon – leading to the rectum. Salts and remaining water are absorbed while undigested materials are packed into feces to be excreted by contractions of the rectum. The colon epithelium lacks villi entirely, and is progressively smoother from the proximal to the distal end [FIGURE 2].

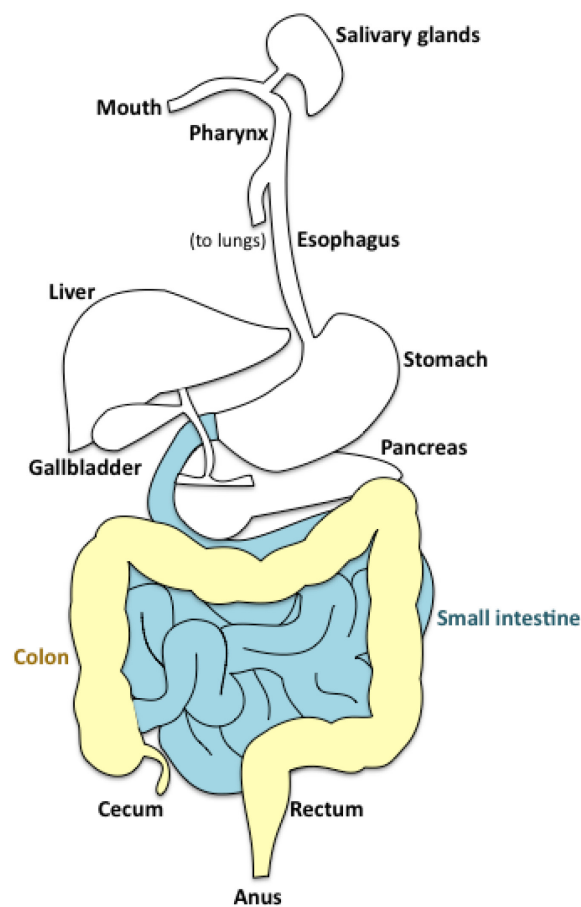


FIGURE 1. The human digestive system.

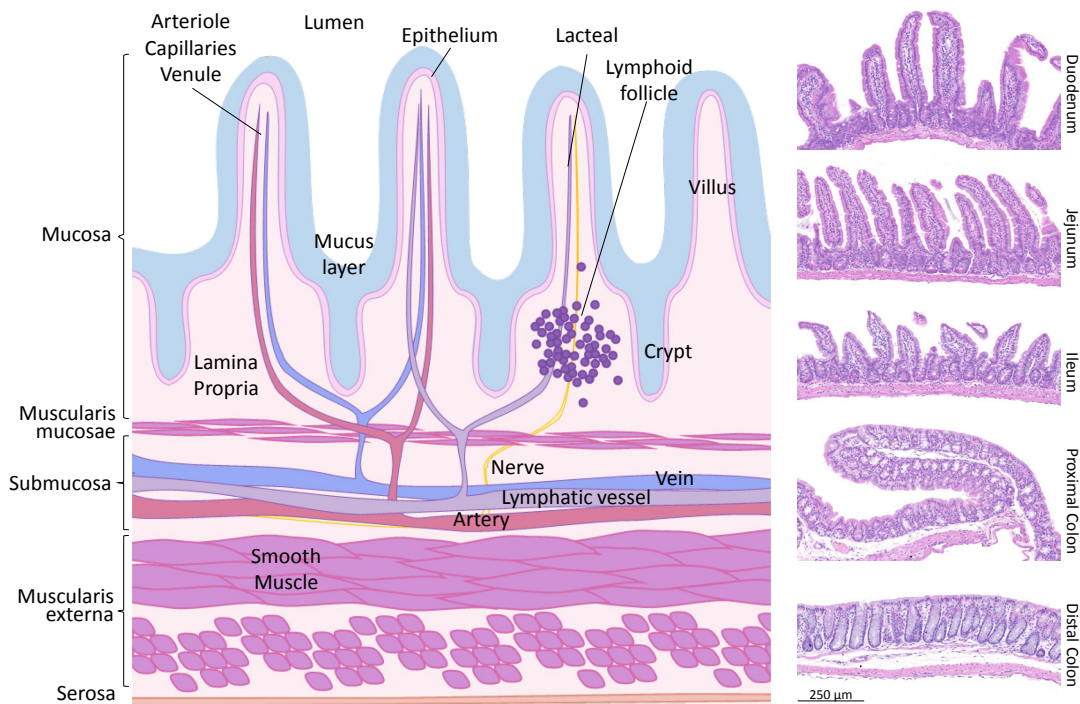


FIGURE 2. Organization of the intestinal wall and anatomy of the mucosa in different segments of the mouse intestine (hematoxylin & eosin staining).

1.2 STRUCTURAL ORGANIZATION OF THE INTESTINE

The different elements that make up the intestinal wall work together to accomplish the two major functions of this organ in digestion and as a barrier.

The intestinal lumen is home to a large amount of microorganisms, altogether known as the gut microbiota, particularly present in the colon. Although technically an environmental factor, the notion of the gut microbiota as an essential symbiont for host development and physiology has become more and more accepted over the last decade. Bacteria dominate the microbiota, which also includes archaea, viruses, fungi, and protozoa. Colonization of the gut at birth is important for the development of the intestinal mucosa, including the maturation of vasculature (Stappenbeck et al., 2002; Reinhardt et al., 2012) and of the gut-associated lymphoid tissue (GALT) (Macpherson et al., 2000; Macpherson, 2004; Bouskra et al., 2008; Uematsu et al., 2008; Gaboriau-Routhiau et al., 2009; Sanos et al., 2009; Atarashi et al., 2011; Kawamoto et al., 2012; Olszak et al., 2012). In the adult intestine, the microbiota notably contributes to digestion as it helps break down undigested products (ie. polysaccharides into short-chain fatty acids). In addition to metabolizing otherwise indigestible nutrients, the microbiota plays an important role in metabolizing xenobiotics – including drugs or pollutants (Koppel et al., 2017). Lastly, the gut commensals, through competition for nutrients and production of virulence factors, protect their host against infection by opportunistic pathogens (Kamada et al., 2012).

Although a single-layered epithelium is advantageous in terms of absorption, it only provides a thin barrier between the luminal contents and the host tissues. The barrier function of the mucosa therefore depends on intracellular junctions, especially tight junctions, that seal neighboring epithelial cells together (Buckley and Turner, 2018). Furthermore, specialized cells

protect the epithelial lining, including mucus-producing goblet cells antimicrobial peptide-producing Paneth cells. As an additional protective measure, the lining of the intestine is continuously regenerated; in fact, the intestinal epithelium renews itself almost entirely every 4 to 5 days (Cheng and Leblond, 1974; Wright and Irwin, 1982) – faster than any other tissue. The regenerative capacity of the gut epithelium depends on a pool of actively dividing intestinal stem cells (ISC) and their highly proliferative transit amplifying (TA) progenitors, all located in epithelial pockets away from the lumen called crypts of Lieberkühn.

The intestinal epithelium is surrounded on the basal side by a connective mesenchyme called the lamina propria. The basal side of intestinal epithelial cells is in direct contact with a network of extra-cellular proteins that form the basement membrane, including type IV Collagen, Laminin and proteoglycans. Basement membrane components are produced both from the epithelium and mesenchymal cells in the lamina propria. Beneath the basement membrane, the mesenchyme comprises a complex extra-cellular matrix (ECM) composed of similar components including type I Collagen and Fibronectin. Embedded in this ECM are various mesenchymal cell types, including various fibroblast populations, myofibroblasts, a layer of smooth muscle called the muscularis mucosa which contributes to peristalsis, endothelial cells of the blood and lymphatic networks, pericytes, and enteric neurons [FIGURE 2]. In addition to these, the lamina propria houses various innate and adaptive immune cells and structures altogether comprising the GALT, capable of mounting an appropriate inflammatory or tolerogenic response to passing antigens. These include B cells (especially immunoglobulin A (IgA)-producing plasma cells), T cells (including pro-inflammatory Th1, Th2 and Th17 cells as well as anti-inflammatory regulatory T cells), neutrophils, natural killer (NK) cells, macrophages and dendritic cells. In addition to these, specialized subsets of T cells (intra-epithelial lymphocytes) and some dendritic cells are found between the intestinal epithelial cells, where they continuously sample luminal contents.

The submucosa contains blood vessels, lymphatic vessels, and nerves embedded in a matrix of fibres and collagen. Beneath those, two layers of smooth muscle, one circular and one longitudinal, also aid in peristalsis. Finally, the intestinal wall is surrounded by a loose layer of connective tissue called the serosa, and the whole of the intestine is held in place in the peritoneum by the mesentery.

1.3 THE INTESTINAL EPITHELIUM

The single-layered mammalian intestinal epithelium is folded upon itself creating finger-like protrusions towards the lumen called villi, beneath which lie small pockets towards the lamina propria called the crypts of Lieberkühn. The small intestinal crypts are almost entirely composed of proliferative cells, with the exception of Paneth cells, a secretory lineage first recognized for their ability to secrete antimicrobial peptides (AMP). The adult mouse intestine contains approximately 1.1 million crypts, with around two-thirds of the cells of each crypt dividing every twelve hours, producing over 300 million new cells each day (Gordon et al., 1992). This astounding regenerative capacity relies on three to sixteen (depending on the study) actively dividing ISC localized at the crypt bottom alongside Paneth cells (in the small intestine)

or related secretory cells (in the colon) (Bjerknes and Cheng, 1981a; Altmann, 1983; Potten and Loeffler, 1987; Sato et al., 2011a; Sasaki et al., 2016). ISC give rise both to new ISC and to TA progenitor cells that will rapidly proliferate and progressively differentiate as they migrate up the crypt. TA daughters will either be absorptive-lineage progenitors, giving rise to enterocytes, or secretory progenitors that will give rise to Paneth cells, goblet cells, or enteroendocrine cells (Schmidt et al., 1985; Bjerknes and Cheng, 1999). In the small intestine, cells become terminally differentiated upon reaching the villus, and then continue their columnar migration to the tip of the villus, where they are expelled into the lumen and die in a process termed anoikis [FIGURE 3]. The entire process of proliferation, differentiation, and expulsion of intestinal epithelial cells takes only 4-5 days (Cheng and Leblond, 1974; Wright and Irwin, 1982).

The villus epithelium therefore contains exclusively differentiated cells, over 80 percent of which are absorptive enterocytes. In addition to these, the differentiated compartment of the epithelium contains the secretory cells mentioned above (with the exception of Paneth cells), as well as three rarer cell types: tuft cells (Gerbe and Jay, 2016), cup cells (Madara, 1982) and microfold (M) cells associated with lymphoid follicles called Peyer's patches (Kraehenbuhl and Neutra, 2000). Each of these differentiated cell types will be discussed in more detail below. The colon has a flat surface with no villi; instead, the bottom third of colonic crypts contains proliferative cells while the upper crypt is differentiated with a considerably high ratio of goblet cells [FIGURE 3]. Paneth cells are notably absent in the colon.

1.3.1 ENTEROCYTES

Enterocytes are the predominant cell type of the intestinal epithelium, making up over 80% of epithelial cells. They are highly polarized absorptive cells characterized by their columnar shape and a brush border of microvilli at the apical pole that increases the surface area of absorption [FIGURE 4]. On the surface of these microvilli, enterocytes express digestive enzymes that play a role in the final breakdown of food particles before they are selectively taken up by the cell and transited to the basal pole. The uptake of nutrients can be active via specific transporters – as is the case for ions, sugars, amino acids, or water-soluble vitamins – or passive – as is the case for water, lipids, and lipid-soluble vitamins. Enterocytes also transport IgA from plasma cells in the lamina propria in the opposite direction; these are endocytosed on the basolateral surface and released into the intestinal lumen.

Differentiation into either the absorptive lineage or secretory lineage depends on NOTCH signaling in the crypt. Expression of the NOTCH target gene *Hes1* is required for enterocyte differentiation, whereas it represses expression of NOTCH ligands and of *ATOH1* (atonal BHLH transcription factor 1, *Math1* in mice), both key to secretory lineage commitment (Yang et al., 2001; Stanger et al., 2005).

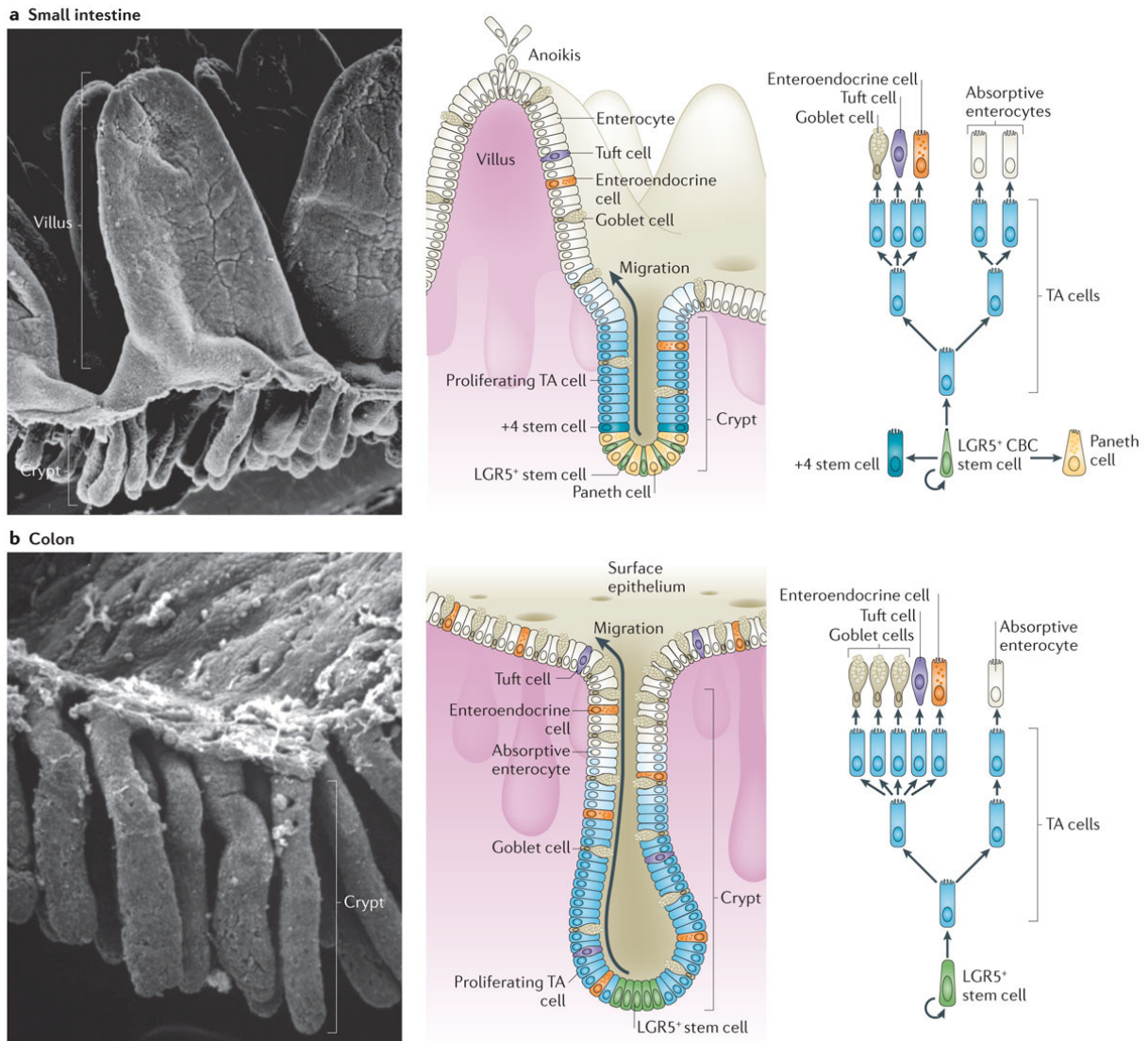


FIGURE 3. Topography of the intestinal epithelium in the small intestine (A) and colon (B). Adapted from Barker, 2014.

1.3.2 GOBLET CELLS

Goblet cells, as previously noted, are secretory lineage cells scattered throughout the epithelium that produce and secrete mucus to both facilitate movement of luminal contents and protect the epithelium. Contrary to enterocytes, these cells depend on *ATOH1* expression early in their lineage commitment. They contain characteristic mucin-containing secretion granules in their apical cytoplasm [FIGURE 4] and release these granules into the lumen by exocytosis.

The main component of the intestinal mucus layer is the mucin MUC2. Before secretion, mucins are highly glycosylated in the golgi apparatus of goblet cells, giving them the capacity to bind water and form a gel-like network once secreted (Johansson et al., 2011). *Muc2*-deficient mice have bacteria in direct contact with the epithelium as well as bacterial infiltration across the epithelium (Van der Sluis et al., 2006; Johansson et al., 2008). This results in inflammation and symptoms of inflammatory bowel disease (IBD). Interestingly, these mice also develop spontaneous intestinal adenomas (Velcich et al., 2002).

The proportion of mucin-producing goblet cells in the epithelium and the thickness of the mucus layer increases from the proximal small intestine (~4%) to the distal colon (~16%) (Cheng, 1974) in direct correlation with the density of the microbiota. In the small intestine, the mucus layer is discontinuous but is supported by the secretion of antimicrobial peptides by Paneth cells. In the colon, two structurally distinct layers of mucus are found: the firm inner layer is considered to be relatively sterile (Johansson et al., 2008; Li et al., 2015a), while the thicker outer layer, made looser by proteolytic dispersion of mucin polymers, houses a distinct subset of bacterial species that can digest complex glycoproteins (Johansson et al., 2011; Li et al., 2015a), therefore creating a unique microbial niche (Li et al., 2015a).

Recent studies have also shown that goblet cells have the ability to endocytose luminal substances and transfer them to antigen-presenting cells in the lamina propria (McDole et al., 2012).

1.3.3 PANETH CELLS

Paneth cells are the major secretory cell type of the small intestinal crypt. Like goblet cells, differentiation of Paneth cells depends upon the expression of the *ATOH1* transcription factor. Paneth cells, unlike the other progenitor lineages, terminally differentiate as they migrate down to the bottom of the crypt and remain at the crypt bottom throughout their uniquely long 6- to 8-week lifespan (Ireland et al., 2005). The position of a Paneth cell is therefore linked to its maturity, with the oldest and more mature Paneth cells found at the crypt base (Bjerknes and Cheng, 1981b). These cells have a characteristic pyramidal shape with prominent, large granules filling their cytoplasm on the apical side. They also have an extensive endoplasmic reticulum and Golgi network, in link with their secretory functions [FIGURE 4].

The distinguishing apical granules of Paneth cells were later shown to contain Defensins, Lysozyme, REG3 (regenerating islet-derived protein 3, REG3A in humans, REG3 γ and REG3 β in mice) and other AMPs, establishing the role of Paneth cells in innate immunity. Although Paneth cells constitutively express Defensins under the control of WNT signaling (van Es et al., 2005a; Andreu et al., 2008), production of other AMPs is up-regulated in response to microbial signals (Mallow et al., 1996; Ayabe et al., 2000; Pütsep et al., 2000; Vaishnava et al., 2008) or pro-inflammatory cytokines like Interferon gamma (IFN γ) (Farin et al., 2014). These secreted AMPs not only protect the crypt epithelium from enteric pathogens (Vaishnava et al., 2008), but also directly affect the composition of the microbiota (Salzman et al., 2010).

Due to their strategic localization at the crypt bottom alongside ISC, a tight functional link between Paneth cells and ISC was proposed early on (Cheng and Leblond, 1974). Indeed, Paneth cells also play an important role in the ISC niche by producing essential trophic factors like WNT ligands, NOTCH ligands, and EGF (Gregorieff et al., 2005; Sato et al., 2011a). The role of Paneth cells in the ISC niche will be discussed in more detail in the following section.

1.3.4 ENTEROENDOCRINE CELLS

Enteroendocrine cells are secretory lineage cells specified by *ATOH1* and Neurogenin-3 expression (Jenny, 2002). They secrete hormones like Serotonin (which notably affects

gastrointestinal motility) and the insulinotropic hormone Glucagon-like peptide-1. There exists up to 15 different subtypes of enteroendocrine cells, depending on their morphology and the hormones they produce. These represent about 1% of epithelial cells, dispersed throughout the differentiated tissue.

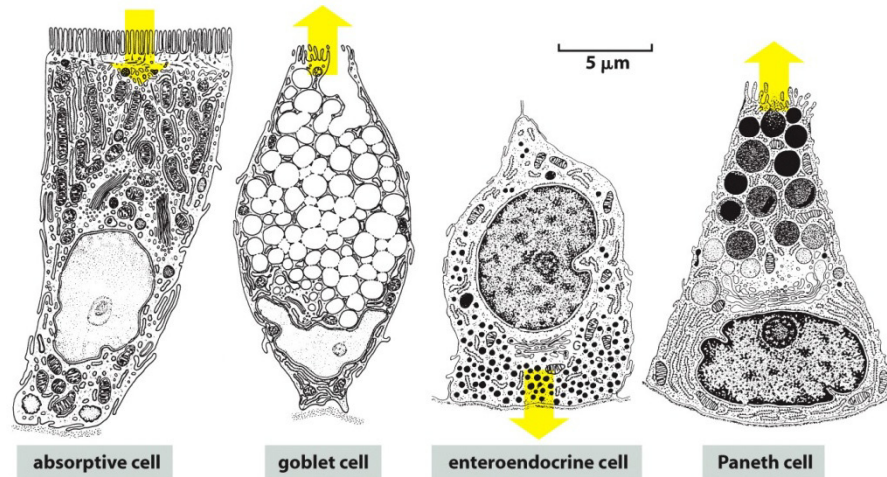


FIGURE 4. The four predominant differentiated cell types of the intestinal epithelium. Adapted from Alberts et al., 2008.

1.3.5 TUFT CELLS

Tuft cells were identified by electron microscopy based on their unique tubulovesicular system and apical bundle of microfilaments connected to a tuft of microvilli that are longer and thicker than those of enterocytes. In light of their morphology, tuft cells were thought to be secretory cells (Sato and Miyoshi, 1997) but whether they are derived from the secretory lineage specified by *ATOH1* is still a matter of debate (Gerbe et al., 2011; Bjerknes et al., 2012). Despite their discovery more than 60 years ago (Jarvi and Keyrilainen, 1956; Rhodin and Dalhamn, 1956), their function was only very recently brought to light. The first identified function of tuft cells was the secretion of opioids (Kokrashvili et al., 2009; Gerbe et al., 2011). Then, in 2016, three independent groups published studies identifying tuft cells as essential to trigger a type 2 immune response in response to parasitic infections in the gut (Gerbe et al., 2016; Howitt et al., 2016; Von Moltke et al., 2016). Mechanistically, this involves signal transduction via the TRPM5 (Transient receptor potential cation channel subfamily M member 5) cation channel and Interleukin (IL) - 25 alarmin production (Gerbe et al., 2016; Howitt et al., 2016; Von Moltke et al., 2016). In the intestinal epithelium, tuft cells are much less frequent than the previous four differentiated cell types, as they make up only about 0.4% of epithelial cells. However, during infections with helminthes *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus* or the protozoa *Tritrichomonas muris*, tuft cell numbers expand dramatically, as do goblet cell numbers and epithelial cell cytokine production, leading to the recruitment of type 2 helper T cells and group 2 innate lymphoid cells (ILC) in the lamina propria. The loss of tuft cells or of their function (by deleting either the *Trpm5* or *Il25* genes) prevents each of these responses and delays the resolution of the infection. More recently, the tuft cell-ILC2 circuit was shown to be basally

activated by commensal protozoan-derived metabolites as a way to prevent infection ([Schneider et al., 2018](#)). Tuft cells therefore play a unique role in the context of parasite infection.

1.3.6 MICROFOLD CELLS

M cells are specialized cells found on follicle-associated epithelium such as that found over Peyer's patches. These cells owe their name to their characteristic apical membrane, which lacks microvilli and instead has a microfold topography ([Kraehenbuhl and Neutra, 2000](#)). M cells are believed to act as sentinels that take up antigens from the lumen and transport them to the underlying GALT. In line with this, their basolateral membrane is invaginated to form pockets that reduce the intracellular distance of antigen transport from the apical membrane and harbor infiltrating lymphocytes. In this way, M cells help the intestinal immune system mount either inflammatory or tolerogenic responses to foreign antigens. M cells are thought to differentiate from absorptive-lineage progenitors in response to stimuli from the underlying lymphoid tissue, namely NF- κ B signaling ([Knoop et al., 2009](#); [Kanaya et al., 2018](#)).

1.3.7 CUP CELLS

Cup cells can be distinguished by light or electron microscopy due to their lighter cytoplasm and shorter brush border compared to neighboring enterocytes, creating the cup-like apical indentation that gave them their name ([Madara, 1982](#)). They are absent in the jejunum but relatively abundant (~6% of villus epithelial cells) in the ileum of rodents, although less so in primates. To date, the function of these cells remains unknown.

II. INTESTINAL STEM CELLS AND THEIR DEFINING NICHE

2.1 MODELS AND MARKERS OF INTESTINAL STEM CELLS

Although the localization of ISC at the crypt bottom has long been established, the identity of ISC has remained a subject of debate over the past decades. Two historical models of ISC have been originally proposed but direct evidence to illustrate their stemness was put forward only in the last decade. Pioneer work by Cheng, Bjerknes and Leblond using electron microscopy, ³H-thymidine incorporation and clonal mutagenesis demonstrated the presence of undifferentiated proliferative cells capable of giving rise to the different intestinal epithelial lineages (multipotent) at positions 1-4 from the bottom of the intestinal crypt, which they called crypt basal columnar cells (CBC) (Cheng and CP., 1974; Bjerknes and Cheng, 1999, 2002). At the same time, works led by Potten's group recognized cells at the fourth position from the crypt bottom (+4) as capable of long-term ³H-thymidine label retention (Potten et al., 1978), an established property of tissue stem cells. Without specific molecular markers to isolate them at the time, it remained unclear whether the mitotically active CBC or the relatively quiescent +4 label-retaining cells (+4SC) ought to be considered as the bona fide ISC.

2.1.1 MARKERS FOR CRYPT BASAL COLUMNAR CELLS

In 2007, the Clevers lab and collaborators identified LGR5 as a marker for CBC. *Lgr5* encodes a leucine-rich repeat-containing G-protein-coupled transmembrane receptor first identified as a Wnt/ β -catenin target gene. Although LGR5 immuno-labelling has proved difficult due to its low expression levels, the development of a mouse model in which an EGFP and a tamoxifen-inducible Cre recombinase cassette was knocked in at the *Lgr5* locus (*Lgr5*-EGFP-*ires*-CreER^{T2}) has allowed characterization of *Lgr5*-expressing cells. In the adult small intestine, *Lgr5*⁺ cells are located at the base of the crypt, intermingled with Paneth cells [FIGURE 5B]. They are rapidly cycling since within 24 hours almost all *Lgr5*⁺ cells of a crypt undergo mitosis. In the colon, *Lgr5*⁺ cells are also found at the crypt bottom interspersed with secretory cells [FIGURE 5F] but have slower cycling kinetics than in the small intestine. The generation of *in vivo* lineage tracing from *Lgr5*⁺ cells using an *Lgr5*-EGFP-*ires*-CreER^{T2}/*Rosa26lacZ* mouse model indicated that *Lgr5*⁺ cells transmit LacZ staining to their progeny and repopulate the entire epithelium during homeostasis [FIGURE 5]. Furthermore, *Lgr5*⁺ cells are long-lived and contribute to tissue renewal over the entire lifetime of the mouse. Therefore, LGR5 is a reliable marker of CBC (Barker et al., 2007), and *Lgr5*-EGFP-*ires*-CreER^{T2} mice a valuable tool to understand their regulation. The ultimate proof of *Lgr5*⁺ CBC as ISC is the capacity of single isolated *Lgr5*⁺ cells cultured *in vitro* in matrigel to generate intestinal organoids that mimic the structure of the *in vivo* epithelium, with *Lgr5*⁺ cells found at the bottom of the proliferative crypt domains [FIGURE 6A-D]. Importantly, these organoids contain the four major differentiated lineages of the intestinal epithelium [FIGURE 6E-H] and self-renew, as shown by continuous passages (Sato et al., 2009).

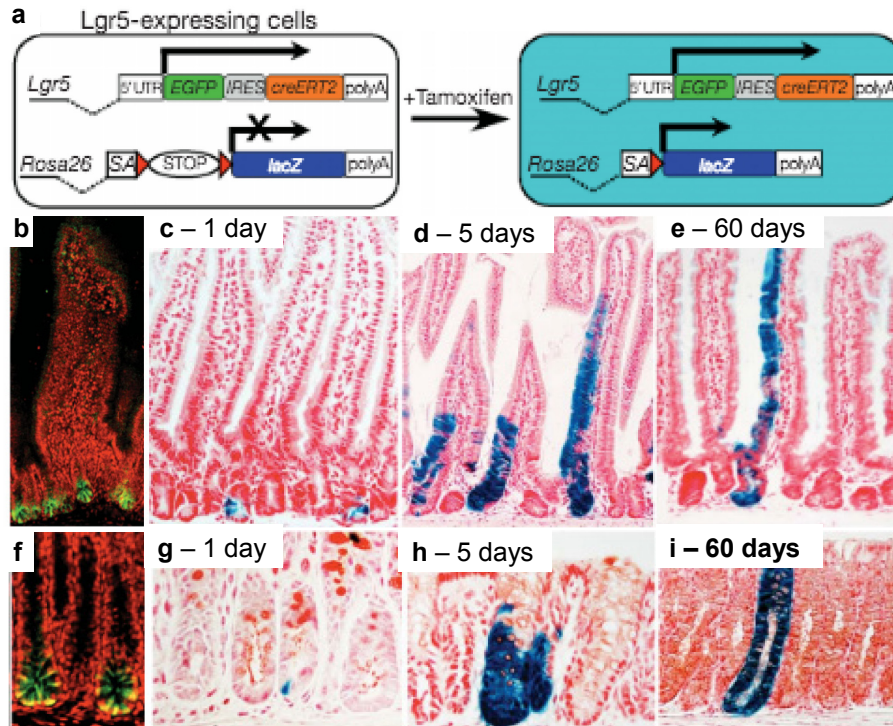


FIGURE 5. Establishment of *Lgr5* as an intestinal stem cell marker (A) Generation of mice expressing EGFP and creERT2 from a single bicistronic message by gene knock-in into the first exon of *Lgr5*. SA, splice acceptor; UTR, untranslated region. (B,F) Confocal GFP imaging counterstained with the red DNA dye ToPro-3 confirming that *Lgr5* expression is restricted to the 6-8 slender cells at the crypt bottom in the small intestine (B) and in the colon (F). (C-E) Histological analysis of LacZ activity in the small intestine 1 day after induction (C), 5 days after induction (D) and 60 days after induction (E). (G-I) LacZ activity in the colon 1 day after induction (G), 5 days after induction (H) and 60 days after induction (I). Adapted from Barker et al., 2007.

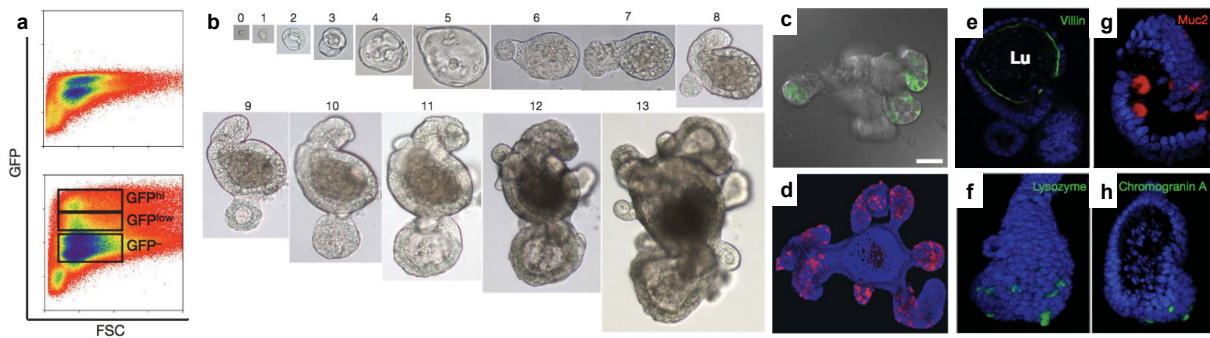


FIGURE 6. Intestinal organoid culture from isolated *Lgr5*⁺ ISC sorted from *Lgr5*-EGFP-IRES-CreERT2 mice (A) Two positive populations, GFP^{high} and GFP^{low}, are discriminated from *Lgr5*-EGFP-IRES-CreERT2 intestines. FSC, forward scatter. (B) Example of a single GFP^{high} cell successfully growing into an organoid. Numbers above the images are the days of growth. (C) 14 days after sorting, organoids from isolated GFP^{high} cells have *Lgr5*-GFP positive cells at the bottom of the crypt domains. (D) Organoids cultured with EdU (red) for 1h incorporate EdU only in the crypt domains. Counterstain, DAPI (blue). (E-H) Confocal image for villin (E, green, enterocytes), lysozyme (F, green, Paneth cells), Muc2 (G, red, goblet cells), and chromogranin A (H, green, enteroendocrine cells). Adapted from Sato et al., 2009.

A CBC stem cell signature using gene expression and proteome profiling has been determined using FACS-purified Lgr5⁺ cells from the Lgr5-EGFP-*ires*-CreER^{T2} model. Indeed, comparison between Lgr5⁺ CBC and their direct TA progeny sorted by different levels of EGFP (high in CBC and low in their TA progeny) provided a comprehensive Lgr5⁺ ISC signature of approximately 500 genes (Muñoz et al., 2012). The signature contains many Wnt/ β -catenin modulators and target genes such as *Lgr5*, *Sox9*, *Ascl2* (achaete-scute homologue 2), *EphB2*, *Troy/Tnfrsf19*, *Axin2*, *Znrf43* (zinc and ring finger 43) (Hao et al., 2012) and *Rnf3* (ring finger 3) (Koo et al., 2012). This and subsequent Lgr5⁺ cell signatures (van der Flier et al., 2009a; Muñoz et al., 2012) along with candidate-approach studies also provided several other verified CBC markers. Among the new markers identified, some have been confirmed as ISC markers by lineage tracing: *Smoc2* (SPARC related modular calcium binding 2) (Muñoz et al., 2012), *Musashi-1* (Potten et al., 2003), *Prominin-1* (Zhu et al., 2009) and notably *Olfm4* (Olfactomedin 4), which is highly expressed in the small intestine compared to other mentioned CBC markers and therefore allows easier detection of CBC (van der Flier et al., 2009b; Schuijers et al., 2014). *Ascl2*, a basic helix-loop-helix transcription factor was identified as a master regulator of CBC maintenance. The conditional deletion of *Ascl2* results in the loss of CBC and its overexpression promotes drastic expansion of the ISC compartment (van der Flier et al., 2009a; Reed et al., 2012; Schuijers et al., 2015).

2.1.2 MARKERS FOR +4 STEM CELLS

The first +4SC marker investigated by lineage tracing was BMI1, a polycomb-repressing complex member essential in hematopoietic and neural stem cell self-renewal. In intestinal homeostasis, *Bmi1* is expressed at the +4 position in a minority of small intestinal crypts (around 10%) and particularly in the duodenum. By *in vivo* lineage tracing using a *Bmi1*-CreER^{T2}/*Rosa26lacZ* mouse model and organoid culture experiments, *Bmi1*⁺ cells were shown to self-renew and give rise to the different epithelial lineages but with much slower cycling kinetics compared to Lgr5⁺ CBC (Sangiorgi and Capecchi, 2008; Yan et al., 2012). In addition, ablation of *Bmi1*⁺ cells using targeted expression of diphtheria toxin (DT) caused crypt loss consistent with a stem cell defect.

Ablation of the Lgr5⁺ CBC using targeted expression of the DT receptor (DTR), on the other hand, caused an increase of *Bmi1*⁺ cells which can regenerate the Lgr5⁺ stem cell population and maintain intestinal homeostasis (Tian et al., 2011). Thus, these relatively quiescent +4SC, have been considered as reserve stem cells in case of damage to the active CBC stem cells. In support of the mobilization of the +4SC following injury, after high dose of radiation (up to 12 Gy), Lgr5⁺ cells are rapidly lost whereas *Bmi1*⁺ cells expand and allow epithelial recovery through replenishment of Lgr5⁺ cells (Yan et al., 2012). In fact, BMI1 has been shown to play a role in DNA damage response signaling; it may therefore play a direct role in the resistance of reserve stem cells to irradiation (Ismail et al., 2010; Ginjalet al., 2011; Pan et al., 2011). Nevertheless, the restoration of a pool of active Lgr5⁺ ISC pool by reserve stem cells appears to be an essential step for epithelial repair following injury, as depletion of Lgr5-expressing cells during radiation-induced damage and subsequent repair is detrimental to the epithelium (Metcalf et al., 2014).

However, BMI1-expressing cells are only present in a small proportion of crypts in the proximal small intestine and are not responsible for the post-injury regeneration throughout the distal

small intestine or colon in these contexts (Tian et al., 2011; Yan et al., 2012). Other markers must therefore be used to identify the reserve stem cells in these regions. Coincidentally, TERT (telomerase reverse transcriptase) also labels single cells at the +4 position that are slowly cycling, label-retaining, resistant to radiation and capable of regenerating the epithelium and Lgr5⁺ cells following injury (Breault et al., 2008; Montgomery et al., 2011). Other reported markers of +4SC include HOPX (homeodomain-only protein) (Takeda et al., 2011), LRIG1 (leucine rich repeats and immunoglobulin like domains 1) (Powell et al., 2012), and, debatably, DCAMKL1 (doublecortin like kinase 1, also an established Tuft cell marker) (May et al., 2008, 2009). Remarkably, cells expressing these markers can give rise to CBC and, conversely, Lgr5⁺ cells can give rise to +4SC (Takeda et al., 2011).

2.1.3 PROGENITORS AS POTENTIAL STEM CELLS: PLASTICITY IN THE INTESTINAL CRYPT

The plasticity of progenitor cells represents another source of potential ISC. In 2012, a study by van Es et al. demonstrated that secretory progenitors expressing Delta-like (*Dll*) 1, a NOTCH ligand, can revert to multipotent stem cells. Using a *Dll1*-GFP-CreER^{T2}/*Rosa26lacZ* mouse model, lineage tracing indicated that secretory progenitors can de-differentiate into Lgr5⁺ ISC upon ablation of CBC by irradiation. Furthermore, these progenitors are able to form Lgr5⁺ ISC-containing organoids *ex vivo* in the presence of WNT signals (van Es et al., 2012a). Similarly, Buczacki and colleagues used an elegant model to mark only label-retaining cells by using a fluorescent-labelled histone H2B reporter construct (H2B-YFP) (Buczacki et al., 2013). They demonstrated that during homeostasis, label-retaining cells express +4SC markers including *Lrig1*, *Tert* and *Hopx*, but also *Lgr5* as well as markers of early Paneth cells and enteroendocrine cells and remain quiescent. However, upon epithelial injury induced by irradiation or chemotherapeutic treatment, label-retaining cells proliferate and act as ISC, giving rise to all differentiated cell lineages. This study therefore indicates that committed secretory precursors are a subpopulation of Lgr5⁺ cells that can act as a potential ISC pool upon injury. More recently, enterocyte progenitors have also been shown to provide an alternative source of potential ISC. Using lineage tracing experiments based on intestinal alkaline phosphatase (*Alpi*) expression, a marker for absorptive progenitors and differentiated enterocytes, Tetteh et al. demonstrated that upon eradication of Lgr5⁺ cells through DT treatment, the proliferating crypt *Alpi*⁺ cells but not differentiated villus *Alpi*⁺ cells were able to de-differentiate and to generate all differentiated cell types, thereby acting as an alternative source of ISC (Tetteh et al., 2016). Two new studies from the groups of Kuo and Shivdasani categorize *Bmi1*-expressing cells as enteroendocrine progenitors, and further demonstrate the capacity of progenitors from this lineage to dedifferentiate and replenish the Lgr5⁺ ISC pool in case of injury (Jadhav et al., 2016; Yan et al., 2017).

Taken together, all these studies call into question the existence of a dedicated reserve stem cell population and rather highlight the plasticity of the intestinal epithelium along with the importance of maintaining the active ISC pool. Questions regarding the molecular drivers and the degree of this plasticity remain open. One predominant hypothesis is that upon injury leading to loss of CBC, surviving reserve stem cells or early progenitors regain direct contact

with the stem cell niche, which drives their de-differentiation back into Lgr5⁺ ISC [FIGURE].

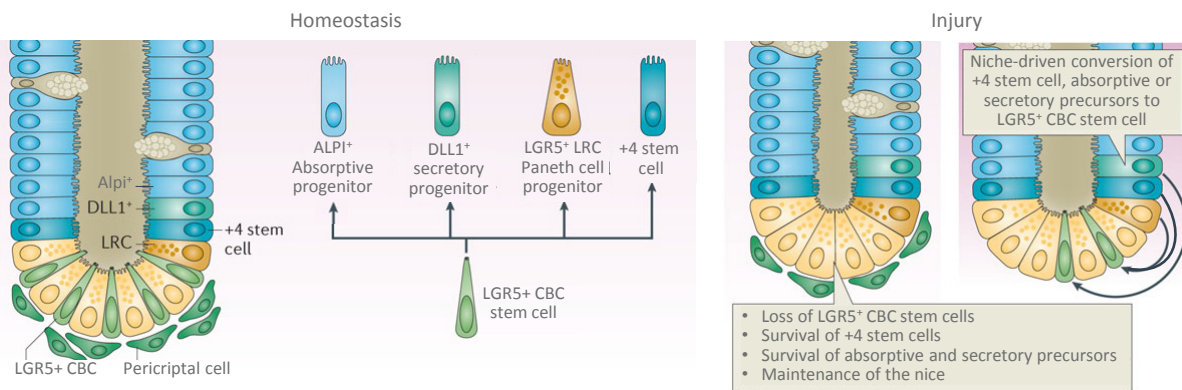


FIGURE 7. Plasticity in the intestinal crypt after injury. Adapted from Barker, 2014.

2.2 SIGNALING PATHWAYS REGULATING CRYPT HOMEOSTASIS

The stem cell niche is defined by a complex network of signals and niche cells that presumably delimit the “positional address” of ISC and support their activity. Proliferation, differentiation and migration along the crypt-villus axis are tightly regulated by an array of interconnected signaling pathways involving both epithelial signals and epithelial-mesenchymal crosstalk. The principal and best-characterized pathways essential to ISC homeostasis and the pathological consequences of their deregulation are discussed below.

2.2.1 WNT/ β -CATENIN SIGNALING

The major regulatory pathway of the ISC compartment is the Wnt/ β -catenin signaling pathway. In the absence of WNT signals, free cytosolic β -catenin is recognized by a protein complex containing the tumor suppressors APC (adenomatous polyposis coli), AXIN1, CK1 α (casein kinase 1 α) and GSK-3 β (glycogen synthase 3 β), called the β -catenin destruction complex. CK1 α and GSK-3 β will phosphorylate serine and threonine residues of the β -catenin N-terminus, leading to its ubiquitination by E3 ubiquitin ligases β -TrCP (β -transducin repeats-containing proteins) and subsequent proteosomal degradation. WNT ligands bind a Frizzled (FZD) receptor and co-receptor lipoprotein-related proteins (LRP5/6 in the intestine). This interaction triggers a cascade of events involving phosphorylation of Disheveled and membrane sequestration of AXIN1, which, through mechanisms that remain unclear, inhibits the destruction complex and prevents β -catenin degradation. β -catenin is therefore free to translocate to the nucleus, where it will bind TCF/LEF (T cell factor/lymphoid enhancer factor) transcription factors to induce Wnt/ β -catenin target genes (Logan and Nusse, 2004), including *c-Myc*, *CyclinD1*, and *Axin2* [FIGURE 8]. Wnt/ β -catenin signal strength is dependent on several agonists and antagonists, which may contribute to the very context-dependent transcriptional response of the pathway. In ISC, LGR5 and its homologs act as receptors for R-spondins, potent WNT signal enhancers that work by neutralizing RNF43 (ring finger protein 43) and ZNRF3 (Zinc And Ring Finger 3), two transmembrane E3 ligases that remove Wnt receptors from the cell surface (de Lau et al., 2011).

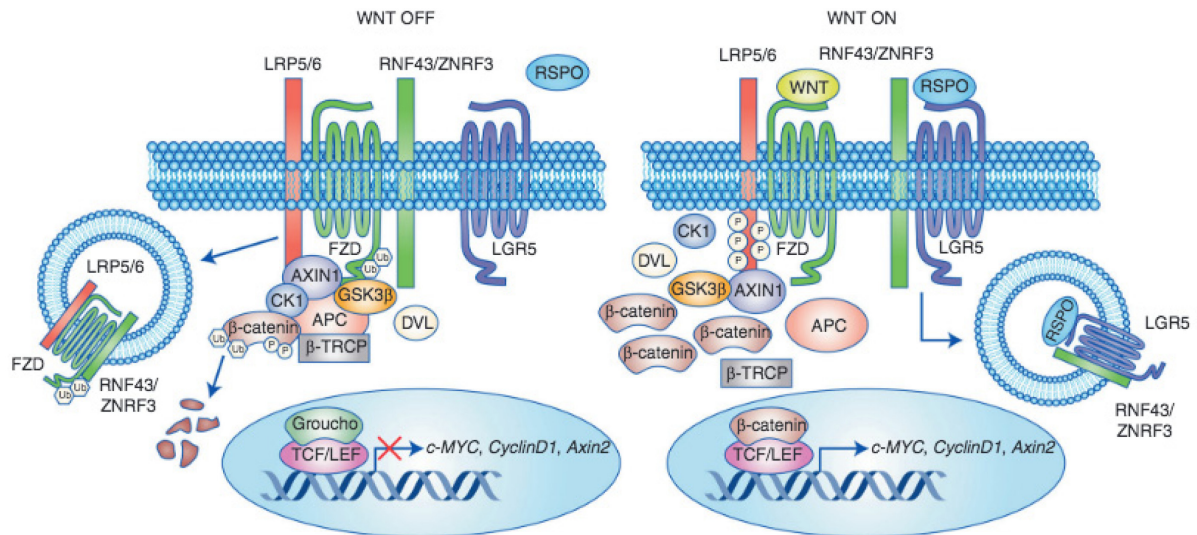


FIGURE 8. *Wnt/β-catenin signaling pathway in the intestine.*
Adapted from Morgan et al., 2018.

β -catenin was first identified as a component of cell-adhesion complexes, linking E-cadherin to α -catenin and the Actin cytoskeleton. Consequently, throughout the intestinal epithelium, β -catenin is mainly localized to the plasma membrane between epithelial cells, but also displays strong nuclear staining at the crypt bottom [FIGURE 9A]. Indeed, Wnt/ β -catenin signaling is particularly active in ISC and Paneth cells and exhibits a decreasing gradient of activity from the crypt bottom up. This Wnt activity gradient is in part established by the localized production of Wnt ligands. Crypt epithelial cells produce WNT3, WNT9b, and WNT6 (Gregorieff et al., 2005; Farin et al., 2012). Of these, WNT3 is specifically expressed in Paneth cells. The intestinal mesenchyme also produces Wnt ligands, namely WNT2b, WNT4 and WNT5a. Furthermore, the mesenchyme near the differentiated epithelium produces Wnt antagonists like DKK (Dickkopf) in a localized manner (Gregorieff et al., 2005). All this combined makes for a rather intricate regulation of Wnt signaling resulting in a Wnt activity-permissive niche in the stem cell and Paneth cell compartment and repression of canonical Wnt signaling in the differentiated epithelium.

Wnt/ β -catenin signaling plays an essential role in regulating proliferation of ISC and their daughters in the crypt. Multiple studies have demonstrated that the Wnt/ β -catenin pathway is essential for the maintenance and proliferation of CBC. In the intestine, disruption of the pathway by deletion of either *Tcf4* or the β -catenin gene *Cttnb1* or more upstream disruption of the pathway by ectopic expression of the Wnt antagonist DKK1 results in a fatal disruption of intestinal architecture with ablation of the proliferative crypts (Korinek et al., 1998; Pinto et al., 2003; Ireland et al., 2004; Kuhnert et al., 2004; Muncan et al., 2006; Fevr et al., 2007; van Es et al., 2012b). Conversely, overstimulation of the pathway by removing *Apc* or by injecting recombinant R-spondin1 into mice induces rapid crypt proliferation involving β -catenin stabilization (Sansom et al., 2004; Andreu et al., 2005; Kim et al., 2005). *Lgr5*⁺ CBC were shown to be highly responsive to such modulations in Wnt signaling, expanding upon stimulation by R-spondin1 or *Apc* loss and dying upon DKK1-mediated inhibition of the pathway (Barker et al.,

2009; Yan et al., 2012).

In addition to proliferation, Wnt/ β -catenin signaling also affects differentiation of ISC daughters. Both the localization of Paneth cells at the crypt bottom and their expression of lineage-specific genes are affected by modulations at different levels of the pathway (Crawford et al., 1999; van Es et al., 2005a; Andreu et al., 2008). Moreover, intestinal tumors in mouse models overexpress Paneth cell-specific genes, and this surprisingly also applies to human colorectal tumors despite the absence of these cells in the healthy colonic mucosa (Crawford et al., 1999; Andreu et al., 2005, 2008; van Es et al., 2005b). Finally, this pathway also affects migration along the crypt-villus axis by promoting expression of EphB receptors and repressing the expression of their Ephrin-B ligands, creating a gradient of their expression along the crypt-villus axis co-inciding with β -catenin activity. Since the interaction between EphB receptors and Ephrin-B ligands has a repellent effect, this mechanism keeps EphB-expressing Paneth and ISC restricted to the crypt bottom and prevents the downward migration of differentiating progenitors (Battle et al., 2002).

The major indicator of the role of Wnt/ β -catenin signaling in the intestinal epithelium is the extensive effects of its deregulation. The conditional deletion of both alleles of *Apc*, the most commonly mutated gene in colorectal cancer (CRC), in the mouse intestinal epithelium leads to β -catenin accumulation and re-localization to the nucleus, intense cell proliferation in the crypt compartment, along with impaired migration and differentiation (Sansom et al., 2004; Andreu et al., 2005). Due to the lack of differentiated cells in the intestinal epithelium, these mice die from malnutrition within days. Genetically engineered mouse models carrying a single *Apc*-mutant allele (*Apc*^{+/ Δ mice) have been useful to modeling CRC as certain crypts will sporadically acquire mutations on the other allele of *Apc* and form polyps and adenomas with high β -catenin activity [FIGURE 9B] (Colnot et al., 2004; Taketo and Edelman, 2009).}

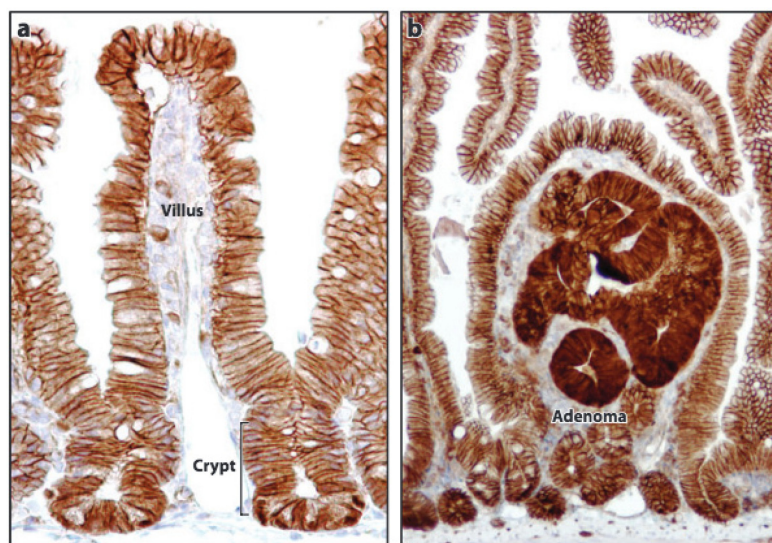


FIGURE 9. β -catenin staining reveals active Wnt signaling (determined by nuclear β -catenin staining) in the crypt bottom in the normal intestinal epithelium (A) and throughout adenomatous tissue of *Apc*^{+/ Δ mice (B). Adapted from van der Flier and Clevers, 2009.}

2.2.2 NOTCH SIGNALING

The Notch pathway is also key to ISC maintenance and daughter cell fate determination. Unlike other signaling pathways, Notch signaling works by lateral inhibition between two adjacent cells. Notch ligands DLL1, DLL4, or JAGGED1 at the cell surface will bind the NOTCH1 or 2 receptors on neighboring cells (Schröder and Gossler, 2002; Sander and Powell, 2004). Upon ligand binding, the receptor is cleaved by a γ -secretase complex, shedding its extracellular portion and releasing its intracellular domain (NICD), which translocates into the nucleus and promotes transcription of target genes involved in proliferation and differentiation. As previously mentioned, the best characterized NICD target, *HES1*, represses transcription of Notch ligands and of *ATOH1*, both of which are key to secretory lineage commitment [FIGURE 10] (Yang et al., 2001; Milano et al., 2004; Stanger et al., 2005).

Lineage tracing experiments reveal active Notch signaling in ISC (Fre et al., 2011; Pellegrinet et al., 2011). Signal inhibition at any step of the pathway converts all proliferative cells in the crypt into post-mitotic secretory cells [FIGURE 10] (Milano et al., 2004; van Es et al., 2005a; Riccio et al., 2008; Pellegrinet et al., 2011; VanDussen et al., 2012; Tsai et al., 2014; Carulli et al., 2016). This includes Lgr5⁺ ISC, which are consequently lost (VanDussen et al., 2012; Carulli et al., 2016), leading to lack of epithelial regeneration, nutrient malabsorption, weight loss, and death. On the other hand, expression of a constitutively active NCID results in increased proliferation and secretory cell depletion (Fre et al., 2005; Stanger et al., 2005). *Math1* deletion also results in depletion of all secretory lineages (Yang et al., 2001; Durand et al., 2012; Kim et al., 2012). NOTCH signaling therefore maintains the proliferating ISC pool and suppresses secretory lineage engagement. Beyond this, however, Notch signaling does not appear necessary for absorptive lineage differentiation as simultaneous *Math1* deletion and Notch disruption does not prevent enterocyte differentiation (Kazanjan et al., 2010; van Es et al., 2010; Kim and Shivdasani, 2011). Furthermore, while the effects of Notch activation on proliferation are only possible in Wnt-activated cells, its effects on differentiation are Wnt-independent (Fre et al., 2009). The current model postulates that Wnt-induced stem cells are maintained in a Notch-high state by neighboring Notch ligand-expressing cells, and that upon exit from the ISC niche, the bimodal nature of the pathway will result in some daughters' expression of *Dll1* and *Math1* and repression of *Notch*, leading to their secretory lineage commitment. These cells will then repress such a commitment in their neighboring TA cells, ensuring an appropriate absorptive-to-secretory lineage ratio. Paneth cells express Notch ligands (Sato et al., 2011a) and thereby sustain active Notch signals in their neighboring ISC, although other sources of Notch ligands also likely exist in the crypt as stem cells are maintained despite the absence of Paneth cells in the colon and in the small intestine of *Math1*-knockout mice (Durand et al., 2012; Kim et al., 2012).

Unsurprisingly, Notch signaling has been detected in both human and mouse intestinal adenomas, and aberrant Notch activation promotes adenoma formation in *Apc*-mutant mice (van Es et al., 2005a; Fre et al., 2009; Peignon et al., 2011). The opposite, however, is not true, as blocking Notch signaling does not prevent the initiation of *Apc*-mutant tumors, suggesting that β -catenin activation overrides the expected forced differentiation of proliferative cells (Peignon et al., 2011). Notably, Notch signaling is weaker in more advanced human carcinomas compared to adenomas, and its activation never leads to adenocarcinoma formation in *Apc*-mutant mice, indicating that Notch activation may be favorable (although not required) for tumor development but not for malignant progression (Fre et al., 2009).

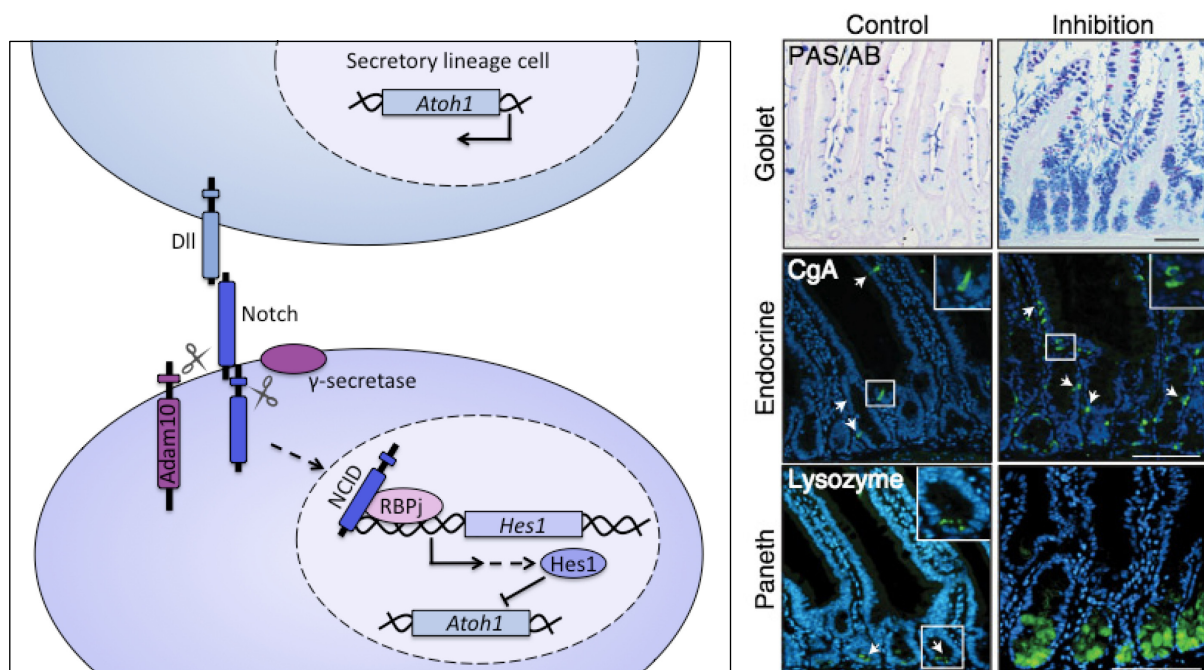


FIGURE 10. Notch signaling pathway in the intestine. Inhibition of Notch signaling favors differentiation into secretory lineage cells, including goblet cells (Periodic Acid Schiff/Alcian Blue staining), enteroendocrine cells (Chromogranin A staining), and Paneth cells (Lysozyme staining). Adapted from Demitrack and Samuelson, 2016.

2.2.3 HEDGEHOG AND BMP SIGNALING

Hedgehog-family proteins and bone morphogenic proteins (BMPs, part of the transforming growth factor beta or TGF- β superfamily) link epithelial homeostasis to mesenchymal signals. The Hedgehog receptor, Patched (PTCH), normally inhibits the constitutively active Smoothened (SMO) receptor, thereby permitting cleavage of GLI transcription factors into their repressive forms. Upon Hedgehog binding, PTCH is repressed, and SMO signaling allows GLI-mediated transcriptional activation of Hedgehog target genes. Hedgehog signaling is exclusively paracrine in the adult intestine; Indian Hedgehog and Sonic Hedgehog (IHH and SHH), the main Hedgehog ligands in the intestine, are secreted by enterocytes and bind PTCH receptors on neighboring mesenchymal cells, inducing BMP production from these cells (Madison et al., 2005; van Dop et al., 2009). Binding of BMP to its type 2 receptor, BMPR2, results in the phosphorylation and activation of its type 1 receptor, BMPR1, which subsequently phosphorylates SMAD proteins 1, 5, or 8. These will then heterodimerize with SMAD4 before translocating to the nucleus to induce

transcription of target genes **[FIGURE 11]**. In the intestine, BMP2 and BMP4 are produced by intravillus and intercryptic mesenchymal cells, and BMPR1 is expressed in differentiated cells and ISC but not in the proliferative TA cells (Haramis et al., 2004; He et al., 2004). BMP antagonists like Noggin or Gremlins are expressed in subcryptic myofibroblasts and smooth muscle (He et al., 2004; Kosinski et al., 2007). As a consequence, BMP activity affects only the differentiated compartment of the epithelium.

The constitutive activation of Hedgehog signaling results in increased BMP signaling and depletion of proliferative cells and ISC (van Dop et al., 2009). Conversely, either inhibition of Hedgehog or deregulation of the BMP pathway results in expansion of the ISC compartment, excessive crypt formation, and the formation of hamatomatous polyps (Haramis et al., 2004; He et al., 2004; Madison et al., 2005; Davis et al., 2015; Qi et al., 2017). Explaining this phenomenon, BMP signaling was shown to directly interfere with Wnt-mediated β -catenin activation through a mechanism involving PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase) and PTEN (Phosphatase and tensin homolog) (Waite et al., 2003; He et al., 2004). Hedgehog and BMP signaling therefore act in conjunction to determine regionalization of the epithelium by restraining proliferation and promoting differentiation.

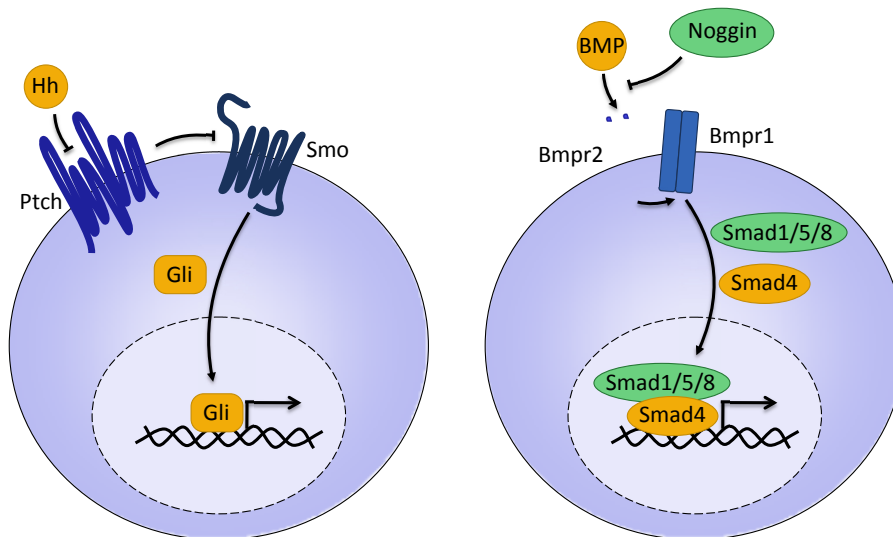


FIGURE 11. Hedgehog and BMP signaling pathways

2.3 ORGANOID CULTURE AND THE EXPERIMENTAL NICHE

Whole small intestinal crypts can be cultured *ex vivo* in matrigel and using a supplemented medium, leading to the formation of intestinal organoids that, as previously noted, mimic the structure of the *in vivo* epithelium. Although this has often been labelled as “niche-independent” growth of ISC, it highlights the importance of a minimal molecular niche for proper ISC function. Among the key supplements to organoid culture medium are Wnt agonist and LGR5 ligand R-spondin1, the BMP antagonist Noggin, and EGF (epidermal growth factor) (Sato et al., 2009). When isolated ISC rather than whole crypts are cultured, exogenous Wnt ligands (typically WNT3a) must be added to the culture medium. Although this allows for ISC maintenance and proliferation, the resulting organoids no longer contain all the differentiated cells of the

homeostatic epithelium but rather seem to consist largely of undifferentiated progenitors that form a spheroid structure (Sato et al., 2011a). This is likely due to the ubiquitous delivery of Wnt ligand, as opposed to the more homeostatic gradient created by co-culture with Paneth cells.

Isolated colon crypts produce insufficient amounts of Wnt ligands to maintain colonic ISC in culture, therefore WNT3a must be added for colonic organoid growth (Sato et al., 2011b). Like small intestinal organoids with exogenous WNT, however, these do not properly differentiate. In the case of human small intestinal and colon crypts, addition of further factors is required for efficient long-term culture – Gastrin, Nicotinamide, an ALK receptor inhibitor and an inhibitor of p38 MAPK (mitogen activated protein kinase) – suggestive of additional important pathways in human ISC function. A final component that must not be neglected for ISC maintenance and organoid growth is the substrate upon which crypts or ISC are plated. *In vivo*, the epithelium is surrounded by an ECM enriched in Laminin at the crypt base. For organoid growth, ISC are embedded in Laminin-rich matrigel. Furthermore, isolated epithelial cells die by anoikis, and the addition of ROCK (Rho-associated protein kinase) signaling inhibitor Y-27632 to the culture medium also improves organoid formation (Sato et al., 2009).

The implication of different signaling pathways in intestinal homeostasis can be tackled *in vitro* as organoids recapitulate the *in vivo* epithelial response. Organoid culture also has the added advantage of easier manipulation of treatments and the possibility to follow epithelial response over time while excluding the mesenchymal response. Recently, Yin et al. found that combined treatment with the GSK3 β inhibitor CHIR99021 and the histone deacetylase (HDAC) inhibitor Valproic acid resulted in organoids containing almost exclusively proliferative Lgr5-EGPF-positive cells. Conversely, different combinations of Wnt or Notch inhibitors can be used to force differentiation into the different lineages of the epithelium (Yin et al., 2014). These models could therefore be used to analyze epithelial cell-type specific responses. Moreover, co-culture of intestinal organoids with lamina propria fibroblasts or lymphocytes has also been used to characterize epithelial-mesenchymal cell interactions in the intestine (Farin et al., 2012; Kabiri et al., 2014).

2.4 THE INTESTINAL STEM CELL NICHE

2.4.1 THE EPITHELIAL NICHE: PANETH CELLS AND DEEP CRYPT SECRETORY CELLS

One epithelial cell type stands out with regards to its involvement in the ISC niche. In addition to AMPs, Paneth cells also produce many of the essential trophic factors regulating the ISC niche, including WNT3, WNT6, WNT9, DLL4, DLL1, EGF and TGF- α (transforming growth factor alpha) (Gregorieff et al., 2005; Sato et al., 2011a). Recently, Farin et al. examined the establishment of the WNT gradient observed *in vivo* from Paneth cells. Surprisingly, they found that WNT3 acts not as a diffusible gradient but as a FZD-bound signal on the basolateral membrane of cells at the crypt bottom that is diluted up the crypt by cell division (Farin et al., 2016). They also report that WNT3 does not localize in Paneth cell granules along with AMPs, implying that it is secreted through another mechanism. This is in agreement with a previous report that put forward a

RAB8a (Ras-related proteins in brain 8a) vesicle-dependent secretion of Wnt ligands (Das et al., 2015).

It has been proposed that the Paneth cell zone defines the ISC niche. As ISC divide and their numbers increase, they will neutrally compete to either remain between Paneth cells and therefore maintain an ISC identity or drift out of the niche and adopt a TA fate (Snippert et al., 2010; Ritsma et al., 2014). An opposing hierarchical model argues that intestinal stem cells divide mostly asymmetrically, giving rise to both a new stem daughter and a TA daughter independently of niche specification. Consistent with Paneth cells delimiting the ISC niche, partial ablation of Paneth cells results in a decrease in the number of Lgr5⁺ ISC, with the remaining ISC crowded around the remaining Paneth cells (Sato et al., 2011a). Surprisingly, however, the total elimination of Paneth cells does not result in the loss of ISC [FIGURE 12A] (Shroyer et al., 2007; Durand et al., 2012; Kim et al., 2012). The space normally occupied by Paneth cells is instead filled by ISC with active Wnt/ β -catenin signaling. In fact, proliferation is increased in Paneth-cell deficient crypts (Shroyer et al., 2007; Durand et al., 2012; Kim et al., 2012) and the regenerative capacity of ISC following irradiation is not affected (Durand et al., 2012). Paneth cells are therefore not required for ISC survival and proliferation. They are also not essential for stem cell localization, as ISC are retained at the crypt bottom despite the lack of Paneth cells. In fact, the small intestines of certain mammalian species, namely dogs and pigs, completely lack Paneth cells (Potten et al., 1997).

In vitro, however, Paneth cell-deficient crypts cannot form organoids (Durand et al., 2012). The same is true of Paneth cell-derived WNT3, the expression of which is dispensable for stem cell maintenance *in vivo* but essential for the growth of intestinal organoids *ex vivo* (Farin et al., 2012). *Wnt3*- or Paneth cell- deficient organoids can be maintained in culture by addition of exogenous WNT3a or by co-culture with intestinal mesenchymal cells [FIGURE 12B] (Sato et al., 2011a; Durand et al., 2012; Farin et al., 2012; Kabiri et al., 2014). The absence of Paneth cells *in vivo* must therefore be compensated by other niche cells likely providing Wnt ligands. WNT2b is the only Wnt ligand normally expressed by the mesenchyme capable of rescuing *Wnt3*- or Paneth cell-deficient organoid growth; it has therefore been suggested as the potential compensatory Wnt signal *in vivo* (Farin et al., 2012). Of course, the existence of another compensatory mechanism should not be excluded. Mesenchymal cell populations capable of compensating for the loss of Paneth cells will be discussed in the next section. Of note, exogenous WNT6 or WNT9b also rescue *Wnt3*-deficient organoid growth, suggesting that the amount produced by the crypt epithelial cells is not sufficient to compensate WNT3 depletion.

Paneth cells have been shown not just to provide growth factors for ISC, but also to orchestrate their response to nutrient availability. It makes sense for the intestinal epithelium to be highly responsive to diet and nutrient availability. In response to calorie restriction, villi shorten along with the TA compartment, while ISC numbers increase. This increase in ISC numbers and proliferation is accompanied by an increase in Paneth cells. Inhibition of mTORC1 (mammalian target of rapamycin complex 1) is a classically described cellular response to nutrient deprivation. In 2012, Yilmaz et al. showed that the augmented ISC renewal following calorie

restriction is dependent on reduced mTORC1 activity in Paneth cells, resulting in increased extracellular cyclic ADP ribose production (Yilmaz et al., 2012). Igarashi and Guarente complemented this study by showing that this paracrine signal activates Sirtuin1 and mTORC1 signaling in ISC, promoting their self-renewal. Paneth cell signaling therefore overrides direct nutrient sensing in ISC to coordinate an adapted response (Igarashi and Guarente, 2016). A new study by Rodriguez-Coman et al. showed that while Lgr5⁺ ISC have high mitochondrial activity, Paneth cells are highly glycolytic. Blocking glycolysis in Paneth cells hindered their ability to support organoid formation in culture, and blocking oxidative phosphorylation in ISC also reduced organoid formation. On the other hand, providing lactate to isolated Lgr5⁺ ISC enhanced organoid formation. The authors therefore propose that Paneth cells, through their high glycolytic activity, provide lactate to ISC, enabling their high mitochondrial metabolism and proliferative capacity (Rodríguez-Colman et al., 2017). Another recent study by Schell et al. challenges this notion, showing that ISC surprisingly do not depend on pyruvate and carbohydrate oxidation; in fact, blocking pyruvate transport into mitochondria *enhances* ISC function, as fatty acid oxidation is presumably turned to as a source of energy (Schell et al., 2017). Similarly, Mihaylova et al showed that fasting promotes ISC function and regeneration by inducing fatty acid oxidation (Mihaylova et al., 2018). Nevertheless, these findings put forward a new potential niche function of Paneth cells in supporting ISC metabolism and orchestrating their adaptation to their metabolic environment.

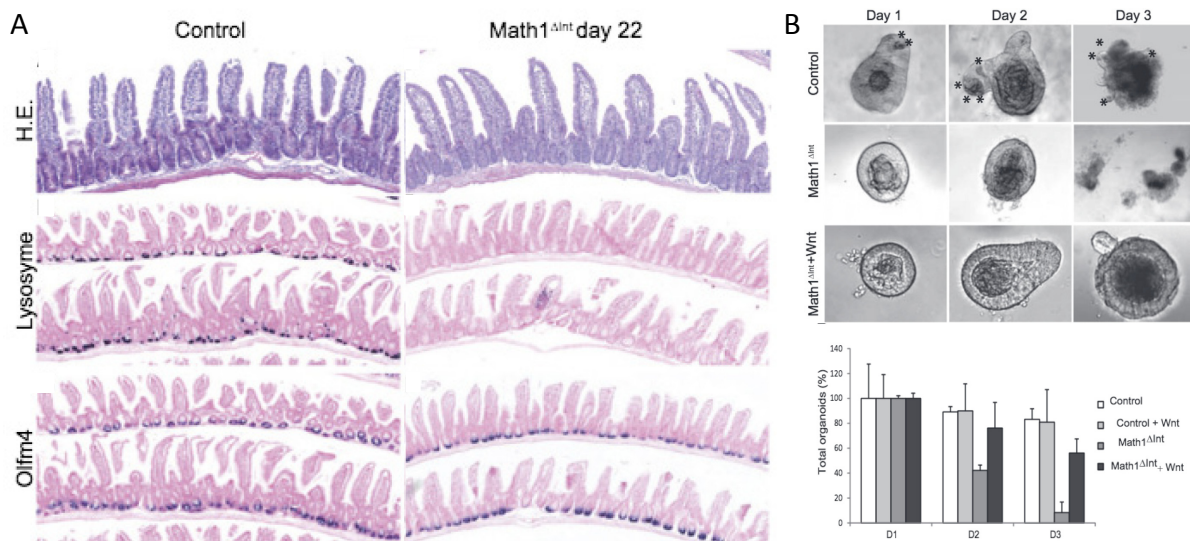


FIGURE 12. The Paneth cell niche is dispensable *in vivo* but essential for ISC function *ex-vivo*. (A) Following *Math1* deletion, the architecture of the intestinal epithelium and ISC pool (*Olfm4* mRNA *in-situ* hybridization) are preserved *in vivo* despite the absence of Paneth cells (*Lysozyme* mRNA *in-situ* hybridization). (B) Paneth cell deficient organoids from isolated *Math1*-knockout crypts rapidly die *ex-vivo*. Addition of exogenous *Wnt* ligand to the culture medium rescues the survival of Paneth cell-deficient organoids.

Adapted from Durand et al., 2012.

Although Paneth cells are absent in the colon, Lgr5⁺ ISC are still found intercalated between secretory cells. These cells, first coined “deep crypt secretory” (DCS) cells in 1983 by G.G. Altmann, are distinct from goblet cells but still contain mucous vacuoles in their cytoplasm (Altmann, 1983). Morphologic analysis of DCS cells reveals many shared features with small intestinal Paneth cells: they have a highly developed endoplasmic reticulum (ER) and Golgi

complex, they mature as they migrate down from the midcrypt to the crypt base, and their numbers vary from the proximal to the distal colon. With this background in mind, Rothenberg et al. identified a new goblet cell type that expresses several factors involved in ISC maintenance, such as DLL1, DLL4 and EGF (Rothenberg et al., 2012). These cells can be recognized by the marker cKIT, also expressed by small intestinal Paneth cells, and intercalate between Lgr5⁺ ISC in the colon. These cKit⁺ cells were also shown to express *Reg4* (regenerating islet-derived 4) and correspond to Altmann's DCS cells. Just recently, Sasaki et al. characterized the gene expression profile of Reg4⁺ DCS cells and found that they express several other Notch and EGF ligands along with goblet cell markers, although their signature matches that of Paneth cells more closely than that of goblet cells (Sasaki et al., 2016). Specific ablation of DCS cells by transgenic expression of DTR and DT treatment led to a loss of Lgr5⁺ stem cells. Consistently, co-culture of Lgr5⁺ stem cells with cKit⁺ and Reg4⁺ DCS cells improves organoid growth while the disruption of DCS cells hinders it. Finally, DCS cell numbers are regulated by Notch signaling, as is the case for Paneth cells (Rothenberg et al., 2012; Sasaki et al., 2016). One key difference stands out between the two cell types: DCS cells, unlike Paneth cells, do not produce any Wnt ligands (Sasaki et al., 2016), although colon Lgr5⁺ cells do depend on them for growth *in vitro* (Sato et al., 2011b). As in the Paneth cell-depleted small intestine, another extra-epithelial source of WNT likely exists in the colon to maintain ISC homeostasis.

2.4.2 THE MESENCHYMAL NICHE

Stromal cells have been shown to play a crucial role in the homeostasis of the intestinal epithelium as well as in its response to different physiological stresses. Several key signaling pathways regulating epithelial proliferation and differentiation involve epithelial-mesenchymal communication, although the specific mesenchymal cell types involved are not yet fully established. Indeed, the identification of different populations of stromal cells in the lamina propria and their functional interactions with the epithelium is a subject of ongoing research. During embryogenesis, interactions between the endodermal epithelium and mesodermal stroma are key to the development of the gastrointestinal tract. In the adult, stromal cells have been shown to provide Hedgehog, BMP, Wnt ligands and Wnt antagonists like DKK, R-spondins, as well as ECM components and other signals important for epithelial homeostasis. Moreover, as small intestinal organoids lack the formation of extruding villi, the lamina propria is likely also necessary for complete crypt-villus axis patterning and morphology.

Stromal cells provide Wnt signals both in the colon, where DCS cells do not produce them, and in the small intestine, in complement to Paneth cells. Similar to the epithelial deletion of *Wnt3* by Farin et al. or Paneth cell ablation by Durand et al., Kabiri and coworkers used epithelium-specific genetic ablation of *Porcn*, encoding the ER O-acetyltransferase Porcupine required for WNT secretion and activity, to demonstrate that epithelial Wnts were dispensable *in vivo* but necessary for organoid growth *ex vivo* (Durand et al. 2012; Farin et al 2012; Kabiri et al., 2014). On the other hand, pharmacologic inhibition of Porcupine (affecting both the epithelium and surrounding mesenchyme) or whole-body deletion of Wntless (another protein required for WNT secretion) results in Lgr5⁺ ISC loss (Kabiri et al., 2014; Valenta et al., 2016). Kabiri et al.

further demonstrate that Porcupine-deficient organoid growth can be rescued by co-culture with intestinal stromal cells (Kabiri et al., 2014). The same group later showed that this was dependent on stromal WNT production (Greicius et al., 2018).

Several studies have since aimed at identifying the WNT-producing mesenchymal cell populations involved in both the small intestine and colon ISC niche, with the inevitable setback of the lack of clearly defined mesenchymal subpopulations and corresponding markers.

Subepithelial myofibroblasts create a layer of stromal cells in very close contact with the crypt epithelium. Different markers have been used to label this population including the smooth muscle markers MYH11 (myosin heavy chain 11) and α SMA (α smooth muscle actin) as well as the PDGF (platelet-derived growth factor) receptor PDGFR α , although none of these markers are exclusive to subepithelial myofibroblasts. Pericryptal myofibroblasts secrete WNT ligands essential for neonatal crypt formation (Valenta et al., 2016; Greicius et al., 2018). However, simultaneous deletion of Porcupine in epithelial cells and myofibroblasts does not disrupt crypt homeostasis (San Roman et al., 2014), suggesting that other WNT-producing cells of the lamina propria exist.

One recent report focused on CD34⁺ GP38⁺ mesenchymal cells, which are negative for endothelial (CD31⁻), hematopoietic (CD45⁻) and myofibroblast (α SMA⁻) markers and surround crypts in both the small intestine and colon. Co-culture of organoids with these cells results in the formation of highly proliferative spheroids, similar to the addition of exogenous Wnt. Moreover, this overrules the need for R-spondin supplementation. Indeed, CD34⁺ GP38⁺ crypt stromal cells express high levels of *Wnt2b*, *Gremlin1* and *Rspo1* as compared to other lamina propria mesenchymal cell types (Stzepourginski et al., 2017). These cells could therefore be an essential mesenchymal component of the ISC niche. Another study found that depletion of mesenchymal cells expressing *Foxl1* (forkhead box L1, previously known as *Fkh6*), disrupted epithelial morphology, including loss of stem and proliferative cells (Aoki et al., 2016). Very recently, the same group characterized Foxl1⁺ cells as telocytes with long processes called telopodes that encompass all crypt cells and are less than micrometers away from the epithelium. Foxl1⁺ cells express numerous crypt-regulating signals in a localized manner: at the crypt bottom, they express WNT2b and R-spondin3, whereas they express BMP and Wnt inhibitors higher towards the crypt-villus junction. Similar to the depletion of Foxl1⁺ cells, blocking WNT secretion specifically from those cells resulted in loss of ISC (Shoshkes-Carmel et al., 2018). Interestingly, the CD34⁺ cells identified in the former study express *Foxl1*, whereas the Foxl1⁺ cells in the latter studies express CD34, suggesting that these may be overlapping populations. A third group approached the question by looking for WNT2b-expressing cells in the lamina propria, and found that subepithelial mesenchymal cells expressing WNT2b are a predominantly Gli1-positive (Valenta et al., 2016). They recently went on to show that blocking WNT secretion from Gli1⁺ cells disrupted colonic stem cell renewal and consequently the integrity of the colonic epithelium. Although not essential at basal levels in the small intestine, these cells expand in numbers and become essential in the small intestine in the absence of

epithelial Wnt ligand production (Degirmenci et al., 2018). Of note, Gli1⁺ cells represent a heterogeneous population including cells expressing CD34 and cells expressing *Foxl1*.

Stromal cells of the lamina propria have also been implicated as key players in epithelial regeneration after injury. Both the CD34⁺ and Gli1⁺ populations mentioned above were shown to play a role in epithelial regeneration following Dextran Sulfate Sodium (DSS) treatment, commonly used to induce colonic epithelium damage and inflammation (Stzpourginski et al., 2017; Degirmenci et al., 2018). Pericriptal fibroblasts have also been shown to drive epithelial response to bacterial infection by producing IL-33, which suppresses Notch signaling and drives differentiation towards secretory lineages (Mahapatro et al., 2016). In addition to stromal cells, microvasculature endothelial cells and enteric neurons have also been shown to play a role in maintenance of the epithelium after injury (Bjerknes and Cheng, 2001; Paris et al., 2001; Maj et al., 2003).

Immune cells in the lamina propria can also interact with the epithelium and its stem cells. Defined subsets of hematopoietic cells such as $\gamma\delta$ T lymphocytes (Komano et al., 1995) or macrophages (Pull et al., 2005; Bansal et al., 2011; Saha et al., 2016) have been reported to affect epithelial homeostasis or recovery following infection or tissue damage, although the signals involved in these interactions remain largely unclear. In one study, stromal macrophages were shown to release extracellular vesicles containing Wnt ligands to support epithelial regeneration upon radiation injury (Saha et al., 2016). Another Wnt-independent mechanism of immune cell support to the epithelium has also been put forward: Co-culture of intestinal organoids with isolated lamina propria lymphocytes stimulates Lgr5⁺ ISC expansion and growth of organoids in a way that is dependent on IL-22 production. The same effect is recapitulated both with isolated group 3 ILC and with recombinant IL-22 in a dose-dependent manner. This process is independent of Paneth cells or altered Wnt, Notch, or EGF activity, instead relying on STAT3 (signal transduce and activator of transcription 3) signaling in ISC (Lindemans et al., 2015). Upon injury *in vivo*, crypt-adjacent ILC, which lack antigen receptors but respond to cytokine signaling, produce IL-22 while epithelial cells upregulate their expression of the IL-22 receptor, overall promoting epithelial regeneration (Hanash et al., 2012; Lindemans et al., 2015). In counterpart, exacerbated IL-22 signals can promote tumorigenesis in the intestine (Huber et al., 2012; Kirchberger et al., 2013).

2.4.3 THE MICROBIAL NICHE

An impressive number of bacteria – considered to be over 10 times the number of human cells in the body – are housed within the human intestine. Colonization begins at birth (Huurre et al., 2008; Biasucci et al., 2010; Dominguez-Bello et al., 2010), but the composition of the microbiota varies throughout the host's lifetime, largely based on environmental factors like diet, lifestyle, use of antibiotics and geography (Rothschild et al., 2018). Generally speaking, anaerobic bacteria of the Firmicutes and Bacteroidetes phyla are the most abundant residents of the intestinal tract (Qin et al., 2010). Similar to the host tissue architecture, the spatial distribution of the gut microbiota varies along two axes. Very few bacterial species survive in the stomach (where bacterial density is around 10¹ bacterial cells per gram) and duodenum (10³ cells per gram) due

to the acidic luminal pH. Bacterial density then increases gradually along the cephalo-caudal axis, with approximately 10^5 cells per gram the jejunum, 10^8 cells per gram in the ileum, and a peak of up to 10^{12} cells per gram of bacteria in the colon [FIGURE 13]. On the other hand, bacterial numbers gradually decrease along the second axis, from the lumen to the crypts of the epithelium. This gradient is dependent on decreasing nutrient availability from food in the lumen, increasing oxygen content and the presence of mucus, AMPs, and IgA from the host tissue. Importantly, not just the density, but the composition of the microbiota also varies along these two axes [FIGURE 13]. The proximal end of the intestinal tract mainly carries oxygen and acid-tolerant species, including Helicobacteraceae and Lactobacillaceae. In contrast, the colon supports anaerobic bacteria like Lachnospiraceae, Bacteroidacea, and Prevotellaceae that actively ferment complex carbohydrates into short-chain fatty acids. Similarly, the conditions along the lumen-crypt axis also favor specific types of bacteria; for example, anaerobic bacteria dominate towards the lumen, while aerobic bacteria are more common towards the crypts (Pedron et al., 2012; Sommer and Bäckhed, 2016).

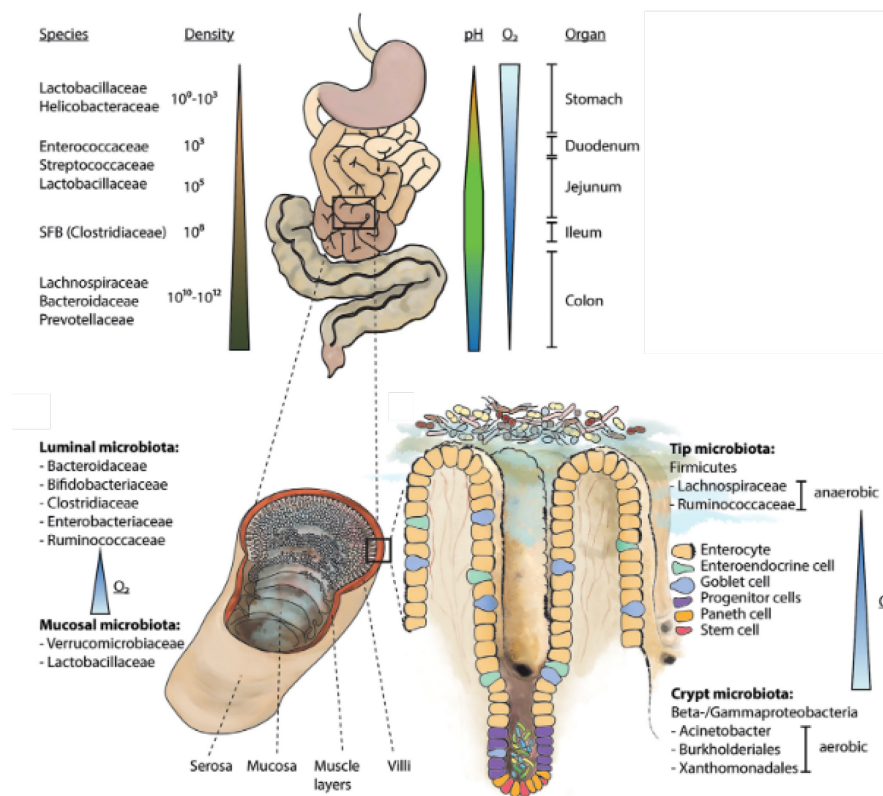


FIGURE 13. Distribution and composition of the gut microbiota along the cephalo-caudal and lumen-crypt axes. Adapted from Sommer and Bäckhed, 2016.

The varying densities and types of bacteria along with the different metabolites they produce can be detected by different host cells in the epithelium and lamina propria. These signals, or combinations of signals, will induce specific responses in different host cell types; for example, microbial signals in the crypt predominantly regulate epithelial cell expression of genes involved in proliferation, cell cycle, DNA replication and repair, whereas microbial signals in the villus tip drive expression of genes involved in immunity and metabolism (Sommer et al., 2015).

Specific molecular patterns unique to a subset of microorganisms (termed microbe-associated molecular patterns, or MAMPs) are sensed by host pattern recognition receptors (PRRs), including transmembrane Toll-like receptors (TLRs) and intracellular NOD-like receptors (NLRs) [FIGURE 14]. These PRRs play an essential role in innate immunity by allowing host cells to distinguish between different types of microorganisms. For example, TLR4 recognizes lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria whereas TLR2 binds lipoteichoic acid (LTA), a major constituent of the cell wall of Gram-positive bacteria. TLR5 distinguishes flagellated bacteria by binding Flagellin, the main structural protein of the flagellum. Most TLRs stimulate central signaling cascades like Nuclear factor κ B (NF κ B) or MAPK signaling through the adaptor protein MyD88 (Myeloid differentiation primary response gene 88), resulting in the production of pro-inflammatory cytokines. As NLRs are intracellular, they detect invasive bacteria. The NLR NOD2 is particularly recognized for its role in intestinal physiology, as mutations of the *Nod2* gene are associated with susceptibility to Crohn's disease (Rioux et al., 2007). NOD2 is expressed in monocytes, Paneth cells, and in ISC (Lala et al., 2003; Nigro et al., 2014) and detects muramyl dipeptide (MDP), a component of peptidoglycan (PGN) common to both Gram-positive and Gram-negative bacteria. Like TLRs, NLRs stimulate NF- κ B signaling but also induce activation of the inflammasome, which promotes the caspase-mediated maturation and secretion of pro-inflammatory cytokines [FIGURE 14].

Both intestinal epithelial cells and immune cells of the lamina propria express these PRRs. To prevent the constant activation of inflammatory responses despite the vast expression of PRR ligands by commensals, several mechanisms are put in place. First, PRR expression is kept low in intestinal epithelial cells and can be upregulated during inflammation (Abreu, 2010). Second, expression of TLRs and their co-receptors can be spatially restricted to the apical or basolateral side of the polarized epithelial cells; this way a distinction could be made between luminal commensals and pathogens that have crossed the epithelial barrier (Abreu, 2010). Third, regulated host expression of molecules that inhibit TLR signaling – like A20 (Vereecke et al., 2014), Tollip (Otte et al., 2004), or PPAR γ (peroxisome proliferator-activated receptor γ) (Neish et al., 2000; Kelly et al., 2004; Petrof et al., 2004) – can also modulate immune responses. Finally, metabolites produced by commensal bacteria have been shown to induce anti-inflammatory signaling (Menard, 2004). The combination of all these mechanisms establishes a balance of tolerogenic and inflammatory host-microbiota interactions in the healthy gut.

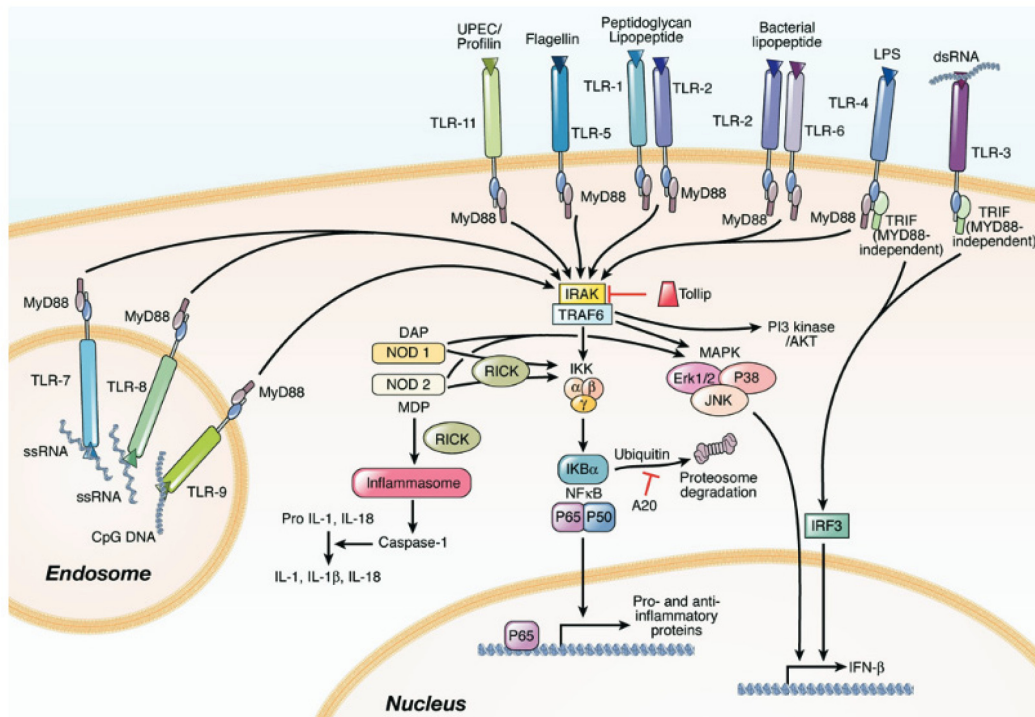


FIGURE 14. Common Pattern Recognition Receptors and downstream signaling pathways. Adapted from Sartor, 2008.

In germ-free animals, the architecture of the intestinal epithelium is affected: crypts are shorter with lower proliferative rates, the microvillus brush border is impaired, and the permeability of the epithelium is higher compared to conventionally raised animals (Abrams et al., 1963; Alam et al., 1994). The epithelium of germ-free animals also have fewer goblet cells and a thinner mucus layer, and this can be reversed in the presence of MAMPs like LPS or PGN (Sharma et al., 1995; Petersson et al., 2011). Similarly, although Paneth cells appear independently of luminal contents, their production of AMPs is stimulated by the presence of the microbiota (Mallow et al., 1996; Falk et al., 1998; Ayabe et al., 2000; Pütsep et al., 2000; Liu et al., 2006; Vaishnava et al., 2008). The microbiota therefore helps maintain the barrier function of the intestinal epithelium. Furthermore, several studies have shown that TLR-mediated recognition of commensals is necessary for the regeneration of the epithelium in response to injury (Rakoff-Nahoum et al., 2004; Fukata et al., 2005).

The fact that epithelial kinetics are slowed in germ-free animals (Abrams et al., 1963; Alam et al., 1994; Reikvam et al., 2011) suggests a direct or indirect effect of the microbiota on ISC dynamics. One mechanism by which the microbiota affects ISC is through microbial metabolites, several of which were discovered to suppress colon stem and progenitor cell proliferation in a recent screen by Stappenbeck's group (Kaiko et al., 2016). Of these, butyrate, a product of bacterial dietary fiber fermentation, had the most drastic effects, suppressing proliferation at low concentrations and inducing apoptosis at concentrations closer to those found in the colon lumen. The authors postulate that the crypt architecture and colonocytes' ability to oxidize and metabolize butyrate create a luminal gradient of butyrate down the crypt regulating stem and progenitor cell proliferation. Interestingly, butyrate-mediated inhibition of proliferation

depends on epigenetic changes involving the histone deacetylase HDAC3, known to link bacterial signals to epithelial response in the intestine (Alenghat et al., 2013).

Because of their localization furthest from luminal contents in the mucus- and AMP-protected crypt bottom, it was long thought that homeostatic ISC benefited from a sterile environment. Challenging this notion, work by Sansonetti's group revealed the existence of a restricted set of bacteria in colonic crypts of healthy mice, which they termed the "crypt-specific core microbiota" (Pedron et al., 2012). Interestingly, this predominantly aerobic population resembles the microbiota found in the midgut of invertebrates, hinting at a potential co-evolutionary selection of commensals favoring proper gut dynamics. Both the commensal microbiota and MyD88 activity modulate crypt proliferation at basal levels and are required for epithelial recovery from DSS-induced colitis, revealing a surprising regenerative effect of microbiota-induced PRR signaling in the gut (Rakoff-Nahoum et al., 2004). These observations were made all the more relevant by reports of direct expression of PRRs TLR4 and NOD2 by Lgr5⁺ ISC directly. The stimulation of these PRRs *in vivo* or in organoid cultures has drastically different effects on ISC; whereas treatment with TLR4 antigen LPS induces apoptosis of small intestinal Lgr5⁺ ISC (Neal et al., 2012) and favors differentiation over proliferation in colonic crypts (Naito et al., 2017), treatment with the NOD2 agonist MDP protects ISC from stress (Nigro et al., 2014). As will be discussed below, the microbiota can also affect stem cell dynamics by affecting their redox state. ISC are therefore in direct communication with the microbiota and can thereby adjust their response to the luminal microenvironment. These studies also bring a new perspective on the reported link between the presence of particular commensals and pathogens and the development of CRC (Machida-Montani et al., 2007; Castellarin et al., 2011; Kostic et al., 2012, 2013; Dejea et al., 2014).

2.5 REGULATION OF INTESTINAL STEM CELL INTEGRITY

Generally speaking, maintaining the genomic integrity of adult stem cells is crucial to prevent a mutation from becoming fixed or passed on to a substantial part of the tissue. For this reason, specific mechanisms are put in place in stem cells that often differ from those existing in downstream progenitors and differentiated cells (Mandal et al., 2011). In several tissues, like the hematopoietic system, adult stem cells are kept quiescent in a hypoxic niche with relatively low metabolic activity and low reactive oxygen species (ROS) production, which is thought protect them from accumulating DNA damage. ISC differ from this scheme in many aspects: they are highly proliferative, metabolically active and highly dependent on oxidative phosphorylation, the main intracellular source of ROS. Remarkably, despite their constant and rapid proliferation, ISC show little deterioration in functional competence as the organism ages. While the signaling pathways responsible for the renewal and differentiation of ISC are well established, the mechanisms controlling their protection and integrity throughout the organism's lifetime are far less understood.

2.5.1 CRYPT MONOCLONALITY & NEUTRAL DRIFT

One of the earliest-described properties of intestinal crypts, even before ISC markers were properly defined, is the tendency of cells within a single crypt to progressively drift towards monoclonality both during development and after a mutagenic event. Using functional mutations at one locus as a read-out, it was shown that shortly after a mutagenic event presumably affecting single ISC within each crypt, both monoclonal and mixed crypts (with cells expressing either the functional or non-functional mutant read-out protein) are present throughout the epithelium. Over time, however, the number of mixed crypts decreases while the number of monoclonal crypts increases (Loeffler et al., 1993). Assuming the acquired mutation is neutral (does not provide any competitive advantage over other stem cells within the same crypt), this means that the chances of a mutation being retained are inversely proportional to the number of stem cells in the crypt. This notion has also been supported by more recent studies giving rise to the previously mentioned neutral drift hypothesis [FIGURE 15], which postulates that ISC within a crypt neutrally compete for space in their defined niche, eventually resulting in a drift towards monoclonality over time (Lopez-Garcia et al., 2010; Snippert et al., 2010; Vermeulen et al., 2013; Ritsma et al., 2014; Huels et al., 2018). In this scenario, a mutated stem cell and its daughters would stochastically be retained in the crypt or not. This remains true even if the mutation at hand were to give it a proliferative advantage and therefore induce a biased drift, as is the case for *Apc* loss or *KRAS* activation – two common mutations in CRC (Lopez-Garcia et al., 2010; Vermeulen et al., 2013; Snippert et al., 2014). This mechanism is therefore thought to protect against the acquisition of deleterious mutations. Interestingly, mutations of *Tp53* – also common in CRC – do not confer a competitive advantage to mutant ISC under normal conditions, but do benefit colon stem cells following colitis-inducing DSS treatment (Vermeulen et al., 2013), hinting at a context-dependent advantage of certain mutations. Very recently, Huels et al. added further support to neutral drift hypothesis by modulating WNT secretion and thereby the size of the niche and number of ISC (Huels et al., 2018). They show that reducing ISC numbers accelerates monoclonal conversion of the crypt and with it the fixation of pro-tumorigenic mutations.

Another hypothesis put forward by Potten et al. in 2002 postulates that ISC preserve their integrity through deterministic asymmetric divisions, where components are selectively sorted to the stem or TA daughter cell (Potten et al., 2002). For example, the mother stem cell's template DNA would be kept in the stem cell daughter whereas the more error-prone newly synthesized DNA would be passed on to differentiating cells. However, this hypothesis has been put into question in the case of ISC, which appear to segregate the majority of their DNA randomly (Escobar et al., 2011). The proposed +4SC population that keeps its genomic material safe by remaining quiescent unless the proliferative population needs to be replaced could represent another way to preserve integrity of the ISC genome.

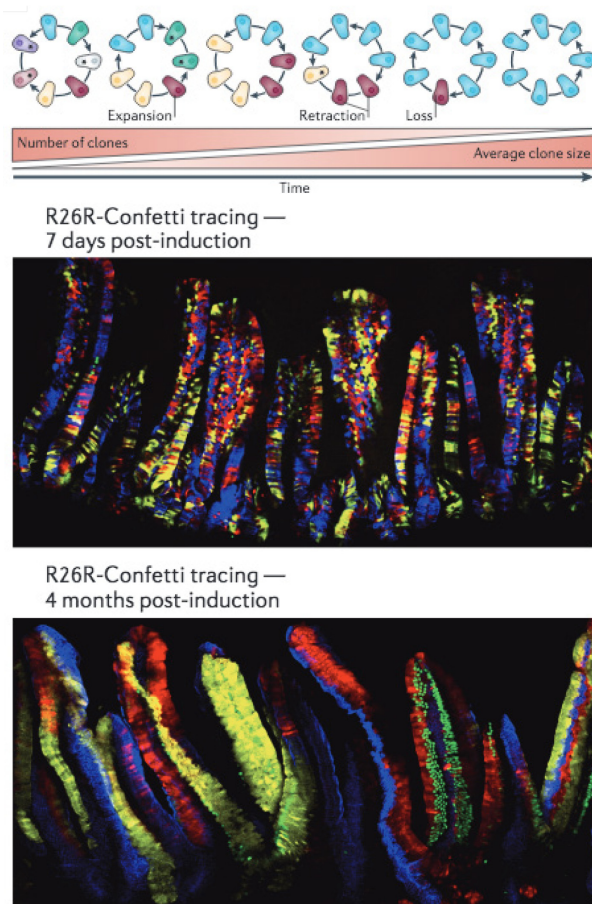


FIGURE 15. Neutral competition between intestinal stem cells contributes to intestinal homeostasis. Due to restricted niche space, intestinal stem cell proliferation leads to displacement of neighboring stem cells from the niche. As a consequence, clonal expansion (arrows) is compensated by the loss of other clones, ultimately resulting in the stochastic fixation of a single clone per crypt. Mouse small intestines expressing the multicolor Cre-reporter R26R-Confetti visually illustrate the competition between individual stem cell clones, each endowed with one of four fluorescent proteins. Over time, most crypts become clonal (single colored). Adapted from Vermeulen and Snippert, 2014.

2.5.2 P53 AND APOPTOSIS

The protein p53, encoded by the gene *TP53*, is a widely recognized tumor suppressor acting as both a transcription factor and an effector protein regulating cell cycle arrest, apoptosis, or senescence in response to stress. In doing so, it either allows damaged cells to recover or, in the face of excessive damage, eliminates the potential threat to tissue integrity (Levine and Oren, 2009). The cellular outcome following stress, such as DNA damage, will therefore depend on the rate of repair and severity of the insult, which will determine the duration and robustness of p53 activation. *TP53* is mutated in a large portion of human cancers, including colorectal cancer, and p53-null mice are known to rapidly develop a wide spectrum of tumors – particularly sarcomas and lymphomas. The vast array of anti-tumoral functions played by p53 in nearly all tissues and cell types has earned it the nickname of “guardian of the genome”.

At basal levels, loss of p53 in the intestinal epithelium does not result in any particular physiological changes: spontaneous apoptosis levels are unchanged (Merritt et al., 1994) and tumors do not spontaneously develop for up to 14 months (Schwitalla et al., 2013a; Chanrion et al., 2014), suggesting that additional mutagenic events are required along with the loss of p53. Also surprisingly, the loss of p53 does not favor tumor initiation or progression in *Apc*^{+/-} mice (Clarke et al., 1995; Demidov et al., 2007). However, p53 deletion prior to treatment with the carcinogen azoxymethane (AOM) prevents damage-induced apoptosis of proliferative cells in the crypt and promotes tumor development (Schwitalla et al., 2013a), indicating that an important function of p53 in the intestinal epithelium is the elimination of damaged, potentially

tumor-initiating cells. Deletion of p53 *after* AOM treatment results in more invasive tumors associated with a defect in barrier function, increased inflammatory signals, and epithelial-to-mesenchymal transition signatures. Likewise, concomitant deletion of p53 and activation of Notch signaling in the intestinal epithelium leads to the development of highly invasive tumors (Chanrion et al., 2014), highlighting an additional role of p53 in tumor progression to malignancy. This is consistent with the fact that *TP53* mutations are found in 50 to 70% of human colorectal carcinomas but not in adenomas, suggesting that loss of p53 function occurs as a late event in CRC that favors genomic instability and malignant progression (Vogelstein et al., 1988; Baker et al., 1989).

As the intestine is one of the most proliferative organs, it is also one of the most sensitive to genotoxic stresses induced notably by ionizing radiation (IR) or chemotherapy. Although low doses of irradiation elicit hematopoietic syndrome, higher doses (>14Gy) result in gastrointestinal syndrome, with heavy cell death in the intestinal crypts resulting in the ultimately lethal loss of intestinal function. IR-induced death in intestinal crypts peaks at around 6 hours after irradiation and is dependent on the rapid induction of p53 and its downstream effectors of apoptosis (Merritt et al., 1994; Qiu et al., 2008). Importantly, both nuclear p53 and IR-induced cell death are concentrated in positions one to 10 from the crypt bottom, affecting primarily ISC and early TA progenitors. The fact that loss of p53 renders intestinal epithelial cells resistant to IR-induced apoptosis supports the aforementioned idea that p53 mediates the removal of damaged cells with carcinogenic potential from intestinal crypts. Interestingly, ISCs were found to repair IR-induced DNA damages more efficiently than TA progenitors or differentiated cells (Hua et al., 2012), highlighting the importance of both preserving the genomic integrity and maximizing the survival of the highly sensitive ISC pool. 24 to 48 hours after irradiation, a second peak of apoptosis occurs that is this time independent of p53 and rather associated with a G2/M cell cycle checkpoint (Merritt et al., 1997). If the damage is not detrimental, the crypts then regenerate [FIGURE 16].

Generally speaking, colonic crypts are more resistant to genotoxic insult (induced either by IR or chemotherapeutic treatment) than small intestinal crypts (Merritt et al., 1994; Tinkum et al., 2015). This has been attributed to higher expression of the anti-apoptotic protein Bcl-2, which moderates the apoptosis of colon ISC not just after irradiation but also at basal levels (Merritt et al., 1995), as well as to more efficient resolution of DNA damages (Hua et al., 2017).

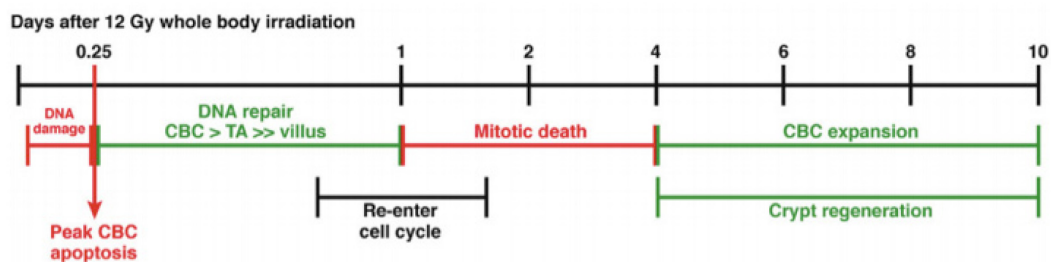


FIGURE 16. Schematic representation of response to 12 Gy irradiation in the small intestinal crypt. Adapted from Lund, 2012.

2.5.3 REACTIVE OXYGEN SPECIES

Initially considered only as a toxic bi-product of cellular metabolism, ROS have since been implicated in various biological processes including proliferation, differentiation and apoptosis. The most common cellular ROS are superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) [FIGURE 17A]. Mitochondrial respiration, the main intracellular source of ROS, produces mainly superoxide during the electron transport across the inner mitochondrial membrane [FIGURE 17B]. Therefore, intracellular ROS levels can be controlled by regulating mitochondrial metabolism and diverting substrates away from oxidative phosphorylation. Membrane-bound NADPH oxidases, which consume NADPH to generate superoxide and subsequently hydrogen peroxide, are another major source of ROS [FIGURE 17C]. In parallel, ROS levels are tightly regulated by various scavenging systems. O_2^- is highly reactive and can be reduced to H_2O_2 by Superoxide dismutase (SOD), and H_2O_2 is subsequently reduced to water (H_2O) and oxygen (O_2) by Catalase or other cellular antioxidants. These include Glutathione, one of the most abundant antioxidants synthesized by the cells, and the Glutaredoxin, Thioredoxin and Peroxiredoxin systems. As H_2O_2 has a longer half-life and can freely diffuse across membranes, it is thought to be the major ROS involved in intracellular signaling. ROS can directly oxidize proteins, lipids, and nucleotides and, if unrestrained, lead to excessive damages and oxidative stress. Certain proteins, called redox sensors, can undergo conformational changes following amino acid oxidation and subsequently induce specific signaling pathways. These notably include p53, ATM (ataxia telangiectasia mutated), p38 MAPK, AKT and Sirtuins, among many others. Conversely, several signaling pathways modulate ROS levels, including the transcription factors NRF2 (nuclear factor erythroid 2) and FOXO (forkhead box family, class O), both of which regulate the expression of proteins implicated in the antioxidant scavenging systems mentioned above (Bigarella et al., 2014).

In the intestine, mitochondrial activity is tightly regulated along the crypt-villus axis. Differentiated enterocytes have a high content of functional mitochondria and depend on oxidative phosphorylation, whereas the highly proliferative crypt progenitors are thought to be more glycolytic (Jeynes and Altmann, 1975; Lin et al., 2010; Stringari et al., 2012). In fact, modulating mitochondrial biogenesis and glycolysis affects villus formation during development and the ratio of proliferative crypt cells to differentiated villus cells in the adult small intestine (Lin et al., 2010; D'Errico et al., 2011; Kumar et al., 2016). As we previously noted, the inverse is true in the ISC niche, where differentiated Paneth cells are highly glycolytic and ISC have high mitochondrial activity – although their metabolic substrates remain a matter of debate (Rodríguez-Colman et al., 2017; Schell et al., 2017). In line with this, ISC have been shown to have high mitochondrial copy numbers, elevated mitochondrial ROS levels and p38 MAPK activation (Myant et al., 2013; Rodríguez-Colman et al., 2017; Schell et al., 2017). Mitochondrial ROS production plays an important role in ISC function as treatment with antioxidants blocks crypt formation in organoids. However, mitochondrial ROS production, mimicked by paraquat treatment, is not sufficient to induce organoid crypt formation (Rodríguez-Colman et al., 2017). Overall, this suggests a unique regulation of mitochondrial activity and ROS production in ISC compared to other crypt cells.

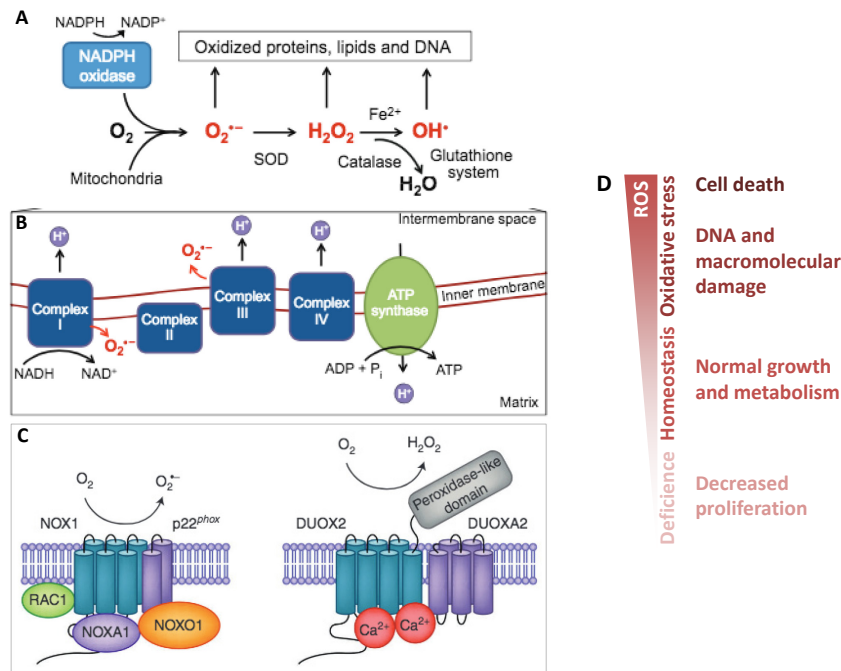


FIGURE 17. Reactive oxygen species production and scavenging. Reactive oxygen species (shown in A) including superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^{\cdot}), are generated from both the electron transport chain during phosphorylative oxidation in the mitochondria (shown in B) and membrane-bound NADPH oxidases (the two major epithelial NADPH oxidases, NOX1 and DUOX2, are shown in C). Various cellular antioxidant systems (shown in A), like superoxide dismutase (SOD), Catalase, and the Glutathione system, control levels of reactive oxygen species and prevent oxidative damage to proteins, lipids, and DNA. Maintaining a balanced cellular redox state is key to intestinal epithelial homeostasis (shown in D). Adapted from Bigarella et al., 2014 and Aviello and Knaus, 2018.

ROS scavenging activity is also variable along the crypt-villus axis, with 2-3-fold higher glutathione reductase, glutathione S-transferase, and glutathione peroxidase activity in the villus compared to the crypt, but nearly 10-fold higher SOD and Catalase activity in the crypt than in the villus (Chu and Steven Esworthy, 1995; Turan and Mahmood, 2007). As a result of both high mitochondrial activity and low antioxidant activity in differentiated villus cells, accumulated ROS are thought to contribute to the induction of anoikis at the villus tip (Turan and Mahmood, 2007; D'Errico et al., 2011). On the other hand, crypt cells are considered to be better protected against oxidative damage.

Finally, intestinal epithelial cells also express NADPH oxidases at their apical membrane, in particular NOX1 (NADPH oxidase 1) and DUOX2 (Dual oxidase 2), that trigger oxidative bursts as an antimicrobial defense mechanism. ROS production from NADPH oxidases has also been shown to contribute to proliferation, regeneration, and differentiation in the intestinal epithelium (Coant et al., 2010; Jones et al., 2013; Leoni et al., 2013; Myant et al., 2013). For example, specific bacterial species (including *Lactobacillus rhamnosis* but not *Escherichia coli*) induce epithelial ROS production and subsequent crypt proliferation in a NOX1-dependent manner (Jones et al., 2013). Jones et al. further showed that this had a cytoprotective effect on crypt cells in the context of irradiation via the activation of NRF2 (Jones et al., 2015). Modulation of ROS production and antioxidant mechanisms is therefore a key mediator of microbiota-crypt interactions. As previously noted, the microbiota can also directly stimulate NOD2 signaling in

ISC, affecting their survival in response to ROS-induced stress (Nigro et al., 2014), although the cytoprotective mechanisms downstream of NOD2 remain to be elucidated.

Fine-tuned regulation of ROS not only plays a role in ISC and epithelial homeostasis, but also in tumor development. For example, the loss of *Apc* has been shown to induce transcription of both RAC1, a GTPase that plays a key role in ROS production as a component of NOX1, and TIGAR, a key enzyme of the pentose phosphate pathway, which in addition to producing nucleotides acts as a major source of reduced glutathione. Although these two enzymes play seemingly opposite roles with regards to ROS, the deletion of either *Rac1* or *Tigar* stunts proliferative responses. The effects of *Rac1* deletion can be rescued by treating mice with paraquat, while those of *Tigar* deletion can be rescued by treatment with ROS scavengers like N-Acetyl Cystein (NAC), illustrating the deleterious effects of either insufficient and excessive ROS [FIGURE 17D] (Cheung et al., 2013, 2016; Myant et al., 2013). Moreover, both *Rac1* and *Tigar* deletion reduce tumor formation in *Apc*^{+/ Δ} mice. Thus, whereas controlled ROS production is required for continued proliferation, ROS scavenging prevents ROS-induced apoptosis in both the homeostatic crypt and tumor cells.

III. COLORECTAL CANCER

3.1 PREVALENCE AND RISK FACTORS

CRC is the third most commonly diagnosed cancer worldwide in both men and women, with an estimated 1.36 million new cases and 694,000 deaths in 2012. In France, CRC is also the third most common cancer (after prostate and breast cancer) with over 40,000 new cases each year, and the second most lethal cancer (after lung cancer) with 17,500 deaths each year [FIGURE 18].

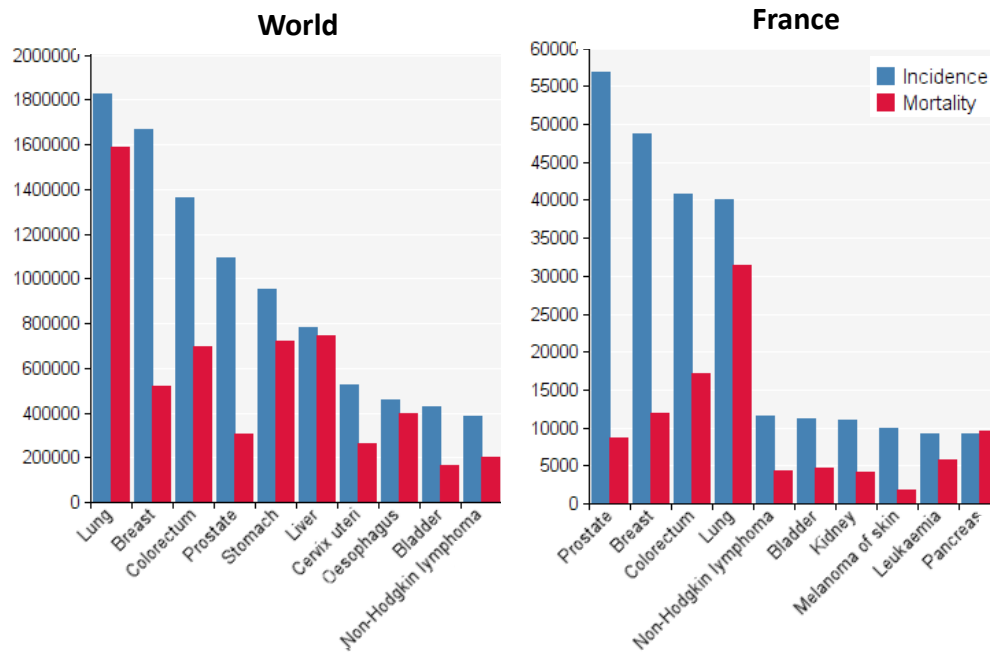


FIGURE 18. Incidence and mortality associated with the ten most common cancer types world-wide and in France. Data from Ferlay et al., 2015.

Several factors contribute to the onset of CRC. Such risk factors include:

- Age: 95% of new cases are detected in patients aged over 50, with the mean detection age around 70 for men and 73 for women.
- Genetics: Although the majority of CRC are sporadic, 20-30% of cases occur in patients with a family history of CRC and 5% of these arise in the setting of inherited syndromes, the two most common being familial adenomatous polyposis (FAP) and Lynch syndrome, also called hereditary non-polyposis colon cancer (HNPCC). FAP, which accounts for approximately 1% of CRC, is due to an inherited mutation on a single allele of the *APC* gene resulting the accumulation of benign adenomatous polyps throughout the intestine and particularly in the colon. In 100% of cases, CRC develops before age 40 (Miyoshi et al., 1992). Mouse models with similar mutations on a single *Apc* allele recapitulate the phenotypes found in FAP patients (Colnot et al., 2004; Taketo and Edelmann, 2009). HNPCC, causing about 2-5% of CRC, is due to inherited mutations on genes related to mismatch repair (MMR), which repairs replication errors like base-base mismatches or insertions/deletions. This results in an 80% lifetime risk of developing CRC. Mutations on genes encoding MMR proteins MSH2 (MutS homolog 2), MLH1 (MutL

homolog 1) or MSH6 (MutS homolog 6) are particularly common. Mice conditionally invalidated for *Msh2* in the intestinal epithelium rapidly and continuously accumulate microsatellite instability over time, and develop tumors with high penetrance within 12 months (Kucherlapati et al., 2010; Keysselt et al., 2017).

- Lifestyle factors like diet, obesity, smoking, and lack of physical activity also contribute to the risk of developing CRC.
- IBD: Patients with Crohn's disease or ulcerative colitis are at higher risk of developing CRC, and this risk increases the longer the patient has the disease. About 1-2% of CRC arise in the context of IBD.

3.2 TUMORIGENESIS AND PROGRESSION

Cancer progression can be seen as a multistep process, where a first mutation drives tumor initiation, followed by additional mutagenic or epigenetic events that may promote growth and malignancy, altogether called tumor progression. CRC is a textbook example of such a stepwise tumor progression. The initial stage is a hyperplasia of the epithelium: the mucosa becomes thicker as a result of crypt hyperproliferation. This can develop into small polyps that can be detected by colonoscopy. The progression of these polyps to later-stage adenomas then to *in situ* adenocarcinomas and eventually malignant carcinomas that invade the basement membrane and can become metastatic is slow, often taking over 10 years. Regular colonoscopies and removal of benign polyps considerably reduces the incidence of CRC, supporting the idea of a step-wise progression of adenoma to carcinoma. Occasionally, distinct, more advanced zones can be distinguished within a single tumor, which has been interpreted as a result of the acquisition of a more malignant phenotype by individual mutant clones within the original adenomatous tumor.

Fearon and Vogelstein initially proposed a model where this sequence of histopathological changes from adenoma to carcinoma is directly associated with certain mutations and molecular alterations [FIGURE 19] (Fearon and Vogelstein, 1990). In this model, tumor initiation is driven by mutations of the *APC* gene, resulting in epithelium hyperplasia. About 80% of sporadic CRC as well as FAP-associated CRC present either inactivating mutations or loss of heterozygosity on both copies of the *APC* gene or, less frequently, mutations on genes encoding other components of the canonical Wnt pathway (Powell et al., 1992; Morin et al., 1997; Fodde et al., 2001; Koo et al., 2012; Seshagiri et al., 2012). Activation of the Wnt/ β -catenin pathway is therefore a central event in CRC. This is consistent with the previously mentioned evidence from models of *Apc* loss in the murine epithelium. The importance of *Apc* deficiency not just as an initiating event but also in driving tumor growth has recently been underlined by Dow et al. (Dow et al., 2015). In their study, restoring *Apc* expression in established tumors, even those carrying additional mutations, leads to their regression and the re-establishment of a normal epithelial architecture. On the other hand, the fact that only a few of the many *Apc*-mutant polyps in FAP patients progress to CRC highlights the need for additional alterations to progress to a more malignant stage. Over the course of tumor progression, additional mutations are indeed acquired; among these are activating mutations of KRAS or other components of the

EGFR (EGF receptor) pathway, loss of function mutations of SMAD2/4 or other factors of the growth-inhibitory TGF- β /BMP signaling, and loss of function of p53. LOH on genes like *SMAD2*, *SMAD4* or *TP53* are increasingly common as tumors become more advanced, suggesting that they are late events in tumor progression. Along with the increasing number of mutations and genetic instability, epigenetic defects and microenvironmental changes also arise, including changes in the tumor-associated stroma, deregulations of the microbiota (dysbiosis), or inflammation.

While the vast majority of CRC develop via this adenoma-to-carcinoma sequence, it is estimated that about 10-20% of CRC develop through a different sequence of morphological changes called the serrated pathway. Serrated polyps and premalignant tumors, characterized by unique saw-tooth pattern morphology, are classified either as traditional serrated adenomas (TSA) or sessile serrated adenomas (SSA). Due to their high molecular heterogeneity, serrated tumors are more commonly distinguished by their unique morphology, though they commonly carry *KRAS* or *BRAF* mutations (more common in TSA and more common in SSA, respectively). Importantly, CRC associated with the serrated pathway are strongly associated with poor prognosis and resistance to therapy.

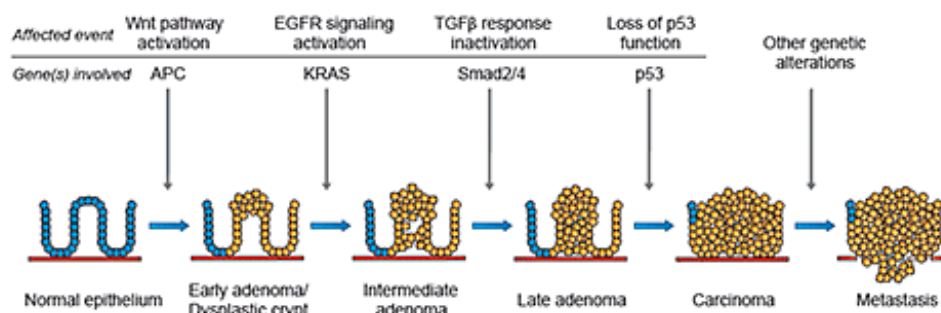


FIGURE 19. Classical sequence of genetic changes underlying the development of colorectal cancer, initially put forward by Fearon and Vogelstein. Adapted from Davies et al., 2005.

3.3 TYPES OF COLORECTAL CANCER

CRC is a heterogeneous disease characterized by genetic instability arising by at least three mechanisms: one involving chromosome instability (CIN, also called microsatellite stable, or MSS), another involving instability at the level of the nucleotide sequence (microsatellite instability, MSI), and a third involving CpG island methylation (CpG island methylation phenotype, or CIMP).

- CIN, involves the presence of numerical or structural chromosomal changes, notably detectable as a high frequency of DNA somatic copy number alterations (SCNA). CIN or MSS tumors represent the vast majority (~84%) of sporadic CRC and all FAP-associated tumors. These tumors typically follow the classical adenoma-to-carcinoma mutation sequence described above.
- MSI, on the other hand, is linked to about 15% of CRC including HNPCC-associated CRC. A key mechanism for MSI is the inactivation, either through germline mutations or epigenetic silencing of genes encoding MMR machinery (called deficient MMR, or

dMMR). This leads to frequent errors in DNA replication, particularly in repeated nucleotide sequences like microsatellite repeats. MSI CRC are further divided into two types, MSI-H and MSI-L, based on their high or low level of MSI, respectively. Generally speaking, patients with MSI-H tumors have a better prognosis and lower risk of metastases than patients with MSI-L or MSS tumors.

- CIMP tumors involve epigenetic mechanisms: CpG clusters in certain promoter regions are generally unmethylated but can become methylated – typically on the promoter of tumor suppressor genes – resulting in the transcriptional inactivation of these genes. This can occur at a low level increasing with age or at a higher level.

These different pathways of CRC progression along with specific recurrent mutations have been used to classify CRC subtypes in several studies, with two notable recent classifications proposed by the cancer genome atlas (TCGA) ([The Cancer Genome Atlas Network et al., 2012](#)) and the CRC subtyping consortium (CRCSC) ([Guinney et al., 2015](#)) [FIGURE 20]. TCGA classification is based on a genome-scale genomic and transcriptomic analysis of 276 samples, divided into two major groups: about 84% of tumors were classified as MSS with a high frequency of SCNA and common mutations in *APC*, *TP53*, *KRAS*, *SMAD4*, and *PIK3CA*. The remaining 16% of tumors were categorized as hypermutated with MSI due either to defective MMR (~13%) or DNA polymerase proofreading mutations (~3%, mainly on the genes encoding DNA polymerase ϵ , *POLE*, or δ , *POLD1*) resulting in an ultramutated phenotype. The CRCSC brought together six independent classification systems based largely on gene expression analysis, which they combined into four core consensus molecular subtypes (CMS): CMS1 (MSI immune, 14%) includes nearly all hypermutated MSI tumors, characterized by strong immune infiltration and activation, while MSS tumors are subdivided into CMS2 CRC (canonical, 37%) with marked Wnt and Myc signaling activation, CMS3 CRC (metabolic, 13%) with clear metabolic dysregulation, and CMS4 CRC (mesenchymal, 23%) with prominent TGF- β activation, stromal invasion and angiogenesis. The remaining 13% of CRC remained unclassified. Importantly, this classification of CRC should allow more accurate prognosis and better-adapted treatment of CRC.

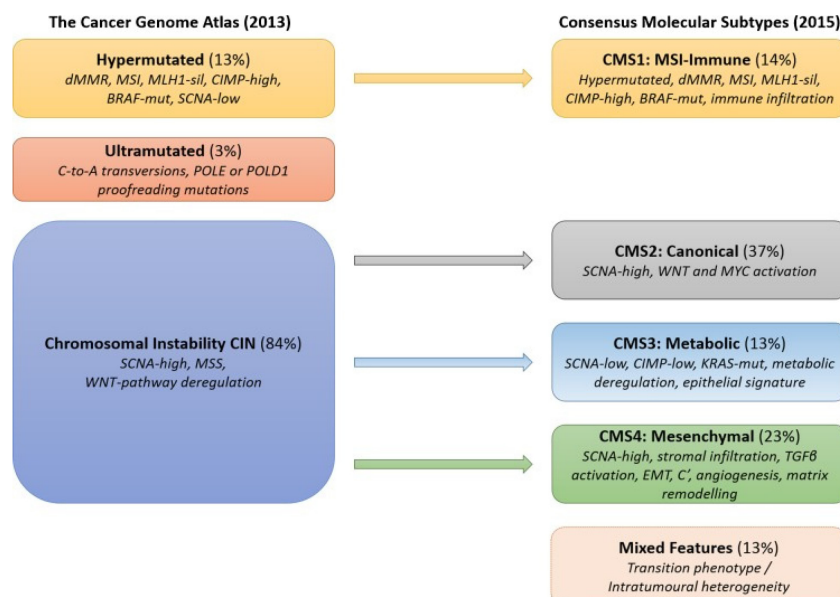


FIGURE 20. Classification of human colorectal cancer. Adapted from Müller et al., 2016.

3.4 TREATMENT

Survival rates in CRC are highly dependent on the stage of diagnosis of the tumor. Over the past 50 years, mortality rates for CRC have improved in France and worldwide due to better detection practices and, consequently, earlier detection.

As of today, the most common approach for treating CRC remains surgical resection. Chemotherapy is the next most common approach, either after surgical resection of the tumor to prevent recurrence or in the case of late-stage tumors. The most commonly used chemotherapies (alone or in combination) for CRC include 5-fluorouracil (5-FU, which acts by blocking thymidine synthesis), oxaliplatin (a DNA intercalating agent), and irinotecan (a topoisomerase inhibitor).

Targeted therapies are of particular interest for advanced CRC, once again either alone or in combination with chemotherapy. Anti-EGFR or anti-VEGFR (vascular endothelial growth factor) antibodies have been used to treat patients with advanced CRC, which has greatly prolonged their overall survival, although tumors often develop resistance to this therapy. Moreover, CRC bearing *KRAS* or *BRAF* mutations do not respond to anti-EGFR therapy. New targeted therapies therefore also represent important therapeutic avenues.

As can be expected, treatments that affect the growth of CRC also have detrimental effects on the highly proliferative non-tumoral intestinal epithelium. Treatments that will potentiate the effects of current therapy in tumors are therefore promising research areas.

Unfortunately, CRC are generally not responsive to novel immune checkpoint-based therapy, with the notable exception of MSI-H CRC (Le et al., 2015; Llosa et al., 2015), for which immune checkpoint inhibitors have recently gained approval.

3.5 INTESTINAL STEM CELLS AND COLORECTAL CANCER

3.5.1 INTESTINAL STEM CELLS AS THE FOUNDER CELLS OF COLORECTAL CANCER

As has been underlined in the previous sections, the different pathways involved in the regulation of homeostatic ISC function are also heavily implicated in the development and progression of CRC. In light of the high number of proliferative cells in the intestine and comparatively low occurrence of spontaneous intestinal tumors, it has long been proposed that only mutagenic hits accumulated in ISC could result in tumor development. Although it is difficult to ascertain which cell types bear the tumor-initiating mutations in humans, studies in mice suggest that ISC are likely to be the CRC cell of origin. Direct evidence has been obtained by the inactivation of *Apc* in *Lgr5*⁺ ISC, leading to the rapid onset of adenomas (Barker et al., 2009). Tumors also rapidly develop upon either loss of function of APC or activation of β -catenin specifically in cells expressing other ISC markers *Bmi1*, *Lrig1* or *Prominin-1* (Sangiorgi and Capecchi, 2008; Zhu et al., 2009; Powell et al., 2012). In contrast, deletion of *Apc* or β -catenin activation in more differentiated cells results only in ectopic proliferative foci, which rarely progress to microadenomas and are generally expelled from the epithelium (Barker et al., 2009;

Schwitalla et al., 2013b). CRC therefore likely arises from ISC having undergone specific mutagenic hits. The monoclonal origin of intestinal adenomas, illustrated by Schepers et al. using R26R-Confetti mouse models, further supports this idea (Schepers et al., 2012).

It should be noted, however, that several studies have since reported that plastic progenitor cells could also de-differentiate and initiate tumorigenesis (Schwitalla et al., 2013b; Metcalfe et al., 2014; Asfaha et al., 2015). For example, the aberrant expression of BMP antagonist Gremlin1 can drive the de-differentiation of villus cells and allow the formation of neoplasias from these cells following the acquisition of additional mutations (Davis et al., 2015). Similarly, the combination of an activated β -catenin with activation of either NF κ B or KRAS signaling could induce the de-differentiation of non-ISC and accelerate tumor initiation (Schwitalla et al., 2013b; Cammareri et al., 2017). However, for the short-lived differentiated cells of the intestinal epithelium to acquire the right amount and combination of mutations is unlikely, unless most of these mutations first arose in the ISC compartment.

3.5.2 COLORECTAL CANCER STEM CELLS

ISC, in addition to acting as the tumor-initiating cells, likely also play an important role in continued tumor growth and progression. Both human CRC and genetically-induced murine adenomas express CBC markers including *Lgr5*, *Musashi-1*, *Ascl2*, *Olfm4* and *EphB2* (Potten et al., 2003; Jubb et al., 2006; Fan et al., 2010; Li et al., 2015b; Jang et al., 2016). In fact, lineage tracing of *Lgr5*⁺ adenoma cells revealed that they fuel the growth of adenomas by generating all the cell types present in the adenoma, indicating that they act as multipotent stem cells of the adenoma in a way that mimics the architecture of the homeostatic intestinal crypt [FIGURE 21] (Merlos-Suárez et al., 2011; Schepers et al., 2012; Cernat et al., 2014; Shimokawa et al., 2017). Furthermore, both *Lgr5* expression alone and the intestinal stem cell signature obtained from sorted *Lgr5*⁺ ISC were established as predictors of poor prognosis in CRC and relapse after treatment (Merlos-Suárez et al., 2011; Chen et al., 2014; Jiang et al., 2016). These findings established that the stem cell-progenitor cell hierarchies found in the crypts of the healthy epithelium are preserved in CRC, and that cells expressing ISC markers act as cancer stem cells in CRC by driving tumor growth, progression, and relapse.

This has led to the idea that LGR5 and other stem cell markers, or at least the cancer cell populations they mark, could represent promising therapeutic targets in the treatment of CRC (Vaipoulos et al., 2012; Morgan et al., 2018). Indeed, several *in vitro* studies on CRC cell lines showed that downregulating *Lgr5* expression reduced the tumorigenic potential of these cells (Chen et al., 2014; Hirsch et al., 2014; Hsu et al., 2014). This was confirmed by different approaches *in vivo*. One study by Gong et al. used an LGR5-targeting antibody-drug conjugate to deliver a drug specifically to the cancer stem cells of xenografted CRC cells. This successfully led to the regression of tumors without harming the healthy intestinal epithelium, although about half the mice subsequently showed tumor relapse (Gong et al., 2016b). Shimokawa et al. and de Sousa e Melo et al. both used transplanted knock-in colorectal cancer organoids to inducibly ablate *Lgr5*⁺ cancer stem cells in the resulting tumors. In both studies, this also halted tumor growth during treatment. However, in both studies, the *Lgr5*⁺ cancer stem cell pool was

replenished and tumor growth re-started after treatment, suggesting that the high plasticity of intestinal crypts is also preserved within tumors (de Sousa e Melo et al., 2017; Shimokawa et al., 2017).

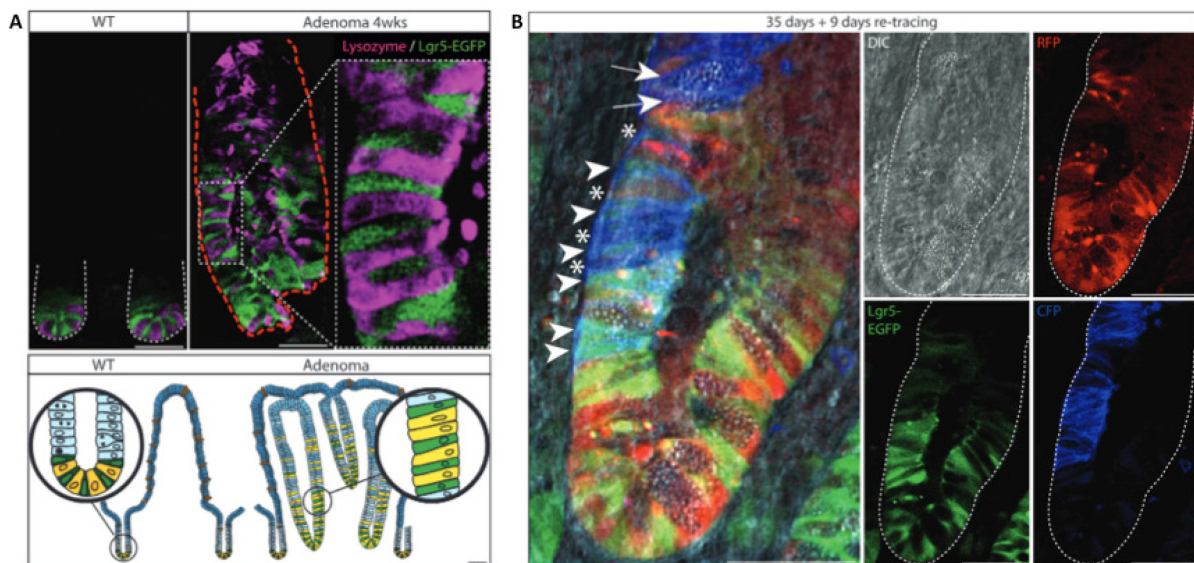


Figure 21. Demonstration of *Lgr5*⁺ ISC as the drivers of colorectal adenoma growth using *Lgr5-EGFP-ires-Cre^{ERT2};Apc^{fl/fl};R26R-Confetti* mice. (A) Distribution of *Lgr5-EGFP*⁺ stem cells and Paneth cells (labelled by Lysozyme staining in purple) at the base of wild-type (WT) crypts and similarly found towards the base of an adenoma segment, illustrated schematically below. (B) *RFP*⁺ R26R-Confetti adenoma traced for 35 days, then re-traced for 9 days after recombination to *CFP*. Within the red segment, a blue clone appears, originating at the *Lgr5-EGFP*⁺ base of the crypt-like structure and giving rise to a clonal ribbon of adenoma cells containing multiple *Lgr5-EGFP*⁺ cells (arrowheads), Paneth cells (arrows) and *Lgr5*⁻ cells (asterisks). Scale bars, 50 μ m.

Adapted from Schepers et al., 2012.

3.5.3 INTESTINAL STEM CELL NICHE COMPONENTS IN COLORECTAL CANCER

Just as dynamics in the normal intestinal epithelial crypt are regulated by microenvironmental factors, these factors also contribute to tumor initiation and progression. In fact, several of the mutations mentioned in the previous sections as promoting the development of adenomas are involved in signaling pathways normally regulated by niche cells, including stromal BMP/TGF- β signaling and microbial or immune cell-induced NF κ B signaling.

Analogous to the ISC niche, mesenchymal cells are found in the CRC stroma, where they are thought to participate in the different stages of tumor progression. Mesenchymal stem cells migrate to and differentiate in the stroma of intestinal tumors, and can promote tumor growth and favor the formation of metastases (Shinagawa et al., 2010). Mechanistically, this could in part be due to growth factors produced by mesenchymal cells. For example, myofibroblast-secreted factors like HGF (hepatocyte growth factor) stimulate Wnt/ β -catenin signaling and subsequently clonogenicity in intestinal tumor cells. In line with this, human colon adenocarcinomas have heterogeneous Wnt activity, and xenografted tumors in mice have the highest β -catenin activity preferentially in tumor cells in close contact with stromal myofibroblasts (Vermeulen et al., 2010). However, it is interesting to note that as the number of CRC-associated mutations increases in genetically-modified organoids or organoids derived

directly from human CRC, the need for growth factors in the culture medium decreases. This suggests a progressive loss of niche-dependence for growth (Drost et al., 2015; Fujii et al., 2016).

In addition to tumor growth, cancer-associated fibroblasts also contribute to invasion and metastasis. Fibroblasts isolated from the tumors of CRC patients increase the invasive capacity of cancer cells through their secretion of matrix metalloproteases, their high contractility, and their ability to interact with ECM components like fibronectin, altogether allowing them to remodel the basement membrane that tumor cells then invade (Attieh et al., 2017; Glentis et al., 2017). Interestingly, this is not systematically the case with fibroblasts isolated from non-tumoral tissue, suggesting that tumor-associated fibroblasts may consist of unique populations of stromal cells or may be primed to behave differently by epithelial tumor cells. Confirming their role in the different steps of tumorigenesis, depletion of tumor-associated fibroblasts in one study significantly reduced tumor growth and metastasis of intestinal tumors. Moreover, removing these fibroblasts also improved response to chemotherapy, presumably by reducing the amount of type I Collagen in the tumor thereby improving drug uptake (Loeffler et al., 2006).

Immune cells also play a role in intestinal tumorigenesis. In humans, chronic inflammation in IBD patients predisposes to cancer initiation. Likewise, DSS administration in either *Apc^{+/-}* mice or AOM-treated mice leads to a strong increase in polyp formation, hinting at an important role of the inflammatory microenvironment in tumor initiation (Tanaka et al., 2003, 2006). Several mechanisms have been proposed to explain this phenomenon. First, inflammation can lead to ROS production and thereby oxidative-stress mediated mutations or proliferation in tumor cells (Hussain et al., 2003). Another mechanism was proposed by Grivennikov et al., who showed that cytokine production from lamina propria myeloid cells and consequent intestinal epithelial STAT3 signaling protect ISC and are important for inflammation-induced tumorigenesis (Grivennikov et al., 2009). Once the tumor is established, immune cells found in the tumor-associated stroma play a key role in tumor growth and progression, although the outcome largely depends on the balance between different immune populations. For example, CD8⁺ cytotoxic T cells or NK cells limit cancer progression and are generally associated with better prognosis for patients, while tumor-associated macrophages and regulatory T lymphocytes are thought to promote tumor growth and immune evasion, respectively (Galon et al., 2006; Swann et al., 2009; Grivennikov et al., 2010).

Thus, the various cell types composing the niche of ISC are also implicated in tumorigenesis and tumor progression, and the microbiota is no exception. First, microbial metabolites like short chain fatty acids can suppress inflammation and cancer, whereas other microbial metabolites like secondary bile acids promote them. Second, TLR-mediated interactions with the microbiota also contribute to tumor initiation, as *Myd88* deletion decreases intestinal adenoma formation in *Apc^{+/-}* mice (Lee et al., 2010). This is largely attributed to microbiota-induced inflammation. Supporting this idea, either antibiotic treatment or the absence of the gut microbiota reduced tumor incidence in colitis-associated CRC mouse models (Garrett et al., 2009; Uronis et al., 2009; Arthur et al., 2012). Moreover, in the context of inflammation-induced CRC, several bacterial species were directly shown to promote inflammatory signals and, consequently, tumorigenesis

(Wu et al., 2009; Arthur et al., 2012). However, IBD-associated CRC represents only a small portion of human CRC. More recently, the commensal *Fusobacterium nucleatum* was shown to be enriched in patients with CRC, and particularly so in the tumoral tissue compared to neighboring healthy tissue. Colonisation with *Fusobacterium* accelerated the onset of colonic tumors in *Apc^{+/-}* mice, but not in inflammation-associated mouse models of CRC (Kostic et al., 2013). A later study confirmed *Fusobacterium* as the most abundant bacterial genus over-represented in human CRC samples compared to neighboring control tissue, and further identified a signature of 57 bacterial genera with differential abundance in CRC tissue (Warren et al., 2013). Such CRC-associated bacterial signatures not only expand our understanding of CRC progression, but also provide new opportunities both for the non-invasive detection of CRC (ie. from stool samples) or determination of prognosis and for therapy.

All in all, ISC function and integrity are highly regulated at homeostasis by a complex network of signaling pathways and niche components, and the deregulation of these same mechanisms come into play in CRC initiation and progression.

CHAPTER 2: AUTOPHAGY

I. MECHANISMS AND REGULATION OF AUTOPHAGY

1.1 OVERVIEW OF AUTOPHAGY

Cell homeostasis and efficient adaptation to cellular or environmental conditions largely depend on two types of pathways: anabolic and catabolic. Indeed, synthesis of new molecules – like proteins, nucleic acids, or lipids – is necessary to replace old cellular components with fresh, better quality ones or provide molecules adapted to a new cellular context. On the other side of the balance is degradation. Degradation of old or dysfunctional molecules ensures proper cell function through quality control and serves as a source of energy and materials. Eukaryotic cells rely on two major degradation systems. The proteasome, as its name indicates, allows the degradation of proteins, and does so in a selective manner mediated by protein ubiquitination. The lysosome, in contrast, is often considered as more “bulk” degradation, taking in a vast array of substrates to be digested by hydrolytic enzymes in this highly acidic vacuole. Lysosomal substrates include extracellular material or portions of the plasma membrane from the endocytic pathway as well as cytosolic materials or organelles through the autophagic pathway.

Autophagy, or “self-eating”, was cleverly coined in 1963 by Christian de Duve, a pioneer in lysosome research and the 1974 Nobel Prize laureate in Physiology or Medicine. He used the term to describe his observations from electron microscopy studies of single- or double-membrane vesicles containing partially digested cytoplasmic content [FIGURE 22]. Over the 30 years that followed, autophagy remained a poorly known and little understood phenomenon. A breakthrough came in the 1990s, when Yoshinori Ohsumi, the 2016 Physiology or Medicine Nobel Prize laureate, performed genetic screens in yeast, isolating mutants defective in autophagy and identifying the first set of many autophagy-related genes (*ATGs*). This and further discoveries skyrocketed our understanding of autophagy in physiology and pathology at both the cellular and organismal level.

Three forms of autophagy have been described [FIGURE 23]. Microautophagy refers to the direct engulfment of cytoplasm and its components through the formation of invaginations into the lysosome directly. Chaperone-mediated autophagy (CMA) involves the recognition of KFERQ-like motifs on cytosolic proteins by the chaperone HSC70 (heat shock cognate 70 kDa protein) and co-chaperones, which target the unfolded protein to the LAMP2 (lysosome-associated membrane protein 2) receptor on the lysosomal membrane that in turn delivers the unfolded protein inside the lysosome through a multimeric translocation complex. Finally, macroautophagy involves the formation of a double-membraned phagophore that will expand and engulf cytoplasmic materials and organelles then close to form a vacuole called the autophagosome. The outer layer of this vacuole will then fuse with the lysosome, allowing the degradation of the inner membrane and its content. Macroautophagy is highly conserved in eukaryotes from yeast to mammals, and can occur both in bulk or target selective substrates for degradation. This type of autophagy is the most extensively studied to date, and will be referred to simply as ‘autophagy’ hereafter.

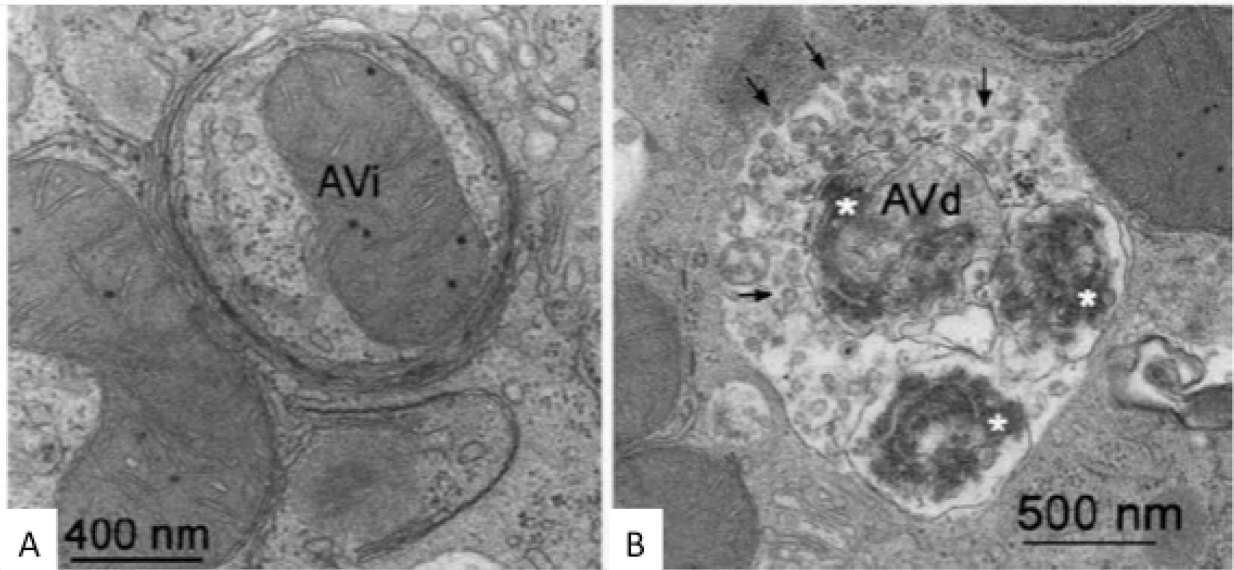


FIGURE 22. Morphology of macroautophagic vacuoles by transmission electron microscopy in mouse hepatocytes. (A) An autophagosome, or initial autophagic vacuole (AVi), containing a mitochondrion, endoplasmic reticulum, and electron-dense ribosomes. The two limiting membranes of the autophagosome are visible at the upper rim of the vacuole. Below, a phagophore seems to be forming and sequestering a peroxisome. (B) A late, degradative autophagic vacuole (AVd) containing partially degraded contents, including the remnants of ribosomes (asterisks). This AV has fused with a multivesicular endosome, as indicated by the numerous small vesicles (arrows). Adapted from the Madame Curie Bioscience Database.

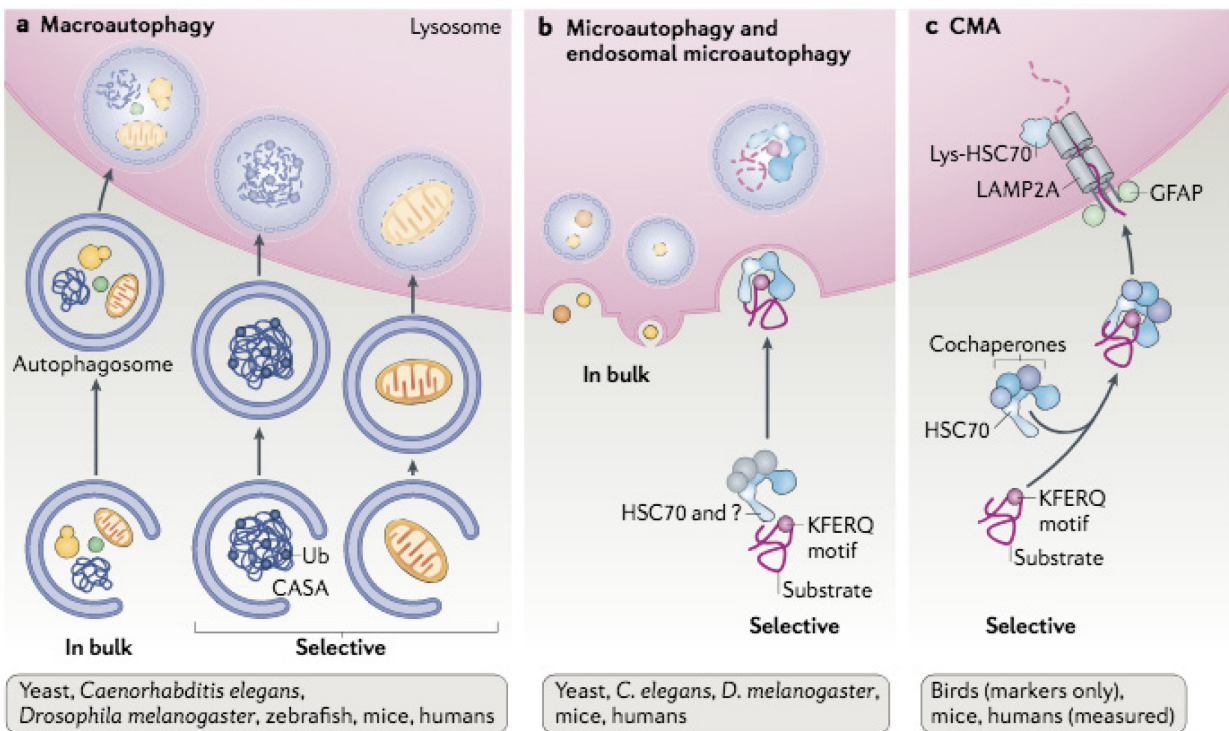


FIGURE 23. Autophagic pathways in mammals. Adapted from Kaushik and Cuervo, 2018.

1.1.1 MOLECULAR MACHINERY OF AUTOPHAGY

The autophagy process can be divided into sequential steps: 1) initiation and nucleation of the autophagosome, 2) expansion of the autophagosome membrane, cargo recognition and selection, 3) closure of the autophagosome and fusion with the lysosome, followed by breakdown of the cargo and release of the degradation products back into the cytosol.

Autophagy is induced through the activation of the ULK1 complex, consisting of ULK1 (Unc51-like autophagy activating kinase 1) or ULK2, ATG13, FIP200 (focal adhesion kinase family-interacting protein of 200 kDa), and ATG101 **[FIGURE 24A]**. mTOR (mammalian target of rapamycin) acts as a central inhibitor of autophagy in nutrient-rich conditions by phosphorylating ULK1 and ULK2, keeping them inactive. Upon mTOR inhibition by starvation or rapamycin treatment, ULK1 and ULK2 are activated, initiating autophagy. The ULK1 complex directly phosphorylates and ubiquitinates the class III PI3K (phosphoinositide 3 kinase) complex. This complex – comprising VPS34 (vacuolar protein sorting 34), VPS15, either ATG14 or UVRAG (UV radiation resistance-associated gene protein), and BECLIN1 – acts as the stage for the nucleation and assembly of the initial phagophore membrane **[FIGURE 24B]**. In addition to its interactions with autophagy proteins, BECLIN1 contains a BH3 domain through which it binds the anti-apoptotic BCL2-family proteins, thereby blocking its function in autophagy. The class III PI3K complex produces PI(3)P (phosphatidylinositol 3-phosphate) that in turn binds and recruits various ATG proteins.

The source of the membrane forming the phagophore is still a matter of debate, although several potential sites have been proposed so far, including ER exit sites (Graef et al., 2013), ER-Golgi intermediate compartments (Ge et al., 2013), ER-mitochondria contact sites (Hamasaki et al., 2013), ER-plasma membrane contact sites (Nascimbeni et al., 2017), and ER subdomains enriched in phospholipid synthesis enzymes (Nishimura et al., 2017). ATG9, the only membrane-spanning ATG protein, and VMP1 (vacuole membrane protein 1) cycle between the phagophore and membranes of other organelles **[FIGURE 24C]**, likely contributing to the delivery of membrane to the expanding phagophore, although how it does so remains unclear (Mizushima, 2018).

Among the proteins recruited through PI(3)P are two ubiquitin-like conjugation systems. Ubiquitin-like ATG12 is first activated by E1-like enzyme ATG7 then transferred to E2-like enzyme ATG10, before forming a covalent conjugate with ATG5 that interacts with ATG16L1. The second ubiquitin-like conjugation system involves orthologs of the yeast protein ATG8, like LC3 (microtubule-associated protein 1 light chain 3) or GABARAP (gamma-aminobutyric acid receptor-associated protein). These are first cleaved by ATG4 (of which four somewhat redundant isoforms called a, b, c and d exist) then activated by ATG7. Then, the E2-like enzyme ATG3, along with the ATG5-12 complex and ATG16L1, conjugates the ATG8 orthologue to the lipid phosphatidylethanolamine (PE) **[FIGURE 24D]**. The PE-conjugated protein is thus bound to the autophagosome membrane (Feng et al., 2014; Bento et al., 2016). Although the PE-conjugated ATG8 proteins are required for the continuation of the pathway, their exact role in

the process remains a matter of debate (Mizushima, 2018). They are, however, clearly involved in the recruitment of selective cargo, as will be discussed later.

The closure of the phagophore remains relatively poorly-understood process, likely involving ESCRT (endosomal sorting complex required for transport) proteins, known to play an important role in vesicle budding and membrane fission in other processes (Filimonenko et al., 2007; Lee et al., 2007; Rusten et al., 2007). The mechanism of fusion between the autophagosome and the lysosome also remains unclear. SNARE proteins (SNAP, or soluble N-ethylmaleimide-sensitive factor attachment protein, receptors) are large complexes known to mediate vesicle fusion. The SNARE protein Syntaxin17 is recruited to the autophagosome and mediates fusion with the lysosome by interacting with SNAP29 and the SNARE VAMP8 on the endosome/lysosome membrane [FIGURE 24E] (Itakura et al., 2012a). Cytoskeletal components, particularly the microtubule cytoskeleton, also play a role in autophagosome-lysosome fusion by mediating autophagosome trafficking towards the perinuclear region, where they meet with lysosomes (Jahreiss et al., 2008; Kimura et al., 2008). Finally, various acid hydrolases within the lysosome break down the inner autophagosome membrane and its contents [FIGURE 24F].

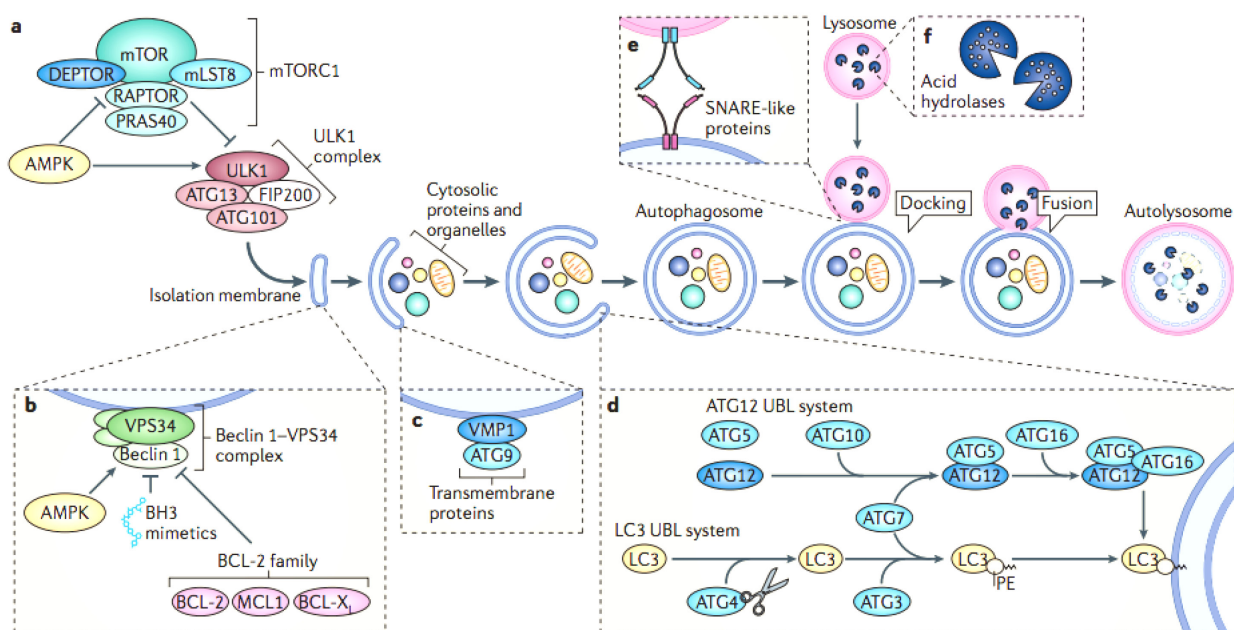


FIGURE 24. Schematic representation of (macro)autophagy. Adapted from Mariño et al., 2014.

It is important to note that many of these proteins are not solely involved in what is called the “canonical” autophagy pathway, and may also be involved in “non-canonical” pathways that do not require the entire repertoire of ATGs described above or play autophagy-independent functions (Subramani and Malhotra, 2013; Dupont et al., 2017; Cadwell and Debnath, 2018).

1.1.2 SELECTIVE AUTOPHAGY

Although initially considered as bulk or nonselective degradation, it has become increasingly apparent that autophagy can be highly selective. Among the growing list of selective autophagy targets are misfolded proteins or protein aggregates (targeted through a process appropriately called aggrephagy), mitochondria (mitophagy), intracellular pathogens (xenophagy),

peroxisomes (pexophagy), lipid droplets (lipophagy), ribosomes (ribophagy), ER (reticulophagy), and glycogen (glycophagy). Selection of substrates depends on the autophagy stimulus, and these targets can be specifically recognized and targeted to the autophagosome through several processes.

First, some selective substrates have domains that interact with the different orthologs of yeast ATG8, LC3 or GABARAP (called LIRs, or LC3 interacting regions) (Noda et al., 2008; Behrends et al., 2010), through which they are directly recruited to the phagophore membrane. Although the different ATG8 orthologs share a high degree of similarity, they are thought to recognize particular cargo through differences in these motifs (Rogov et al., 2017). Other selective substrates bind ATG8 proteins indirectly by recruiting autophagy receptors such as p62 (also called SQSTM1 or Sequestosome1), NBR1 (neighbor of BRCA1 gene 1), NDP52 (nuclear dot protein 52), or Optineurin. In addition to a LIR motif, adaptor proteins have other domains allowing recognition of the cargo directly or through post-translational modifications like ubiquitination. For instance, p62 contains a LIR, essential in its autophagic degradation, and a ubiquitin-associated domain that binds ubiquitinated targets. In this way, p62 contributes to aggrephagy, mitophagy, xenophagy, and pexophagy among other forms of selective autophagy. Taking aggrephagy [FIGURE 25] as an example, before p62 was recognized as a LIR-containing adaptor protein in autophagy (Ichimura et al., 2008), it was detected in misfolded and ubiquitinated protein aggregates called inclusion bodies, associated with liver injury or neurodegenerative diseases (Zatloukal et al., 2002). It was only later that p62 was shown to participate in the formation of these inclusion bodies and target them for autophagic degradation (Bjørkøy et al., 2005; Komatsu et al., 2007; Pankiv et al., 2007). Another example of this mechanism is xenophagy [FIGURE 25], which collectively describes the selective autophagy of intracellular pathogens, including viruses, bacteria or fungi. Following *Salmonella typhimurium* infection and release into the cytosol, bacterial proteins are rapidly ubiquitinated then recognized by p62 but also other adaptors like NDP52, and Optineurin (Thurston et al., 2009; Zheng et al., 2009; Wild et al., 2011), which then promote the sequestration of the pathogen in the autophagosome. Although several of these receptors can bind the same bacterium, they appear to play separate functions as the individual knockout of each of these proteins enhances bacterial replication.

Second, recognition of selective substrates can happen independently of interactions with ATG8 proteins. One such way is to recruit the ULK1 complex to the target (Kamber et al., 2015).

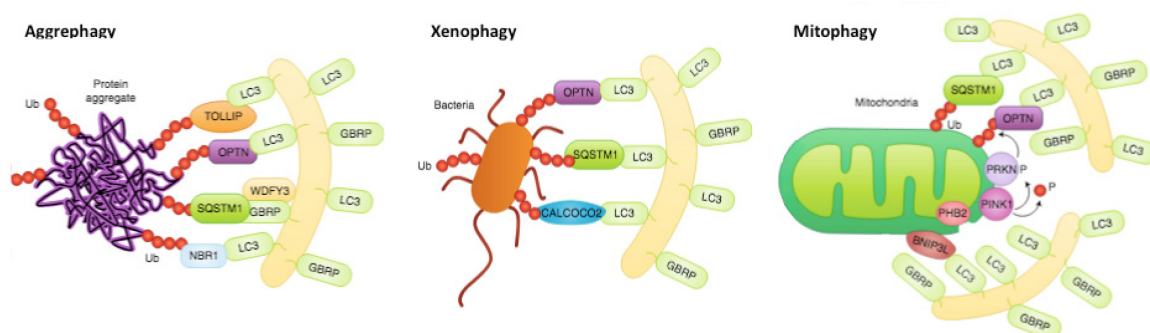


FIGURE 25. Examples of selective autophagy in mammals. Adapted from Gatica et al., 2018.

Sometimes several of these mechanisms cooperate, as is the case for mitophagy [FIGURE 25]. Mitochondria are a major site of intracellular ROS production and a hub for apoptotic signals. Therefore, a healthy population of mitochondria is critical for cellular homeostasis. Mitophagy ensures steady-state mitochondrial turnover, selectively removes damaged mitochondria and can adjust mitochondrial numbers to adapt to changing metabolic needs (Youle and Narendra, 2011; Esteban-Martínez et al., 2017). The best characterized mechanism for induction of mitophagy is PINK1 (PTEN-induced kinase 1) / PARKIN signaling. PINK1 acts as a sensor of mitochondrial damage. It contains a mitochondrial targeting sequence, and is imported through the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) by translocases (TOM and TIM, respectively) in a way that is dependent on the mitochondrial transmembrane potential. In healthy mitochondria, the transmembrane potential is maintained by efficient oxidative phosphorylation, and PINK1 is cleaved in the IMM, leading to its release into the cytosol and rapid proteasomal degradation. Damaged mitochondria fail to import PINK1 to the IMM, so it accumulates on the OMM (Jin et al., 2010; Meissner et al., 2011; Greene et al., 2012; Lazarou et al., 2012). There, it phosphorylates ubiquitinated OMM proteins at Ser65, leading to the recruitment of PARKIN, which is also phosphorylated by PINK1 (Lazarou et al., 2012; Kane et al., 2014; Koyano et al., 2014; Ordureau et al., 2014; Shiba-Fukushima et al., 2014). PARKIN is an E3 ubiquitin ligase that, in turn, ubiquitinates various proteins on the OMM. Although PARKIN is not required for mitophagy, as PINK1-mediated Ubiquitin Ser65 phosphorylation can recruit autophagy adaptor proteins in its absence (Lazarou et al., 2015; Ordureau et al., 2015), it is thought to amplify the PINK1 signal by increasing the concentration of Ubiquitin on the OMM. This both initiates the degradation of mitochondrial proteins by the proteasome, and recruits five different autophagy adaptor proteins: Optineurin, NDP52, TAX1BP1 (Tax-1 binding protein 1), p62 and NBR1. Indeed, the deletion of all five proteins efficiently blocks mitophagy, and re-expression of NDP52, Optineurin and, to a lesser extent, TAX1BP1 rescues mitophagy (Lazarou et al., 2015). Optineurin and NDP52 receptors promote mitophagy not only by interacting with LC3, but also by recruiting more upstream autophagy-initiation factors including ULK1 to the mitochondria (Itakura et al., 2012b; Wong and Holzbaur, 2014; Lazarou et al., 2015). LC3- and ULK1-independent recruitment of ATG9 to mitochondria for mitophagy has also been reported (Itakura et al., 2012b). p62 and NBR1, on the other hand, have been suggested to support mitophagy by clustering damaged mitochondria rather than targeting them directly to the phagophore (Narendra et al., 2010). Finally, regulated expression of BNIP3L (BCL2 interacting protein 3 like, also called NIX), which localizes to the OMM through a trans-membrane region, allows the clearance of damaged mitochondria through LIR-mediated interactions with GABARAP (Sandoval et al., 2008; Novak et al., 2010; Esteban-Martínez et al., 2017). Altogether, these sometimes-redundant pathways allow cell- and context- specific regulation of mitophagy and speak to the importance of this process in homeostasis.

1.2 REGULATION & FUNCTIONAL CONSEQUENCES OF AUTOPHAGY

Although autophagy occurs constitutively at a basal level in all cells, it is also upregulated in response to various intracellular or environmental stresses. Some of the best-characterized pathways regulating autophagy are described below, along with the functional role of autophagy

in the cellular response to these stresses. Some physiological implications of both basal and stress-induced autophagy will then be discussed in the next subchapters.

1.2.1 GROWTH FACTORS, NUTRIENTS, AND ENERGY LEVELS: mTOR SIGNALING

Autophagy was first recognized as a cellular response to starvation. Indeed, autophagy is activated in response to various metabolic stresses, including nutrient deprivation, growth factor depletion, or in the case of high metabolic needs. In these scenarios, bulk autophagy can serve as an alternative source of building blocks for synthesis and energy production. The pro-survival function of autophagy in the case of starvation is highly conserved, as is its regulation by the mTOR signaling pathway.

mTOR, a central regulator of cell growth, proliferation and survival, is a core component of two distinct functional complexes, mTORC1 and mTORC2. The mTORC1 complex, consisting of the mTOR catalytic subunit RAPTOR (regulatory-associated protein of mTOR), PRAS40 (proline-rich Akt substrate of 40 kDa), mLTS8 (mammalian lethal with SEC13 protein 8), and DEPTOR (DEP-domain containing mTOR-interacting protein), is sensitive to rapamycin and acts as the major regulator of autophagy. mTORC1 integrates signals from several pathways in response to intracellular amino acid and ATP levels as well as extracellular growth factors, and in turn stimulates nucleotide synthesis, ribosome biogenesis, protein synthesis and lipid synthesis through various targets including p70-S6K (ribosomal protein S6 kinase-1) and 4E-BP1 (inhibitor of the eukaryotic initiation factor 4E, or eIF4E, binding protein 1). In parallel to stimulating these different anabolic pathways, it inhibits autophagy. Thus, in nutrient-rich conditions, mTOR stimulates cell growth and proliferation, whereas in starvation conditions, mTOR is inhibited and autophagy is induced.

mTORC1 is activated downstream of many growth factors, particularly insulin. Growth factor receptors like the insulin receptor induce class I PI3K activation and consequent PI(3,4,5)P₃ production. Of note, the tumor suppressor PTEN can counteract this step. PIP₃ recruits the kinase AKT to the plasma membrane, which in turn inhibits autophagy in two ways. First, AKT phosphorylates the TSC1/2 (tuberous sclerosis complex) heterodimer, thereby inactivating it. TSC1/2 normally acts as a GTPase-activating protein (GAP) for and inhibitor of RHEB (Ras homolog enriched in the brain), which directly interacts with and activates mTORC1 (Long et al., 2005a). Thus, AKT activation activates RHEB and consequently mTORC1. Second, AKT can phosphorylate the transcription factor FOXO3, sequestering it in the cytoplasm and preventing it from activating transcription of autophagy genes like *LC3* and *GABARAP*, *VPS34* and *ULK1* (Mammucari et al., 2007; Zhao et al., 2007).

Amino acids are the basic building blocks for protein synthesis. Essential amino acids are imported into the cells by solute carriers (SLCs) in a glutamine-dependent manner and are sensed by RAG (Ras-related GTP-binding protein) GTPases heterodimers, which become activated in the presence of amino acids and interact directly with RAPTOR. This enables the recruitment of the mTORC1 complex to the lysosome, where RHEB can in turn activate mTORC1 [FIGURE 26] (Long et al., 2005b; Sancak et al., 2008). In the case of amino acid deprivation,

autophagy, through the degradation of cytoplasmic proteins, replenishes the pool of free amino acids and allows cell survival.

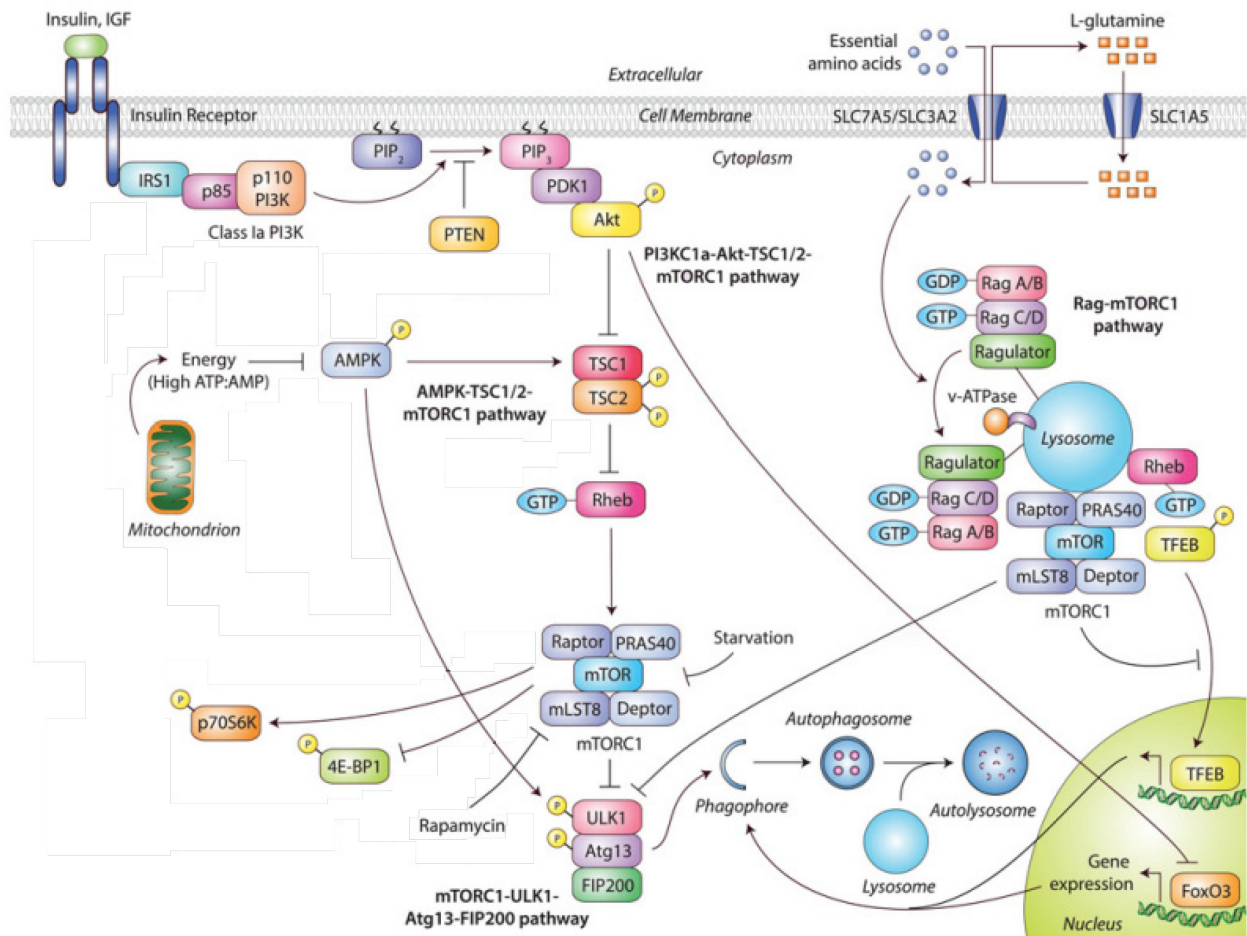


FIGURE 26. mTOR-dependent regulation of autophagy. Adapted from Sarkar, 2013.

Most of the anabolic mechanisms activated downstream of mTOR require energy in the form of ATP, the production of which is largely dependent on glucose metabolism. AMPK (AMP-activated protein kinase) acts as a major sensor of cellular energy status; as its name indicates, it becomes activated in response to decreasing ATP-to-AMP ratios. AMPK regulates mTOR either directly or through TSC1/2. Unlike AKT, AMPK activates TSC1/2, thereby inhibiting mTORC1 and activating autophagy (Inoki et al., 2003). It also inhibits mTORC1 activity by directly phosphorylating RAPTOR (Gwinn et al., 2008). Additionally, AMPK can activate autophagy independently of mTOR through direct activating phosphorylation of ULK1 (Egan et al., 2011; Kim et al., 2011).

Finally, mTORC1 also regulates autophagy by phosphorylating the transcription factor EB (TFEB), thereby maintaining it in the cytoplasm. In starvation conditions, TFEB translocates to the nucleus and positively regulates expression of lysosomal and autophagy genes (Settembre et al., 2011, 2012; Roczniak-Ferguson et al., 2012).

Thus, mTORC1 acts as a central hub regulating autophagy in response to converging signals relating the levels of growth factors, nutrients, and ATP.

1.2.2 APOPTOTIC SIGNALS: BCL2 AND P53

Many of the stimuli that ultimately trigger cell death also induce autophagy, suggesting that cells initially try to adapt to intrinsic or extrinsic stress, and resort to cell death in the face of insurmountable damage.

BECLIN1 was initially identified in a yeast-two-hybrid screen for proteins interacting with the antiapoptotic protein BCL2. Several BCL2-family members, including BCL2, BCL-XL (Bcl extra large), and MCL1 (myeloid cell leukemia sequence 1) interact with a BH3 domain on BECLIN1, thereby disrupting its interaction with VPS34 and preventing the initiation of autophagy (Patingre et al., 2005; Feng et al., 2007a; Maiuri et al., 2007; Oberstein et al., 2007). On the other hand, several BH3-only proteins – including BAD (Bcl2 antagonist of cell death), BID (BH3-interacting domain death agonist), BNIP3 (Bcl2 interacting protein 3), NOXA and PUMA – are thought to promote autophagy by competitively displacing BECLIN1 from BCL2-family proteins [FIGURE 27A] (Maiuri et al., 2007). One exception, BIM, has instead been described to sequester BECLIN1 and inhibit autophagy [FIGURE 27A] (Luo et al., 2012). Furthermore, JNK (c-Jun N-terminal kinases) –mediated phosphorylation of BCL2 enhances this displacement, favoring the induction of autophagy [FIGURE 27B] (Bassik et al., 2004; Wei et al., 2008).

p53 also regulates autophagy in parallel to apoptosis in a context-dependent manner. Generally speaking, the subcellular localization of p53 dictates its effect on autophagy; nuclear p53 stimulates autophagy, while cytoplasmic p53 inhibits it. Nuclear p53 induces transcription of the pro-apoptotic BH3-only proteins, but can also favor autophagic activity by up-regulating transcription of other targets. These targets include both genes encoding core autophagy proteins, including ULK1 and ULK2 (Gao et al., 2011; Kenzelmann Broz et al., 2013), and proteins involved in the regulation of autophagy. For example, p53 regulates transcription of both AMPK subunits and regulators of AMPK activity (Feng et al., 2007b; Budanov and Karin, 2008), as well as expression of PTEN, which counteracts class I PI3K-mediated stimulation of mTOR (Stambolic et al., 2001; Feng et al., 2007b). In addition, p53 activates transcription of the gene encoding DAPK1 (death-associated protein kinase 1), which phosphorylates BECLIN1 on its BH3 domain thereby releasing it from its interaction with BCL2 and stimulating its association with the class III PI3K complex to initiate autophagy (Zalckvar et al., 2009a, 2009b; Eisenberg-Lerner and Kimchi, 2012). Lastly, p53 induces transcription of DRAM (damage-regulated autophagy modulator), a lysosomal protein involved in the later stages of autophagy (Crighton et al., 2006). Cytoplasmic p53, on the other hand, has been reported to stimulate mTOR activity (Tasdemir et al., 2008) and interact with FIP200 (Morselli et al., 2011; Yu et al., 2013b), thus blocking the activity of the ULK1 complex and the initiation of autophagy [FIGURE 27C].

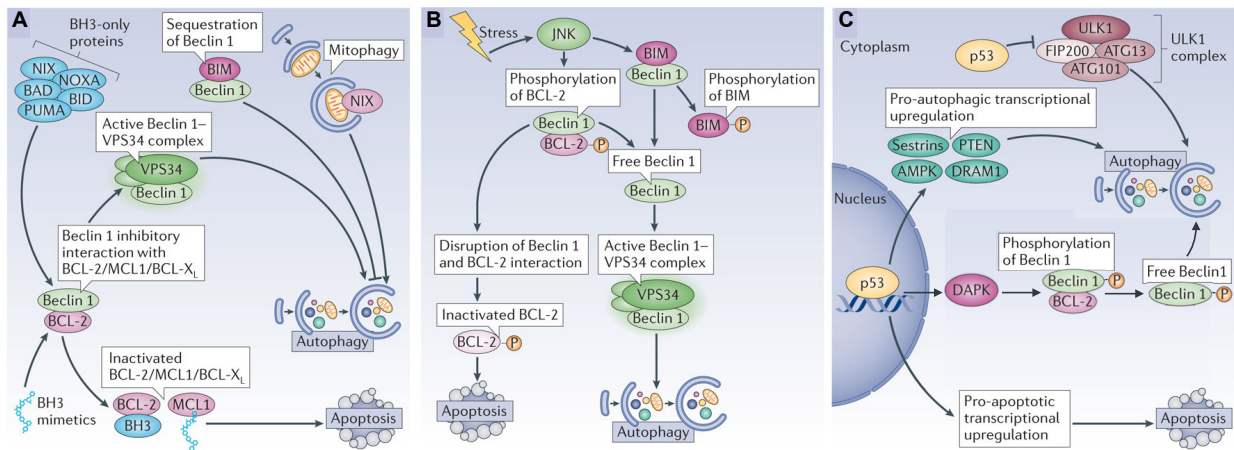


FIGURE 27. Coordinated stimulation of apoptosis and autophagy. Adapted from Mariño et al., 2014.

In line with the idea of a pro-survival role for autophagy, rapamycin treatment was shown to protect cells against pro-apoptotic treatment in an autophagy-dependent manner (Ravikumar et al., 2006). There have since been numerous examples in the literature of the protective role of autophagy against apoptosis. Mitophagy is one of the key mechanisms allowing this. By removing damaged mitochondria, autophagy prevents the release of pro-apoptotic mitochondrial intermembrane proteins, including Cytochrome c, which trigger a cascade of Caspase cleavage resulting the activation of effector Caspase-3 and 7 and ultimately in cellular demise. As will be discussed below, autophagy additionally helps alleviate different pro-apoptotic stresses, such as ER stress, oxidative stress, or DNA damage. Furthermore, various interactions between autophagic and apoptotic proteins have been reported and likely contribute to the balance between the two pathways in a context-dependent manner (Mariño et al., 2014; Cooper, 2018).

1.2.3 ENDOPLASMIC RETICULUM STRESS

The ER is the major site of protein synthesis and folding, and the compartment where vesicular transport throughout the cell initiates. Furthermore, the ER serves as a major reservoir of intracellular Ca^{2+} . Various cellular stresses – including perturbations in Ca^{2+} homeostasis, oxidative stress, altered protein glycosylation or defects in protein folding – can result in the accumulation of misfolded proteins in the ER lumen, a state known as ER stress. In response to ER stress, the cell induces the unfolded protein response (UPR), a network of signaling pathways involving three signal transducers embedded in the ER membrane: IRE1 α (inositol-requiring enzyme 1 α), ATF6 (activating transcription factor 6) and PERK (protein kinase RNA-like ER kinase).

- IRE1 α , the most conserved branch of the UPR, acts as an endonuclease on the cytosolic end, regulating the unconventional mRNA splicing of the UPR-specific transcription factor XBP1 (X-box binding protein 1) when activated. XBP1 in turn regulates transcription of factors involved in protein folding and secretion, lipid synthesis and ER-associated degradation (Lee et al., 2003; Acosta-Alvear et al., 2007). Upon higher levels of

ER stress, IRE1 α can also cleave other mRNAs at specific sequences, inducing mRNA decay (Hollien and Weissman, 2006).

- ATF6, a transcription factor normally sequestered in the ER, is relocalized to the Golgi apparatus and cleaved during ER stress, releasing its cytosolic fragment to activate expression of UPR target genes like the protein-folding chaperone BiP (binding immunoglobulin protein) (Haze et al., 1999; Yamamoto et al., 2007).
- Lastly, PERK oligomerizes upon ER stress, activates itself, and subsequently inhibits translation by phosphorylating eIF2 α (eukaryotic translation initiation factor 2 α). Hyperactivation of PERK favors the eIF2 α -independent translation of factors like ATF4, which notably drives the expression of the transcription factor CHOP (c/EBP homologous protein).

ER stress and the UPR have been shown to induce autophagy through several mechanisms. First, PERK-induced transcription factors like ATF4 and CHOP induce transcription of several *ATG* genes, including *LC3*, *ATG12* and *ATG5* (B'chir et al., 2013). Furthermore, both ATF4 and CHOP induce transcription of factors that will inhibit AKT and mTOR, resulting in the induction of autophagy (Du et al., 2003; Ohoka et al., 2005; Whitney et al., 2009). In addition, CHOP upregulates ERO1 α (ER oxidoreductase 1 α), which leads to the release of luminal Ca²⁺ stores into the cytoplasm, activating CaMKII (calcium calmodulin kinase II), a direct positive regulator of AMPK (Høyer-Hansen et al., 2007; Li et al., 2009a). Second, several UPR factors stimulate autophagy by acting on BECLIN1. CHOP induces transcription of genes encoding BH3-only proteins and represses transcription of those encoding anti-apoptotic BCL2 proteins, favoring BECLIN1 release from BCL2. IRE1 α also favors dissociation of BCL2 from BECLIN1 through JNK-mediated phosphorylation of BCL2 proteins, which additionally affects Ca²⁺ efflux from the ER (Bassik et al., 2004; Ogata et al., 2006; Høyer-Hansen et al., 2007). Finally, DAPK1 is also activated by ER-stress through unknown mechanisms, and also blocks the interaction between BCL2 proteins and BECLIN1 by phosphorylating BECLIN1 (Gozuacik et al., 2008; Zalckvar et al., 2009a).

Autophagy can then help alleviate ER stress by removing unfolded protein aggregates or controlling mitochondrial ROS production. In some contexts, however, ER stress-induced autophagy is deleterious as it results in a rare form of cell death termed autophagic cell death, characterized by large-scale autophagic vacuolization of the cytoplasm (Ding et al., 2007; Adolph et al., 2013).

1.2.4 HYPOXIA

Normoxia for mammalian cells is around 2-9% oxygen; oxygen levels below 1% are considered insufficient for the cell, which enters a state called hypoxia. This can occur physiologically during embryonic development, or in pathological conditions like insufficient blood flow to a tissue (ischemia) or in the core of solid tumors. The transcription factor HIF1 (hypoxia-inducible factor 1) is acutely induced in response to hypoxia to promote survival in oxygen-low conditions. HIF1 notably drives the transcription of genes involved erythropoiesis and angiogenesis to stimulate the local supply of oxygen, genes that decrease mitochondrial biogenesis and respiration to

avoid excessive ROS production, and genes that favor glycolysis to allow anaerobic ATP production. Among these, HIF1 targets include both the pro-apoptotic BH3-family protein BNIP3, which can induce autophagy by disrupting the interaction between BCL2 and BECLIN1, and BNIP3L, the previously mentioned inducer of mitophagy (Zhang et al., 2008; Bellot et al., 2009; Mazure and Pouyssegur, 2009). Indeed, increased autophagy, and particularly increased mitophagy acts as an adaptive response to hypoxia by reducing mitochondrial respiration and ROS levels. Furthermore, the low ATP levels during hypoxia result in AMPK activation and mTORC1 inhibition (Arsham et al., 2003; Liu et al., 2006). In addition to inhibiting protein synthesis, this could also activate autophagy, providing a compensatory source of materials for energy production.

1.2.5 OXIDATIVE STRESS

Excessive ROS levels are also a trigger of autophagy. The only ATG proteins known to be directly oxidized are ATG4 orthologues, which in addition to cleaving LC3 or GABARAP prior to PE-conjugation can also cleave away the PE conjugate. Oxidation of ATG4 by H₂O₂ inhibits its de-lipidating activity, thus favoring elongation of the phagophore (Scherz-Shouval et al., 2007). Both extracellular H₂O₂ and mitochondrial superoxide induce autophagy through AMPK- and BECLIN1- dependent mechanisms (Chen et al., 2009; Zhang et al., 2009a; Zmijewski et al., 2010; Chen et al., 2011; Kalyanaraman et al., 2012; Li et al., 2013). ROS and reactive nitrogen species (RNS) have also been described to indirectly activate autophagy through the DNA damages they induce, as described in the next section (Alexander et al., 2010; Rodríguez-Vargas et al., 2012; Tripathi et al., 2013). Upon oxidative stress, autophagy exerts protective effects and prevents ROS-mediated cell death (Gonzalez-Polo et al., 2007; Kaminsky et al., 2012; Dutta et al., 2013) through several mechanisms:

First, autophagy can degrade by-products of oxidative stress, including oxidized proteins and lipids. Macroautophagy is not the only player in the degradation of oxidized proteins, as the proteasome and CMA also play a role. The proteasome can degrade mildly oxidized proteins but necessitates these proteins to be unfolded to do so. More heavily oxidized proteins therefore rely on lysosomal degradation. Moreover, prolonged or chronic oxidative stress can result in oxidative damage to proteasome components (Kriegenburg et al., 2011). Expression of *LAMP2* is upregulated, HSC70 activity is stimulated, and CMA is activated during oxidative stress (Callahan et al., 2002; Kiffin et al., 2004). However, these two systems can only handle protein degradation. Autophagy is therefore likely turned to for the degradation of other oxidized substrates and of oxidized protein aggregates.

Second, autophagy regulates cellular ROS levels through mitophagy and pexophagy, thereby targeting two major sites of ROS production. In this way, autophagy not only prevents oxidative stress but can also respond to it; excessive mitochondrial superoxide production or inefficient ROS scavenging in the mitochondria (ie. by SOD2) result in membrane depolarization and induction of PARKIN-mediated mitophagy (Wang et al., 2012).

Third, autophagy exerts its protective effects by mediating the antioxidant response, notably via the transcription factor NRF2. NRF2 is normally sequestered in the cytoplasm by the E3

ubiquitin ligase adaptor protein KEAP1 (kelch-like ECH-associated protein 1) and subsequently degraded by the proteasome. As a consequence, the constitutive levels of NRF2 are very low. Oxidative stress-induced modifications of cysteine residues in KEAP1 induces a conformational change that blocks its association with NRF2, allowing NRF2 to translocate to the nucleus and induce expression of its target antioxidant response genes (Hayes and McMahon, 2009; Lau et al., 2010; Taguchi et al., 2011). In 2010, Komatsu et al. and Lau et al. independently showed that defective autophagy resulted in NRF2 stabilization and induction of antioxidant response genes through the accumulation of p62. Indeed, p62 interacts with the NRF2-binding site on KEAP1, stabilizing NRF2 and inducing expression of its target genes (Komatsu et al., 2010; Lau et al., 2010). In turn, NRF2 positively regulates p62 expression, creating a positive feedback loop (Jain et al., 2010). Furthermore, a later study by Komatsu's group showed that phosphorylation of p62 by mTORC1 or other kinases markedly increases its affinity for KEAP1 both in the case of defective autophagy or upon activation of selective autophagy (ie. mitophagy or xenophagy), revealing a regulated coupling between the KEAP1-NRF2 system and autophagy [FIGURE 28] (Ichimura et al., 2013).

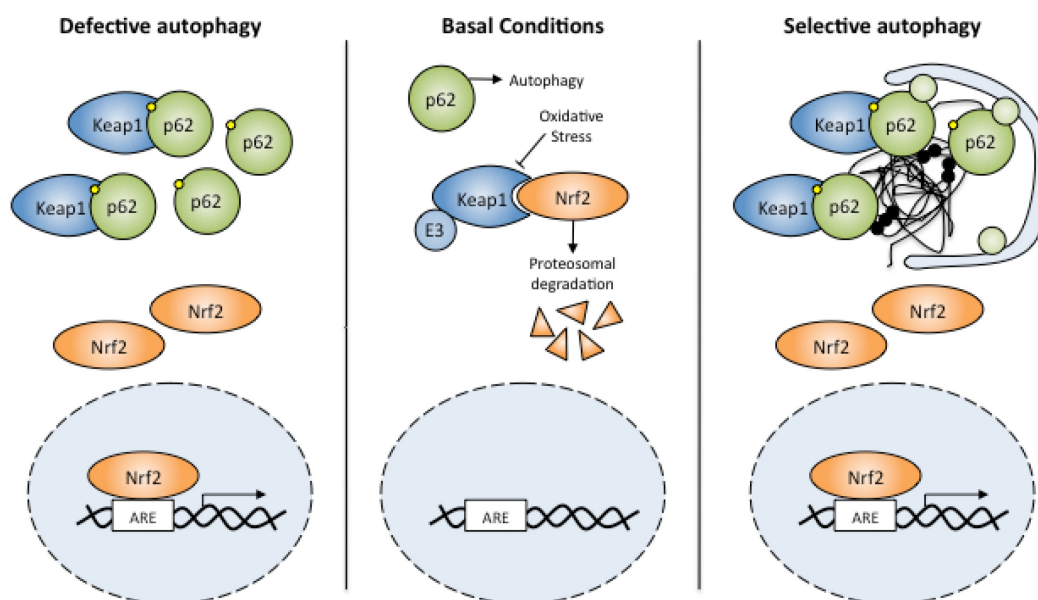


FIGURE 28. Keap1-Nrf2 regulation by autophagy. ARE: Antioxidant response element.

1.2.6 DNA DAMAGE

Over the course of a lifetime, DNA damage constantly arises from errors in DNA replication, spontaneous chemical reactions or exposure to external and cellular metabolism-derived agents. Different DNA damage repair (DDR) pathways will come into play depending on the type of lesion and phase of the cell cycle. Three main repair pathways are extensively described for single-stranded lesions. Small base alterations – including oxidized bases, alkylation and single-stranded breaks – are handled by base-excision repair (BER) [FIGURE 29]. Bulkier lesions that distort the DNA structure and block the progression of replicating or transcribing polymerases – such as those induced by UV rays – are repaired by nucleotide-excision repair (NER) [FIGURE 29]. Lastly, replication errors like base-base mismatches or insertions/deletions (which

frequently occur at repeated sequences like microsatellites) are handled by the aforementioned MMR pathway [FIGURE 29]. Double-stranded breaks (DSBs) resulting from IR, genotoxic agents, or free radicals are generally repaired either by either homologous recombination (HR) or non-homologous end joining (NHEJ). In HR, DSBs are first recognized by the MRN complex (composed of MRE11, RAD50, and NBS1) and ATM (ataxia telangiectasia mutated), which phosphorylates downstream targets involved in DSB repair and cell cycle arrest to allow sufficient time for repair (including the histone H2AX called γ H2AX when phosphorylated, BRCA1, and effector checkpoint kinases 1 and 2, or CHK1 and CHK2). The extremities of the DSBs are resected by exonucleases to create 3' single-stranded overhangs, which then interact with proteins like RAD51 and BRCA2 to search for and invade the homologous chromosome, used as a template to repair the break [FIGURE 29]. Using the homologous DNA as a template for repair means two things: first, that HR can only occur during S, G2, or M phase of the cell cycle, when a homologous chromosome is present, and second, that the damaged DNA will be accurately recovered. NHEJ, on the other hand, can occur at any stage of the cell cycle but is more error-prone. DSBs are recognized by KU70-KU80 heterodimers at the two broken ends, which allows the recruitment of other proteins including DNA-PK (DNA-dependent protein kinase) and LIG4/XRCC4 (ligase4/X-ray repair cross complementing 4) proteins involved in the ligation of the two ends [FIGURE 29]. The choice between HR and NHEJ at DSBs is thought involve a mutual antagonism between the HR factor BRCA1 and NHEJ-promoting protein 53BP1 (tumor protein p53 binding protein 1).

A growing body of evidence suggests that autophagy can be activated upon DNA damage.

ATM, a major sensor of DSBs, acts as a central link between DNA damage and autophagy by directly activating AMPK (Alexander et al., 2010). This can notably be the case in response to ROS- or RNS-induced damages (Alexander et al., 2010; Tripathi et al., 2013). Oxidative stress can also activate ATM independently of DSBs, and cells lacking ATM are more sensitive not just to DNA damage but also to oxidative stress (Guo et al., 2010), although autophagy has not been studied in link with these observations.

AMPK can additionally be activated in response to ROS-induced damages through the NAD⁺ dependent enzyme PARP1 (poly [ADP-ribose] polymerase 1), which plays a key role in several DNA repair pathways (Rodríguez-Vargas et al., 2012; Chaudhuri and Nussenzweig, 2017). PARP1 activity results in depletion of the NAD⁺ and ATP pool, which in turn can be sensed by AMPK to induce autophagy (Rodríguez-Vargas et al., 2012). By depleting cellular NAD⁺, PARP1 can also impair the activity of other NAD⁺ dependent enzymes, like Sirtuins. Notably, SIRTUIN1 deacetylates and thereby activates PGC1 α (peroxisome proliferator-activated receptor α coactivator 1 α), a transcriptional regulator of mitochondrial biogenesis. Reduced SIRTUIN1 activity during DNA damage repair could therefore also favor mitochondrial clearance and ROS control over mitochondrial biogenesis (Fang et al., 2014).

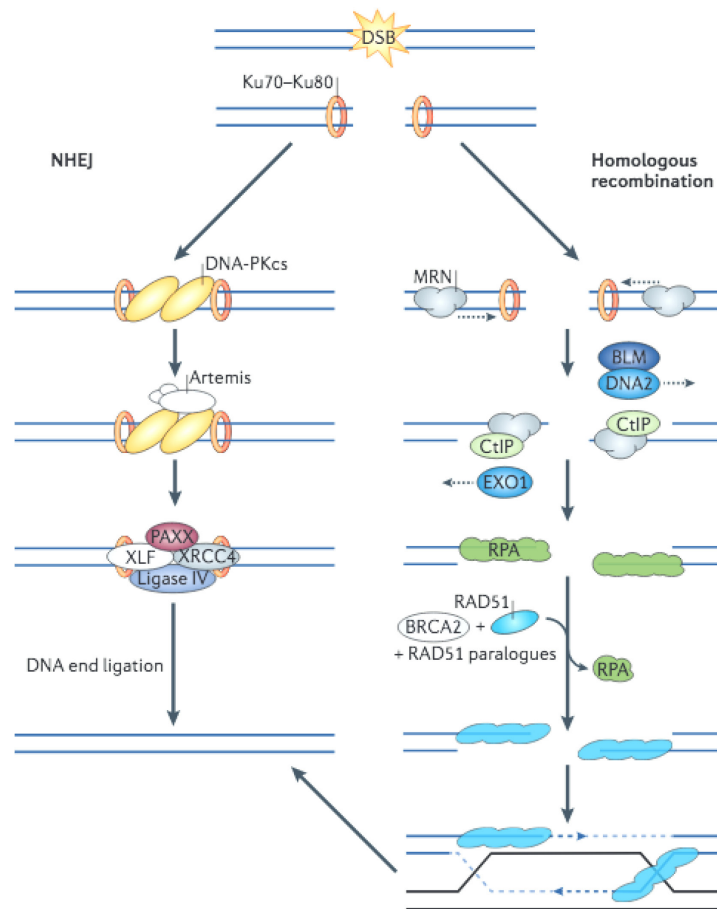
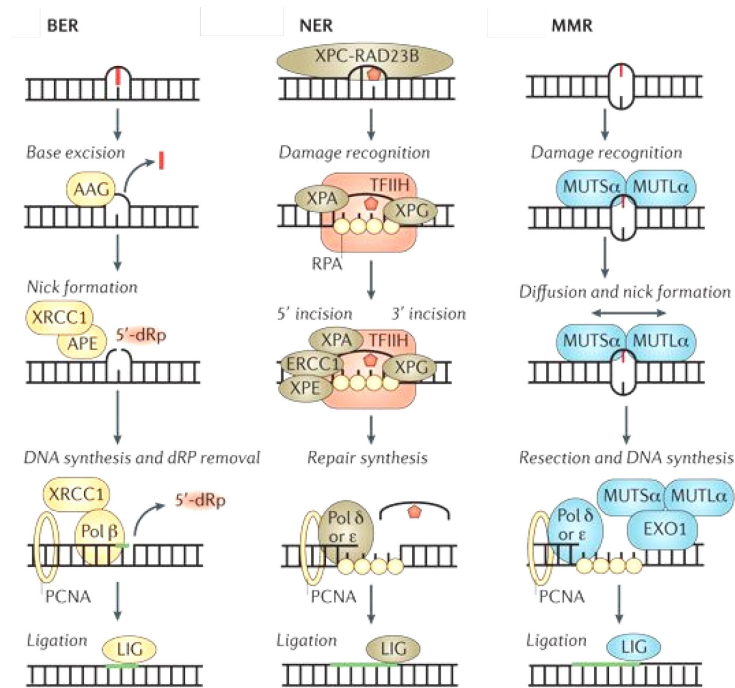


FIGURE 29. Single-stranded (top) and double-stranded (bottom) DNA damage repair mechanisms. Adapted from Fu et al., 2012 and Schwertman et al., 2016.

Finally, DNA damage induces p53, thus favoring its pro-autophagic nuclear function through the expression of genes encoding BH3-only proteins, AMPK subunits and AMPK regulators (Feng et al., 2007b; Budanov and Karin, 2008), PTEN (Stambolic et al., 2001; Feng et al., 2007b), DAPK1 (Zalckvar et al., 2009a, 2009b; Eisenberg-Lerner and Kimchi, 2012), or DRAM (Crichton et al., 2006).

In return, autophagy helps preserve genomic stability. BECLIN1 has long been established as a tumor suppressor gene, particularly in breast cancer (Aita et al., 1999; Yue et al., 2003). In light of this, it was shown that allelic loss of *BECLIN1* or defective autophagy sensitized cells to metabolic stress and promoted chromosome instability, with increased DNA damage, gene amplification, and aneuploidy (Karantza-Wadsworth et al., 2007; Mathew et al., 2007; Dou et al., 2015). Further supporting its protective role, the upregulation of autophagy following genotoxic treatments, like irradiation or chemotherapy, improves cell survival. This is of particular interest with regards to cancer treatment, as will be discussed in the next subchapter. However, the mechanistic implication of autophagy in the maintenance of genomic stability has only recently started to be explained.

Once activated, autophagy can regulate levels of DNA-damaging ROS, as mentioned previously, and can provide a supply of energy and nucleotides to help fuel DNA repair. Autophagy can also selectively remove micronuclei, or nuclear membrane-enclosed damaged chromosome fragments (Rello-Varona et al., 2012; Adams et al., 2013). More recently, several studies showed a direct implication of autophagy in regulating the DDR machinery.

- One study by Qiang et al. demonstrated that autophagy positively regulates NER by enhancing DNA damage recognition through the sensor proteins XPC (xeroderma pigmentosum, complementation group C) and DDB2 (DNA damage-binding protein 2) (Qiang et al., 2016).

Furthermore, defects in DSB repair when autophagy is compromised have been vastly reported (Bae and Guan, 2011; Chen et al., 2015; Lin et al., 2015; Hewitt et al., 2016; Wang et al., 2016d). A few recent studies have pinpointed mechanisms explaining this phenomenon:

- Liu et al. described the first mechanistic link between the inhibition of autophagy and DNA repair. They show that the inhibition of autophagy stimulates proteasomal activity leading to increased degradation of CHK1, a key factor for HR [FIGURE 30A]. As a consequence, HR is critically impaired and NHEJ is turned to for DSB repair, resulting in diminished genomic integrity (Liu et al., 2015).
- Chen et al. further showed that autophagy-mediated degradation of heterochromatin protein 1 α (HP1 α) allows access of HR factors to the site of DNA damage (Chen et al., 2015).
- Wang et al. found that inhibition of autophagy suppresses DNA-damage-induced H2A ubiquitination. This effect is dependent on p62 accumulation in autophagy-deficient cells, as the overexpression of p62 alone reduces chromatin ubiquitination while p62 knockdown enhances it. Mechanistically, they found that p62 binds the E3 ligase RNF168 (ring finger protein 168) in the nucleus and inhibit its activity, which is

normally essential for H2A ubiquitination and the recruitment of DDR factors like RAD51 and BRCA1 to DSBs [FIGURE 30B]. As a result, autophagy-deficient cells have reduced efficiency of HR, but not NHEJ, and have higher sensitivity to irradiation (Wang et al., 2016f).

- Hewitt et al., showed a similar effect of nuclear p62 on DSB repair through distinct mechanisms. They found p62 to dynamically interact with the cytoskeleton protein FilaminA at DNA damage foci. FilaminA was previously shown to be important for RAD51 recruitment to DSBs and efficient HR (Jingyin et al., 2009; Velkova et al., 2010). p62 stimulates the proteasomal degradation of both FilaminA and RAD51 directly in the nucleus, thus favoring NHEJ over HR and slowing the rate of repair. Inhibition of autophagy therefore similarly slows DNA repair kinetics in a p62-dependent manner [FIGURE 30C] (Hewitt et al., 2016).
- Finally, Xu et al. showed that by targeting the STAT3 repressor KAP1 (Krüppel associated box associated protein 1) for degradation, autophagy led to the upregulation of STAT3-mediated *BRCA1* transcription, thereby promoting HR and maintaining the genomic integrity of hematopoietic cells (Xu et al., 2017).

Although each of these studies indicate different mechanisms for autophagic regulation of DSB repair, they all support a role for autophagy in ‘error-proof’ HR over ‘error-prone’ NHEJ, and therefore a key role of autophagy in preserving genomic integrity.

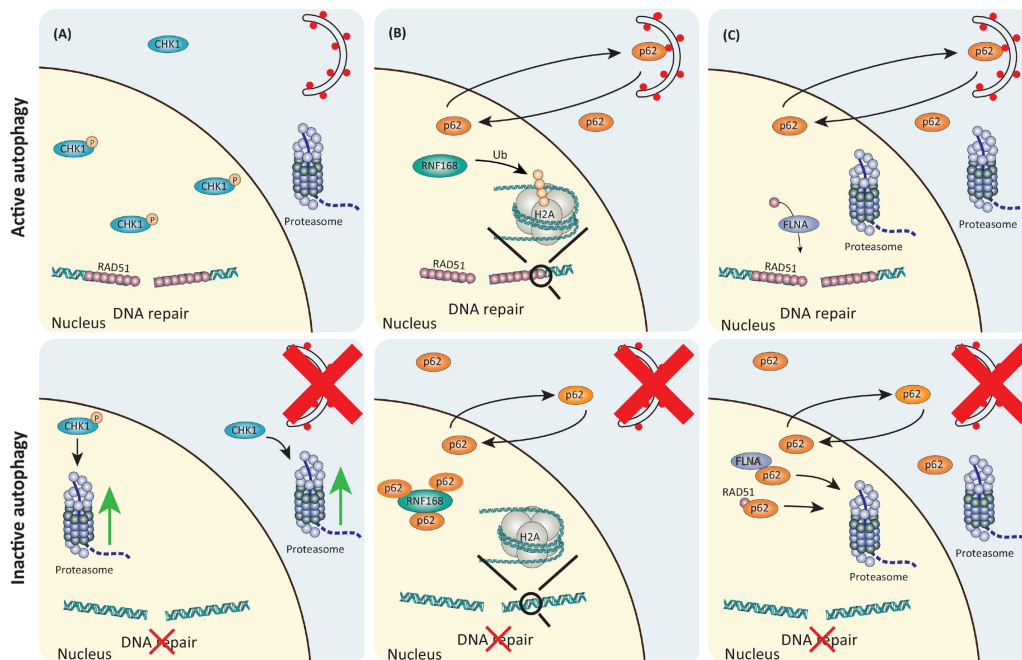


FIGURE 30. Autophagy regulation of double-stranded break repair. Adapted from Hewitt and Korolchuk, 2017.

1.2.7 INFECTION, INFLAMMATION & IMMUNITY

Autophagy additionally plays an essential role in innate immunity. As previously mentioned, xenophagy acts as a key cellular defense against invasive pathogens (Gutierrez et al., 2004; Nakagawa et al., 2004). The precise mechanisms inducing xenophagy are a matter of ongoing research and often dependent on the pathogen at hand. One way to target autophagy to an

invading pathogen is the ubiquitination of the pathogen's proteins or their direct recognition by the autophagic machinery (Ogawa et al., 2005; Thurston et al., 2009; Zheng et al., 2009; Wild et al., 2011). As a more general mechanism, remnants of ruptured membranes following pathogen entry into the cytosol also induce autophagy (Dupont et al., 2009; Thurston et al., 2012; Kumar et al., 2017). Viruses can also stimulate autophagy through antiviral PKR (protein kinase R) signaling, which inhibits viral translation by phosphorylating the translation initiation factor eIF2 α (Talloczy et al., 2002; Tallóczy et al., 2006). Some viruses, like the Hepatitis C virus, induce ER stress, which in turn triggers autophagy (Sir et al., 2008). Highlighting the long-standing role of autophagy in innate immune defense, some pathogens have developed ways to escape autophagy. This is the case for the Herpes simplex virus 1, which encodes a protein capable of sequestering BECLIN1 (Orvedahl et al., 2007), or the enteric bacteria *Shigella*, which secretes factors that mask its proteins from recognition by the autophagy machinery (Ogawa et al., 2005). Some pathogens even hijack autophagy for their own benefit, using the vacuole as a replicative niche or stimulating autophagy to provide them with nutrients (Gutierrez et al., 2005; Birmingham et al., 2008; Pujol et al., 2009; Moreau et al., 2010).

Besides directly recognizing pathogens to target them for xenophagy, autophagy can also be activated by cytosolic NLRs. NOD1 and NOD2 both drive autophagosome formation upon peptidoglycan detection by interacting directly with ATG16L1 (Cooney et al., 2010; Travassos et al., 2010; Chauhan et al., 2015). As will be discussed later, this is of particular interest in IBD as allelic variants of the *NOD2* gene or in autophagy genes like *ATG16L1* and *IRGM* (IFN-inducible immunity-related GTPase family M member 1) predispose to Crohn's disease. Autophagy can also be activated by extracellular or membrane-enclosed (phagocytosed) pathogens, as TLR signaling also stimulates autophagosome formation via MyD88 and TRIF (Toll-IL-1 receptor domain-containing adapter-inducing IFN) (Sanjuan et al., 2007; Xu et al., 2007; Delgado et al., 2008; Shi and Kehrl, 2008, 2010).

Aside from NOD1 and NOD2, many NLRs are inflammasome components that can be stimulated by diverse stimuli, including bacterial toxins or mitochondrial danger-associated molecular patterns (DAMPs) like ATP, ROS or mitochondrial DNA. These drive the activation of Caspase 1, which subsequently cleaves pro-IL-1 β and pro-IL-18 to their mature form. IL-1 β and IL-18 are then either secreted or pass through permeabilized membranes (Cullen et al., 2015), driving an inflammatory response. Several NLRs and inflammasomes have been reported to induce autophagy (Lei et al., 2012; Byrne et al., 2013; Wlodarska et al., 2014). However, the role of autophagy in this pathway remains somewhat ambiguous. On the one hand, autophagy attenuates inflammasome activity by preventing bacterial product or DAMP accumulation (Nakahira et al., 2011; Zhou et al., 2011; Kreibich et al., 2015) and by targeting inflammasome subunits and pro-IL-1 β for degradation (Harris et al., 2011; Shi et al., 2012). This is consistent with studies showing that autophagy defects lead to inflammasome hyperactivation (Saitoh et al., 2008; Nakahira et al., 2011; Plantinga et al., 2011; Lee et al., 2012b; Buffen et al., 2013), and suggests that autophagy acts to dampen inflammatory responses. On the other hand, autophagy has also been reported to drive the unconventional secretion of IL-1 β (Dupont et al., 2011). As with IL-1 β , autophagy is also involved in the immunogenic secretion of DAMPs like ATP and

HMGB1 (high mobility group box 1, a chromatin-associated protein) from dying cells (Tang et al., 2010a; Martins et al., 2014).

In addition to bacterial signals and the inflammasome, DAMPs can directly stimulate autophagy (Biswas et al., 2008; Tang et al., 2010b, 2011), as can several inflammatory cytokines like IL-1 β , TNF, and IFN γ (Gutierrez et al., 2004; Singh et al., 2006). Conversely, autophagy has been shown to limit production of several pro-inflammatory cytokines other than IL-1 β , including IL-1 α , IL-18, IL-23, IL-6, TGF β , and type I IFNs (Castillo et al., 2012; Peral de Castro et al., 2012; Marchiando et al., 2013; Ding et al., 2014; Mathew et al., 2014; Grimm et al., 2016). Autophagy also attenuates inflammatory NF κ B signaling (Paul et al., 2012), adding an additional layer to its many immuno-modulatory functions.

Besides innate immunity and inflammatory signaling, autophagy contributes to adaptive immunity by mediating antigen presentation. More specifically, autophagy delivers cytosolic antigens or antigens from targeted pathogens to class II MHC (major histocompatibility complex)-loading compartments in dendritic cells, B cells, and epithelial cells. In this way, autophagy affects both the development of naïve T cells in the thymus (Nedjic et al., 2008) as well as adaptive T cell responses (Paludan et al., 2005; Schmid et al., 2007; Cooney et al., 2010; Ireland and Unanue, 2011). Likewise, autophagy can affect the presentation of antigens by class I MHC (Tey and Khanna, 2012; Wenger et al., 2012; Fiegl et al., 2013). Autophagy plays an additional role in antigen presentation by mediating the degradation of immunological synapses; thus, defective autophagy results in hyperstable interactions between dendritic cells and T cells, enhancing T cell activation (Wildenberg et al., 2012).

Lastly, autophagy within hematopoietic cells has proven to be important for the proper differentiation and function of certain lineages. Loss of certain ATGs is detrimental to immunosuppressive regulatory T cells (Kabat et al., 2016; Le Texier et al., 2016; Marcel and Sarin, 2016; Wei et al., 2016), whereas it conversely promotes differentiation towards pro-inflammatory T_H2 and T_H9 profiles (Kabat et al., 2016; Rivera Vargas et al., 2017). Deletion of *Atg5* or *Atg7* also impairs the differentiation and immunity of natural killer cells (O'Sullivan et al., 2015, 2016; Wang et al., 2016c) macrophages (Esteban-Martínez et al., 2017), and effector or memory CD8⁺ T cells (Puleston et al., 2014; Xu et al., 2014; Schlie et al., 2015).

II. AUTOPHAGY IN PHYSIOLOGY AND PATHOLOGY

2.1 AUTOPHAGY IN HEALTH AND DISEASE

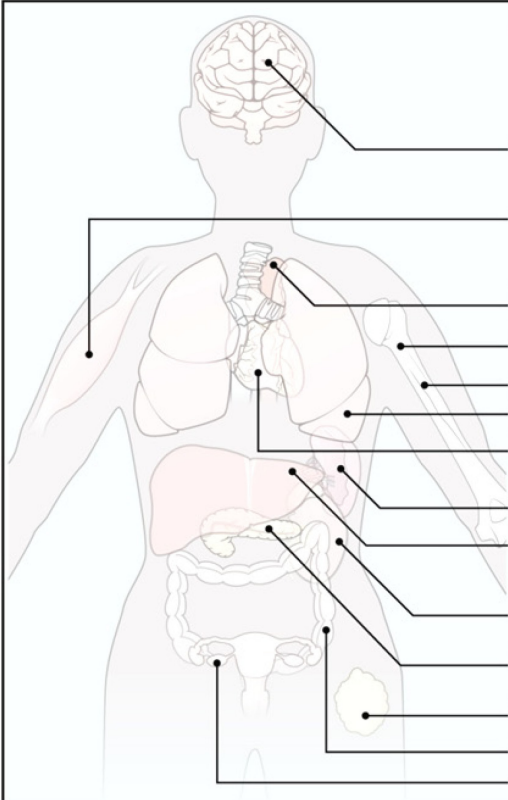
In light of the vast array of functions of autophagy in cellular homeostasis, it comes as no surprise that various links have been established between autophagy and physiology – or defects in autophagy and disease – both in human tissues and murine models. A few examples are discussed below and illustrated in **FIGURE 31**.

2.1.1 DEVELOPMENT

The fact that many systemic *Atg*-knockout mice die either in utero or within 1 day of birth highlights the importance of the autophagic pathway during development. Interestingly, for reasons that remain to be elucidated, mice deficient in genes not involved in the conjugation systems (ie. *Beclin1*, *Vps34*, *Atg9a*, *Atg13*) are embryonic lethal, while mice deficient in nonredundant *Atg* genes involved in conjugation systems (ie *Atg3*, *Atg5*, *Atg7*, *Atg12*, *Atg16l1*) are neonatal lethal (Kuma et al., 2017). From a few hours following fertilization, autophagy is massively upregulated, and is required for the survival of the blastocyst (Tsukamoto et al., 2008). Although the exact role of autophagy at this point remains unclear, it has been postulated to act as a source of nutrients until implantation of the embryo (Tsukamoto et al., 2008), or to mediate the degradation of paternal mitochondria (Rojansky et al., 2016) and unnecessary maternal proteins. During early development of the embryo, autophagy was also shown to play an essential role in energy production allowing the detection and clearance of apoptotic cells (Qu et al., 2007).

The neonatal period represents another phase where autophagy is crucial (Kuma et al., 2004; Komatsu et al., 2005; Kuma et al., 2017). Using GFP-tagged LC3 to visualize autophagosomes in mice, autophagy was shown to increase shortly after birth and remain elevated for 24 to 48 hours (Kuma et al., 2004; Tritarelli et al., 2004). Again, autophagy is thought to provide necessary nutrients to the infant following the loss of maternal supply and until breastfeeding begins. In line with this idea, while the acute systemic deletion of *Atg7* in adult mice is not immediately lethal in fed conditions (although these mice eventually die of neurodegeneration or bacterial infection), it becomes lethal upon fasting (Karsli-Uzunbas et al., 2014). However, nutrient supply may not be the only important function of autophagy in this context; Lee et al. founds that starved *Atg7*-deficient mouse embryonic fibroblasts induced p53 in response to accumulated DNA damage, and that blocking the DNA damage response by deleting the checkpoint kinase CHK2 partially rescued the neonatal lethality of *Atg7*-knockout mice (Lee et al., 2012a).

Furthermore, conditional ablation of autophagy genes revealed the importance of the pathway in the proper development of several tissues, notably the central nervous system (Hara et al., 2006; Komatsu et al., 2006; Maria Fimia et al., 2007) and the hematopoietic system (Pua et al., 2007; Miller et al., 2008; Nedjic et al., 2008; Mortensen et al., 2011).



Organ	Roles of Autophagy	Diseases
General function	Starvation-induced amino acid production; constitutive turnover of cytoplasmic contents; selective degradation of p62, damaged mitochondria, microbes, etc.; life-span extension/antiaging	Tumor suppression and progression
Brain	Prevention of aggregate formation; Parkinson-dependent mitophagy; regulation of food intake and energy balance	Parkinson disease Alzheimer disease
Muscle	Maintenance of muscle mass	Myopathies : (Danon disease, XMEA, Bethlem myopathies, UCMD) Lysosomal storage diseases
Thymus	Negative selection	
Bone marrow / Hematopoiesis	Erythropoiesis; maintenance of hematopoietic stem cell	
Bone		Paget disease?
Lung	Regulation of airway responsiveness	Cystic fibrosis
Heart	Adaptation to hemodynamic stress; prevention of age-dependent dysfunction	Cardiac hypertrophy
Immune/ lymphoid system	Regulation of cytokine production; development of T cells and B cells	Infection
Liver	Prevention of hepatocellular degeneration; suppression of hepatic tumors; lipid droplet elimination; gluconeogenesis	α_1 -antitrypsin deficiency Hepatocellular carcinoma?
Kidney	Maintenance of podocyte and tubular cell integrity	
Pancreas	β -cell adaptation to high-fat diet; prevention of trypsin autoactivation	Diabetes Acute pancreatitis
Adipose tissue	Adipogenesis	Obesity
Intestine	Maintenance of Paneth cell function	Crohn disease
Embryos	Preimplantation development	

FIGURE 31. Roles of autophagy in physiology and pathology. Data illustrated in this figure have been obtained from both mouse and human studies. XMEA: X-linked myopathy with excess autophagy, UCMD: Ullrich congenital muscular disorder. Adapted from Mizushima and Komatsu, 2011.

2.1.2 METABOLIC DISEASE

One of the most drastic phenotypes of tissue-specific autophagy deletion can be seen in the liver, where *Atg7* loss causes hepatomegaly, hepatocytic hypertrophy, and eventually hepatitis in mice (Komatsu et al., 2005). This is associated with an accumulation of swollen and deformed mitochondria, increased numbers of peroxisomes and lipid droplets, as well as p62- and Ubiquitin-positive protein aggregates. The lack of proper p62 turnover partially explains these phenotypes, as they are largely attenuated by the simultaneous deletion of the *Sqstm1* gene (Komatsu et al., 2007). Several other studies reported lipid accumulation in the liver following pharmaceutical inhibition of autophagy or deletion of key autophagy genes (Singh et al., 2009a; Jaber et al., 2012; Settembre et al., 2013), indicating a role for autophagy in hepatocyte function and lipid metabolism. Thus, it has been suggested that defective autophagy in the liver may contribute to the incidence of metabolic syndrome (Singh et al., 2009a; Yang et al., 2010).

In the pancreas, autophagy plays a particularly important role in the function of insulin-producing β cells, as β cell-specific *Atg7* deletion results in islet degeneration, in part attributed to increased susceptibility to ER stress and ROS accumulation (Ebato et al., 2008; Jung et al., 2008; Quan et al., 2012). This results in impaired insulin secretion, hyperglycemia, and also prevents the physiological expansion of β cells in response to a high-fat diet (Ebato et al., 2008; Jung et al., 2008). Defective autophagy in this tissue could therefore be linked to type 2 diabetes (Kaniuk et al., 2007; Masini et al., 2009; Quan et al., 2012), while the beneficial effects of

metformin, a drug commonly used to treat type 2 diabetes, could be linked to the induction of autophagy (Masini et al., 2009).

On the contrary, deletion of either *Atg5* or *Atg7* in adipose tissue leads to impaired adipogenesis and prevents lipid accumulation, with drastically increased mitochondrial numbers and high rates of fatty acid oxidation in the few remaining adipocytes. This results in increased insulin sensitivity and a lean, obesity-resistant phenotype (Baerga et al., 2009; Singh et al., 2009b; Zhang et al., 2009b). Autophagy therefore plays a key role in systemic metabolism and in the proper function of the different organs involved.

2.1.3 NEURODEGENERATION

The fact that the majority mice die of neurodegeneration within 2 to 3 months following the systemic deletion of *Atg7* speaks to the importance of autophagy in the central nervous system (Karsli-Uzunbas et al., 2014). Germline neuron-specific deletion of *Atg5*, *Atg7*, or *Fip200* leads to the accumulation of autophagy substrates like protein aggregates and damaged organelles within neurons. These mice display motor and behavioral deficits along with neurodegeneration and eventually die within 6 months of birth (Hara et al., 2006; Komatsu et al., 2006; Liang et al., 2010).

In line with these observations, defective autophagy was observed in human neurodegenerative diseases (Nixon et al., 2005; Pickford et al., 2008). Autophagy can notably degrade aggregate-prone proteins responsible for various neurodegenerative diseases in humans, including TAU aggregates in Tauopathies like Alzheimer's disease, α -SYNUCLEIN aggregates in Parkinson's disease, and aggregates of mutant Huntingtin found in Huntington's disease (Ravikumar, 2002; Ravikumar et al., 2004; Berger et al., 2006). In line with these observations, pharmacological stimulation of autophagy helps eliminate these aggregates and improves neurological symptoms in animal models of neurodegenerative disease (Ravikumar et al., 2004; Tanaka et al., 2004; Berger et al., 2006; Ma et al., 2007; Spencer et al., 2009; Kickstein et al., 2010; Ozcelik et al., 2013). Moreover, genetic mutations in the genes encoding the mitophagy proteins PARKIN and PINK1 cause early-onset familial and sporadic Parkinson's disease as damaged mitochondria accumulate, culminating in the degeneration of dopaminergic neurons (Cookson, 2012). This indicates an important role not just for autophagy, but also for mitophagy in neurodegenerative diseases.

2.2 AUTOPHAGY IN ADULT STEM CELLS

As the implication of autophagy in intracellular quality control and cellular remodeling grows more and more evident, several recent studies have started to approach the question of its role in homeostatic adult stem cells. Adult stem cells are pluripotent cells capable of both self-renewal and differentiation and are responsible for the homeostatic maintenance and damage-induced regeneration of the organism's tissues. Among the best-studied adult stem cells are hematopoietic stem cells (HSCs), neural stem cells (NSCs), and muscle stem cells (more commonly called satellite cells). Autophagy has been studied in each of these, revealing

important contributions to stem cell maintenance, renewal, and differentiation with both convergent and unique roles depending on the tissue.

Adult HSCs are kept quiescent in a hypoxic niche in the bone marrow and can differentiate into multipotent progenitors either physiologically to replenish the normal loss of blood cells or in response to haematological stress. Autophagic flux is particularly high in HSCs compared to their mature progeny (Watson et al., 2015). Inhibition of autophagy in the adult HSCs of mice by genetic ablation of *Atg7*, *Atg5*, or *Atg12* resulted in severe impairments in HSC function (determined by HSC numbers, colony-formation capacity, and capacity to replenish the haematopoietic system when transplanted into irradiated mice), increased proliferation, and myeloid lineage expansion (Mortensen et al., 2011; Watson et al., 2015; Ho et al., 2017). In each of these studies, increased numbers of mitochondria and increased levels of ROS were reported following the loss of autophagy. High autophagy could therefore maintain HSCs in a quiescent state within their hypoxic niche in the bone marrow by limiting mitochondrial numbers and thereby ROS production. In line with this, silencing of *Pink1* or *Parkin* expression also impairs HSC maintenance (Ito et al., 2016). Downregulation of autophagy and mitophagy could also serve as a key regulatory step to HSC differentiation, which requires an oxidative switch (Yu et al., 2013a; Kohli and Passequé, 2014). Mitophagy further contributes to the terminal differentiation of macrophages (Esteban-Martínez et al., 2017) and erythrocytes (Sandoval et al., 2008).

Adult NSCs also reside in hypoxic niches in different regions of the brain. Although they are relatively quiescent, they can give rise to neural progenitors that allow the maintenance and reorganization of the brain circuitry. Just as loss of autophagy in HSCs impairs hematopoiesis, loss of some (but not all) autophagy genes in NSCs impairs neurogenesis (Yazdankhah et al., 2014; Lv et al., 2015; Wang et al., 2016a). Loss of *Ambra1* (an *Atg* encoding a protein that stimulates BECLIN1 activity in the class III PI3K complex), *Beclin1*, *Fip200*, or *Atg13* reduces proliferation and increases apoptosis of NSCs, ultimately leading to stem cell exhaustion (Wang et al., 2013, 2016a; Yazdankhah et al., 2014). Mechanistically, *Fip200* deletion results in superoxide accumulation due not to an increase in mitochondrial mass, but rather to a p62-dependent relocation of cytoplasmic SOD1 to the nucleus (Wang et al., 2016a). In line with the importance of ROS regulation in NSCs, FOXO proteins were previously shown to be essential for NSC maintenance by driving expression of antioxidant defense genes (Paik et al., 2009). Notably, these phenotypes are in stark contrast with those reported following the loss of autophagy in embryonic NSCs, where *Ambra1* or *Atg5* knockdown instead increases proliferation and inhibits differentiation. In this context, increased autophagy is through to promote differentiation by supporting the high energy demands of differentiating neural progenitors (Vázquez et al., 2012; Lv et al., 2015) and by favoring a glycolytic switch through BNIP3L-mediated mitophagy (Esteban-Martínez et al., 2017). This suggests that the need for autophagy differs between foetal and adult NSCs.

Autophagy induction is also required to support the bioenergetic demands of activated satellite cells (Tang and Rando, 2014). The loss of *Atg7* in satellite cells leads to oxidative stress, DNA

damage, protein aggregates, damaged mitochondria and markers of senescence (García-Prat et al., 2016). Interestingly, these are also phenotypes of aged satellite cells, along with decreased autophagic flux, which can be rescued by rapamycin or spermidine treatment, two drugs known to stimulate autophagy.

Clearly, the function of autophagy in adult stem cells varies greatly between tissues. Nevertheless, common trends arise when comparing the role of autophagy in the maintenance and differentiation of each of these stem cell populations: particularly ROS regulation, mitophagy, and energy homeostasis (Boya et al., 2018). It should be noted, however, that all three of these adult stem cell populations are relatively quiescent and only periodically exit their quiescent state in response to specific signals that indicate the need to repopulate the tissue. This comes in contrast to the continuously proliferating intestinal stem cell populations described in the previous chapter.

2.3 AUTOPHAGY & AGING

Strong evidence exists linking impaired autophagy to functional aging in invertebrate model organisms, and arising evidence in mammals hints at a similar pattern (Hansen et al., 2018).

First, as discussed above, autophagy plays a major role in several age-related diseases, including neurodegenerative diseases, metabolic syndromes, and – as will be discussed in the next section – cancer.

Second, autophagy decreases with age in several tissues. Notably, protein expression of LC3 and ATG7 has been shown to decline with age in the mouse hypothalamus and in the muscle of both mice and humans (Kaushik et al., 2012; Carnio et al., 2014). Furthermore, turnover of autophagic vacuoles and rates of autophagic proteolysis are also decreased in the livers of aged rats (Terman, 1995; Donati et al., 2001; Del Roso et al., 2003). Protein levels of lysosomal LAMP2A also decrease with age in the rat liver, and this is associated with decreased levels of other types of autophagy as well, like CMA, which has also been linked to aging (Cuervo and Dice, 2000; Cuervo and Wong, 2014). Altogether, this illustrates a decrease in autophagy with age in various tissues. Interestingly, the opposite pattern has been shown for mTOR activity and could be at least partially responsible for these observations (Baar et al., 2016).

Third, impaired or diminished autophagy has been associated with various classical hallmarks of aging, including loss of proteostasis, mitochondrial dysfunction, genomic instability, or somatic stem cell exhaustion (López-Otín et al., 2013).

Lastly, regimes or pharmaceutical treatments that extend lifespan, such as caloric restriction or spermidine, induce autophagy. Importantly, the effects of these treatments on lifespan have clearly been shown to depend on autophagy in invertebrate models (Hansen et al., 2018), and evidence is beginning to be put forward in mice as well (Eisenberg et al., 2016). Moreover, enhancing autophagic activity through genetic alterations in mice – either through ubiquitous *Atg5* overexpression or through a point mutation on *Beclin1* that disrupts the interaction of its protein product with BCL2 – not only increases their lifespan compared to wild type littermates, but also promotes healthspan: *Atg5*-overexpressing mice are leaner with increased insulin

sensitivity, and improved motor function. Furthermore, isolated MEFs from these mice demonstrate higher resistance to oxidative stress. Meanwhile, *Beclin1*-mutant mice show fewer age-related renal or cardiac pathological changes and diminished spontaneous tumorigenesis (Pyo et al., 2013; Fernández et al., 2018).

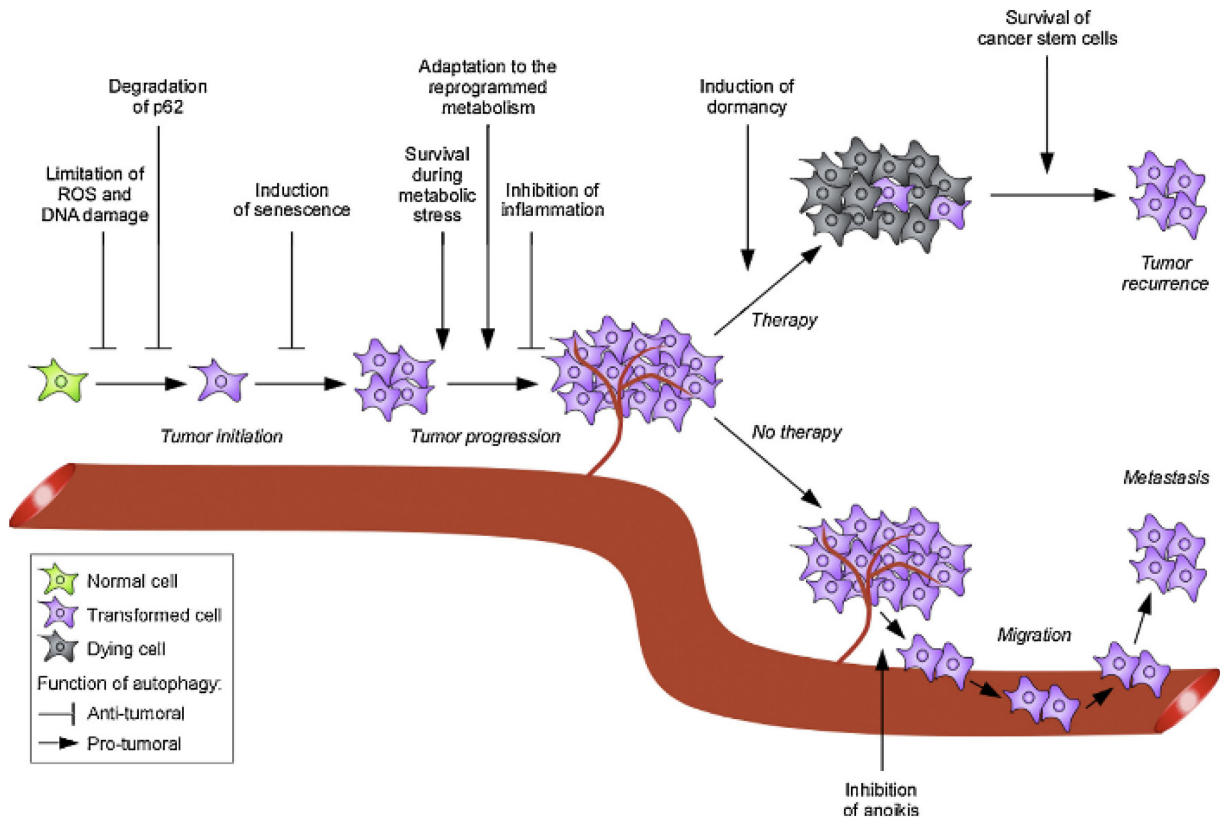


FIGURE 32. Tumor-suppressive and tumor-promoting roles of autophagy at different stages of tumorigenesis. Adapted from Lorin et al., 2013.

2.4 AUTOPHAGY IN CANCER

The first link between the autophagic machinery and cancer was made in 1999 by Beth Levine's group when the gene encoding BECLIN1, was identified as a candidate tumor suppressor gene (Liang et al., 1999). Indeed, *BECLIN1* is monoallelically deleted in a large proportion of human breast, ovarian, and prostate cancers. A few years later, the tumor-suppressive role of BECLIN1 was confirmed in two independent studies showing that mice deleted for one allele of *Beclin1*, which display reduced autophagy at the whole-body level, develop various sporadic tumors including lymphoma, lung cancer, and hepatocellular carcinoma (Qu et al., 2003; Yue et al., 2003). A wave of research regarding the role of autophagy in cancer followed, resulting in the general dogma put forward today: that autophagy plays a tumor-suppressive role in healthy tissue, whereas it promotes the growth and survival of established tumor cells [FIGURE 32].

2.4.1 AUTOPHAGY IN PRE-MALIGNANT CELLS

Cell-intrinsically, autophagy suppresses tumorigenesis by preventing the accumulation of ROS, preserving genomic integrity, or by eliminating invading pathogens with transforming activity. Autophagy is also thought to suppress tumorigenesis in a non-cell autonomous manner in some

tissues by limiting chronic inflammation, which can be a strong oncogenic driver. Several studies confirmed the results obtained with *Beclin1* using deletion of other autophagy genes, including allelic loss of *Ambra1*, which leads to the development of tumors primarily in the liver and lungs of mice (Cianfanelli et al., 2015), or mosaic *Atg5* deletion, which results in the formation of hepatic adenomas (Takamura et al., 2011). Likewise, the hepatocyte-specific deletion of either *Atg5* or *Atg7* led to the spontaneous formation of benign tumors (Inami et al., 2011; Takamura et al., 2011). Interestingly, in the case of either *Beclin1*- and *Ambra1*- heterozygous mice, the tumors that develop do not display loss of heterozygosity, suggesting that impaired autophagy does not drive tumorigenesis alone but instead creates a permissive cellular environment for transformation.

In line with this idea, several of the most common or potential driver mutations found in human cancers are predicted to result in the downregulation of autophagy. This includes, for example, the inactivation of PTEN, the cytoplasmic accumulation of p53, or the oncogenic activation of PI3K or AKT signaling (Kandoth et al., 2013). Furthermore, the inhibition of autophagy has been shown to favor chemically- or genetically- induced tumorigenesis in several tissues. For example, ablation of *Atg7* in a BRAF^{V600E}-driven mouse model of lung tumorigenesis accelerated the early stages of tumorigenesis (Strohecker et al., 2013). Similarly, decreased ATG5 expression was observed in human melanoma samples compared to benign nevi or normal skin cells, and suppressing *Atg5* in primary melanocytes expressing either oncogenic BRAF^{V600E} or HRAS^{G12V} prevented oncogene-induced senescence (Liu et al., 2013b). As a final example, while complete loss of *Atg5* impairs HSC function, the heterozygous loss of *Atg5* in HSCs enhances development of acute myeloid leukemia when associated with a driver mutation (MLL-ENL fusion) (Watson et al., 2015). Thus, generally speaking, decreased levels of autophagy favor tumor initiation in a cell-intrinsic manner.

2.4.2 AUTOPHAGY SUPPORTS TUMOR GROWTH AND METABOLISM

Despite the suppressive effect of autophagy in tumor initiation, autophagy genes are only rarely mutated in human cancer, and even *BECLIN1* deletions have been suggested to be passenger mutations to deletions of the nearby tumor suppressor gene *BRCA1* (Laddha et al., 2014). In fact, several tumors actually show an increase in basal autophagy levels. As in normal tissues, autophagy can help tumor cells cope with metabolic stress, growth factor deprivation, hypoxic conditions or oxidative stress. In line with this pro-tumorigenic role of autophagy, inhibition of autophagy in various tumor tissues or cancer cell lines hinders their growth.

Once again, the work of Strohecker et al. (Strohecker et al., 2013) using a mouse model of BRAF^{V600E}-induced lung cancer can be taken as an example. In this study, while the loss of *Atg7* favored tumor initiation, the developing tumors are predominantly benign oncocytomas instead of adenomas, and the mice bearing these tumors survive much longer than those with autophagy-proficient tumors. The authors propose that autophagy-supplied substrates like glutamine sustain BRAF^{V600E} tumor metabolism and growth. A similar reduction in tumor burden and extension of lifespan was observed upon *Atg7* or *Atg5* deletion in KRAS^{G12D}-driven lung tumors (Guo et al., 2013; Kim et al., 2014) despite an increase in tumor initiation (Rao et al.,

2014). In all three cases, the reduced tumor progression was associated with p53 activation, although deletion of p53 only partially relieved tumor progression (Guo et al., 2013; Strohecker et al., 2013; Rao et al., 2014). This suggests that autophagy contributes to tumor growth in part by restraining p53 activation and in part through p53-independent mechanisms. In link with an effect of autophagy on tumor cell metabolism, Rao et al. found impaired oxidative phosphorylation and ATP production in *Atg5*-deficient *Kras*^{G12D} tumor cells, while Guo et al. show that *Atg7* deficiency impaired the ability of tumor cells to turn to lipid catabolism and fatty acid oxidation as a source of energy in *KRAS*^{G12D}-mutant and p53-deficient tumors (Guo et al., 2013; Rao et al., 2014). The idea that *Kras*-driven lung tumors rely on autophagy to provide metabolic substrates was further supported by a study in human tumor-derived cell lines (Guo et al., 2016). While these studies address the issue of tumor cell-autonomous autophagy inhibition, Karsli-Uzunbas et al. (Karsli-Uzunbas et al., 2014) mimicked the effect of autophagy-inhibiting treatments in the clinic by deleting *Atg7* at the whole-body level once *KRAS*^{G12D} and p53-null lung tumors were established. Systemic autophagy inhibition not only caused tumor cell proliferative arrest and death, but the kinetics and magnitude of these events were accelerated compared to tumor-specific *Atg7* deletion, hinting at additional non-cell autonomous effects of autophagy inhibition in this tissue. Moreover, the beneficial anti-tumoral effects of *Atg7* deletion occurred prior to any deleterious systemic effects, providing hope for the therapeutic use of acute autophagy inhibition in lung cancer.

Autophagy also plays an important role in the progression of pancreatic ductal adenocarcinoma (PDAC). In humans, 90% of PDAC possess *KRAS* mutations, a driver oncogene in PDAC formation, while mutations in the gene encoding p53, *TP53*, occur in approximately 75% of PDAC. Interestingly, autophagy was found to be elevated in primary PDAC tumors and cell lines (Yang et al., 2011; Perera et al., 2015). These high levels of autophagy were shown to maintain a lysosome-derived pool of amino acids allowing PDAC cell growth (Perera et al., 2015). As in the lung, loss of *Atg7* or *Atg5* in *Kras*-mutant mouse pancreas leads to a marked accumulation of early pre-cancerous pancreatic intraepithelial neoplasias (PanINs). However, these undergo very limited progression to PDAC and mice bearing these autophagy-deficient tumors have a prolonged lifespan compared to autophagy-proficient cases (Rosenfeldt et al., 2013; Yang et al., 2014). Furthermore, autophagy-deficient tumors show reduced oxygen consumption, diminished proliferation, increased DNA damage, and higher levels of apoptosis; similar to what was previously found in primary pancreatic tumors and cell lines following silencing of *ATG* genes (Yang et al., 2011, 2014; Rosenfeldt et al., 2013). Similar results were also found using Chloroquine (CQ), an antimalarial drug that blocks autophagosome fusion with lysosomes (Yang et al., 2011). Following pancreatic loss of *Atg7* or *Atg5*, mice eventually become sick and die, but this is instead due to endocrine dysfunction consistent with the aforementioned role of autophagy in pancreatic function (Rosenfeldt et al., 2013; Yang et al., 2014). These studies diverge, however, on their analysis of the effect of autophagy in *Kras*-mutated and p53-deficient tumors [FIGURE 33]. Rosenfeldt et al. used an embryonic homozygous deletion of *Tp53* in the pancreas along with the *Kras* mutation. Contrary to p53-proficient tissue, tumors formed irrespective of autophagy status and, surprisingly, *Atg7* loss or hydroxychloroquine (HCQ, a

derivative of CQ) treatment accelerated PDAC formation in p53-null tissue (Rosenfeldt et al., 2013). Yang et al., on the other hand, used a *Kras*-mutant and p53 loss-of-function mouse model to show that *Atg* deletion or HCQ treatment could still suppress tumor progression and prolong mouse survival in a p53-null context (Yang et al., 2011, 2014). They further confirmed this using HCQ treatment on *KRAS*- and *TP53*- mutant patient-derived pancreatic cancer xenografts (Yang et al., 2014). Although the differences responsible for the opposing response between the embryonic and adult models of p53 loss remain uncertain, it seems that the second model is more clinically relevant and ongoing clinical trials for autophagy inhibition in PDAC will determine its efficiency in patients.

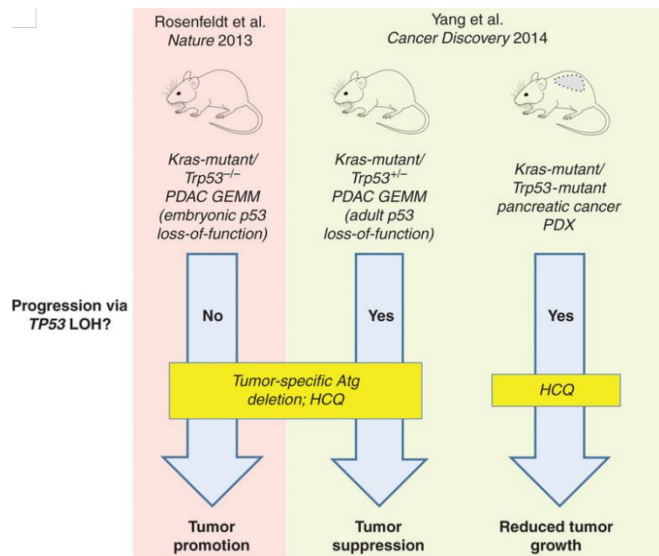


FIGURE 33. Effects of autophagy inhibition in mouse models of pancreatic cancer. The differences observed upon autophagy inhibition, either by *Atg* gene deletion or hydroxychloroquine (HCQ) treatment, in genetically-engineered mouse models and patient-derived xenografts (PDX) of *Kras*-mutant PDAC are likely a result of the developmental stage at which p53 function is lost and whether PDAC progression involves *Trp53* loss of heterozygosity (LOH). Adapted from Amaravadi and Debnath, 2014.

Atg gene deletion has also proved to have anti-tumoral effects in other mouse models of *Kras*- or *Braf*- mutant tumors, including melanoma (Xie et al., 2015) and glioblastoma (Gammoh et al., 2016), as well as in several RAS-activated cell lines (Guo et al., 2011b; Lock et al., 2011). This also applies to tumors driven by other mutations, like PTEN-depleted prostate tumors (Santanam et al., 2016), Philadelphia-chromosome-positive chronic myeloid leukemia (Karvela et al., 2016) or classical Hodgkin lymphoma (Birkenmeier et al., 2016).

In addition, autophagy may promote the later stages of cancer progression including tumor cell dissemination and metastasis as it protects tumor cells from undergoing anoikis (Fung et al., 2007), facilitates migration (Kenific et al., 2016; Sharifi et al., 2016), and promotes the survival of disseminated dormant cells that will initiate secondary tumors (Vera-Ramirez et al., 2018).

Although these studies strongly support the dogma where autophagy suppresses tumor initiation but promotes tumor progression, it should be noted that this is not always the case. In fact, the role of autophagy in cancer actually appears to be quite tissue- and context- dependent. Despite the *BECLIN1* being monoallelically lost in 40-75% of sporadic breast and ovarian carcinomas, heterozygous loss of *Beclin1* did not further promote oncogene-driven mammary carcinogenesis (Lozy et al., 2014). In another model of mammary carcinogenesis, allelic loss of *Beclin1* actually reduces tumorigenesis in a p53-dependent manner (Huo et al., 2013). Clearly, a trending “context” that modulates the effects of autophagy in cancer is the presence of functional p53.

2.4.3 AUTOPHAGY & RESISTANCE TO THERAPY

In addition to supporting tumor cell metabolism and stress response, autophagy also protects tumor cells from cytotoxic agents commonly used to treat cancer. Indeed, autophagy is activated in response to several forms of cancer therapy (including chemotherapy, targeted inhibitors or radiation) and in various tumor types. In many cases, the upregulation of autophagy was shown to act as a pro-survival response that promotes therapeutic resistance. This was initially demonstrated by Amaravadi et al. (Amaravadi et al., 2007), who showed that the inhibition of autophagy by CQ or by silencing *Atg5* expression both enhanced tumor regression upon alkylating drug therapy and delayed tumor recurrence. A vast number of studies *in vitro*, using genetically engineered mouse models or using patient-derived xenografts have since corroborated the beneficial effects of autophagy inhibition when combined with anticancer treatment (Ojha et al., 2015; Rebecca and Amaravadi, 2016). Mechanistically, in response to cytotoxic treatments, autophagy could suppress p53, allow ER stress or oxidative stress resistance, contribute to DNA damage repair or induce a state of senescence or “dormancy” allowing cells to survive treatment and later re-establish tumor growth. The latter supports an additional role for autophagy in tumor relapse following treatment, as suggested by the delayed tumor recurrence in the study by Amaravadi et al, and in other studies (Amaravadi et al., 2007; Gupta et al., 2010; Sosa et al., 2013; Beyaz et al., 2016). On the contrary, enhanced autophagy in tumor cells following treatment appears to be beneficial for the anti-tumoral effects of immunogenic chemotherapy or radiotherapy (Li et al., 2008; Sompolinsky et al., 2011). This has essentially been linked with the role of autophagy in antigen presentation or in immunogenic ATP release from dying tumor cells.

Various clinical trials to modulate autophagy in cancer are currently under way – with 61 ongoing trials reported on www.clinicaltrials.gov as of August 2018 [FIGURE 34], the vast majority focused on inhibiting autophagy. Currently, the only clinically approved drugs to inhibit autophagy are CQ and HCQ, and these have proved effective in combination therapy to treat various cancers (Levy et al., 2017). It should be noted, however, that these drugs also have anti-tumoral properties independent of their effect on autophagy (Maes et al., 2014; Eng et al., 2016).

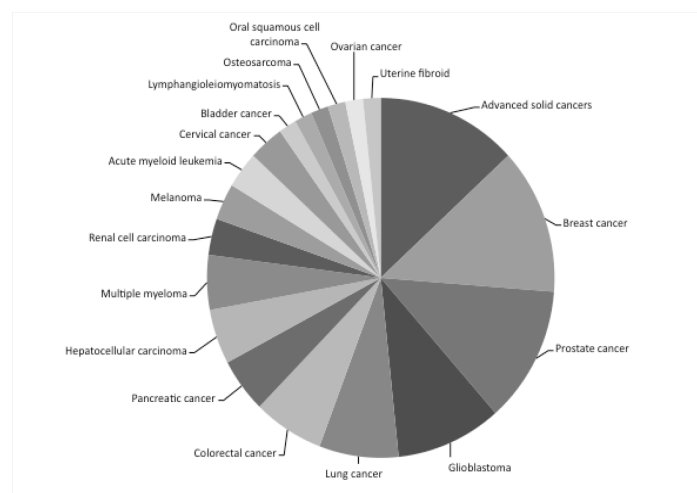


FIGURE 34. 61 ongoing clinical trials on autophagy and cancer by cancer type as of August 2018. Data from www.clinicaltrials.gov.

2.4.4 AUTOPHAGY & CANCER STEM CELLS

Similar to tissue stem cells, a subpopulation of cells with stem-like properties called cancer stem cells are thought to drive the long-term growth of tumoral tissue. This distinct subset of cancer cells holds the tumorigenic potential of the tumor (as shown by their ability to form organospheres *in vitro* or establish tumors in immunocompromised mice). Like normal tissue stem cells, cancer stem cells are dependent on their microenvironment – typically hypoxic, nutrient-poor niches within the tumor – to sustain the population. They are also considered as more resistant to therapy and may drive tumor regrowth following treatment. Moreover, cancer stem cells express mesenchymal traits and could drive metastasis. Although the notion of cancer stem cells remains controversial, cancer stem cell populations have been identified in numerous tumor types, and evidence for a stem- and progenitor- like organization within tumors *in vivo* has been put forward for intestinal tumors (as described in the previous chapter) as well as brain and skin tumors (Chen et al., 2012; Driessens et al., 2012; Schepers et al., 2012).

As autophagy plays an important role in normal tissue stem cells, and in line with its role in tumor progression, recurrence and metastasis, it has been postulated to play a role in the maintenance of cancer stem cells. The role of autophagy has been most extensively described in breast cancer stem cells, where autophagy markers are overexpressed and autophagic flux is enhanced. Moreover, inhibition of autophagy in these cancer stem cells has been shown to block their tumorigenicity, their ability to go into dormancy and survive cytotoxic treatment, and their ability to form metastases (Espina et al., 2010; Chatterjee and van Golen, 2011; Cufí et al., 2011; Gong et al., 2013; Yue et al., 2013; Maycotte et al., 2015; Vera-Ramirez et al., 2018). The nutrient- and oxygen- deprived conditions of the cancer stem cell niche could be inductive to autophagy. In line with this idea, in pancreatic tumors, HIF α and autophagy were shown to play a role in the equilibrium between non-stem cancer cells and cancer stem cells (Zhu et al., 2013). Similarly, the importance of autophagy in the resistance of cancer stem cells to cytotoxic therapy was notably demonstrated in chronic myeloid leukemia and bladder cancer (Bellodi et al., 2008; Ojha et al., 2014). Interestingly, not just bulk autophagy but mitophagy specifically was shown to play a role in the maintenance of cancer stem cells in both hepatocellular carcinoma and esophageal squamous cell carcinoma, by suppressing nuclear p53-mediated repression of stemness genes (Liu et al., 2017) and by regulating redox homeostasis (Whelan et al., 2017), respectively.

2.4.5 AUTOPHAGY AND THE TUMOR MICROENVIRONMENT

Aside from its cell-intrinsic functions in tumor cells, autophagy also plays an important role in both autocrine and paracrine interactions between tumor cells and with their microenvironment. For one, ATP secretion by melanoma cells through the autophagic machinery was shown to play an essential role in tumor cell migration and drug resistance (Martin et al., 2017). Tumor cell autophagy was also shown to assist in immune evasion by preventing exposure of Calreticulin (an immunogenic cell death signal) in cancer cell lines (Garg et al., 2013) and suppressing melanoma cell secretion of chemokines that drive tumor infiltration by natural killer cells, which play a fundamental role in anti-tumoral immunity (Mgrditchian et al., 2017).

In solid tumors, various mesenchymal cell types – including fibroblasts, hematopoietic cells, and cells of the vascular and lymphatic vessels – are found within the tumor mass and can support tumor growth by providing metabolic substrates, antioxidants, growth factors and blood supply. In breast cancer, high levels of LC3 and high autophagic activity in cancer-associated fibroblasts have been linked with a more aggressive phenotype (Zhao et al., 2017). Several other studies have put forward evidence for a tumor-promoting role of autophagy in cancer-associated fibroblasts, either by simply protecting the tumor-supporting fibroblasts themselves from oxidative stress (Wang et al., 2016b), by driving the secretion of growth-promoting cytokines like IL-6 or IL-8 (Valencia et al., 2014; New et al., 2017), or by allowing fibroblasts to provide metabolic substrates to fuel energy production in the tumor cells (Capparelli et al., 2012; Sousa et al., 2016). A worthy example of the latter is that of pancreatic stellate cells, specialized fibroblasts that stimulate tumor growth *in vivo* and *in vitro* by secreting non-essential amino acids like alanine in an autophagy-dependent manner. The secreted alanine is then taken up by tumor cells and used to fuel mitochondrial metabolism, reducing tumor cell dependence on limited microenvironmental glucose (Sousa et al., 2016). In each of these cases, deletion of autophagy in cancer-associated fibroblasts suppressed tumor growth.

In addition to tumor stromal fibroblasts, autophagy can also play an important role in immune cells involved in tumor immune surveillance. On top of to the globally immunomodulatory effects of autophagy, autophagic activity in T lymphocytes in the context of cancer seems to favor populations linked with immune evasion (ie. regulatory T lymphocytes) over those acting against tumor progression (ie. T_H9 cells), as discussed in section 1.2.7 of this chapter.

Targeting autophagy in the tumor microenvironment in addition to tumor cells may therefore present a promising anticancer strategy. Once again, the effects of inhibiting autophagy in both tumor cells and the microenvironment could be tissue- and context- dependent. While whole-body autophagy inhibition appears to exert a greater antitumor activity than inhibiting autophagy only in tumor cells (Karsli-Uzunbas et al., 2014), caloric restriction or mimetics, which rather induce autophagy, have also been shown to enhance anticancer immunosurveillance and the efficiency of treatment (Saleh et al., 2013; Di Biase et al., 2016; Pietrocola et al., 2016). These ambiguous results highlight the need for a better understanding of tumor-cell extrinsic effects of autophagy.

Overall, these studies reveal an important role of autophagy in various aspects of mammalian physiology and disease, with both general and tissue-specific roles of autophagy. In the following subchapter, the known functions of autophagy specifically in the intestine will be discussed, with particular attention attributed to its functions in the intestinal epithelium.

III. AUTOPHAGY & THE INTESTINAL EPITHELIUM

As covered in the previous chapter, the homeostasis of the intestinal epithelium is ensured by a variety of mechanisms and signaling pathways. Among these, the implication of autophagy is growing increasingly evident. In fact, autophagy appears to have very specific roles in the different cell types of the intestinal epithelium.

3.1 AUTOPHAGY IN SECRETORY CELLS OF THE INTESTINAL EPITHELIUM

The best-established function of autophagy in the intestinal epithelium is in the secretory cell lineages. Through their secretion of AMPs and mucus, respectively, Paneth cells and goblet cells protect the intestinal epithelium and the organism by limiting colonization and infection and regulating the composition of the microbiota (Vaishnava et al., 2008). Several studies have revealed a role of the autophagic machinery in the secretory function of these cell types.

Indeed, the deletion of *Atg5*, *Atg7*, *Atg16l1*, or *Atg4b* or even the expression of a hypomorphic allele of *Atg16l1* (*Atg16l1^{HM}*) in mice results in malformed secretion granules within Paneth cells and a defect in AMP production and release (Cadwell et al., 2008, 2009; Cabrera et al., 2013; Lévy et al., 2015; Burger et al., 2018). Specifically, autophagy-deficient Paneth cells have fewer and more disorganized granules [FIGURE 35] and a diffuse intracellular Lysozyme staining. Moreover, autophagy-deficient intestines lack Lysozyme staining in the mucus surrounding the epithelium, highlighting a defect in the secretion of this major AMP. As will be discussed below, an allele of *ATG16L1* carrying a point mutation (T300A) resulting in reduced expression of the protein was identified as a key susceptibility allele for Crohn's disease. Interestingly, Paneth cells from patients carrying the *ATG16L1^{T300A}* allele display similar defects in secretion granule structure and Lysozyme secretion.

This phenotype appears to be dependent on other factors as well. Intriguingly, *Atg16l1^{HM}* mice raised in germ-free conditions present a normal Paneth cell morphology. However, chronic viral infection using the murine norovirus (MNV) strain CR6 re-establishes the secretory defects of autophagy-deficient Paneth cells (Cadwell et al., 2010). The authors therefore put forward the idea that a pathogen-plus-susceptibility gene interaction is required for the establishment of these Paneth cell secretory defects. In line with this idea, a recent study by Bel et al. (Bel et al., 2017) established secretory autophagy as an alternative mechanism for the secretion of lysozyme in the case of bacterial infection. Reliance on this alternative mode of secretion was triggered by bacteria-induced ER stress (a way for bacteria to interfere with the classical secretion route) but also required extrinsic signals from innate lymphoid cells.

Aside from its effects on AMP secretion granules, loss of *Atg16l1* or *Atg7* in Paneth cells also results in ER stress [FIGURE 35] and the activation of the UPR (Adolph et al., 2013). The opposite is also true: blocking the UPR by deleting *Xbp1*, variants of which are also linked to Crohn's disease, stimulates autophagy. Loss of both *Xbp1* and autophagy results in severe inflammation and spontaneous Crohn's disease-like ileitis. Autophagy and the UPR therefore act in conjunction within Paneth cells to maintain inflammatory homeostasis in the intestine.

Unlike what is seen in Paneth cells, the morphology of mucin-containing secretion granules in goblet cells is not particularly altered by the inhibition of autophagy, nor is the mRNA expression of mucins. Nevertheless, autophagy-deficient goblet cells also present secretory defects; they accumulate mucin-containing granules within their cytoplasm [FIGURE 35] (Patel et al., 2013; Lassen et al., 2014; Tsuboi et al., 2015). Mucin-containing vacuole secretion was thus found to implicate autophagy proteins, endocytosis, and ROS production by NADPH oxidases (Patel et al., 2013).

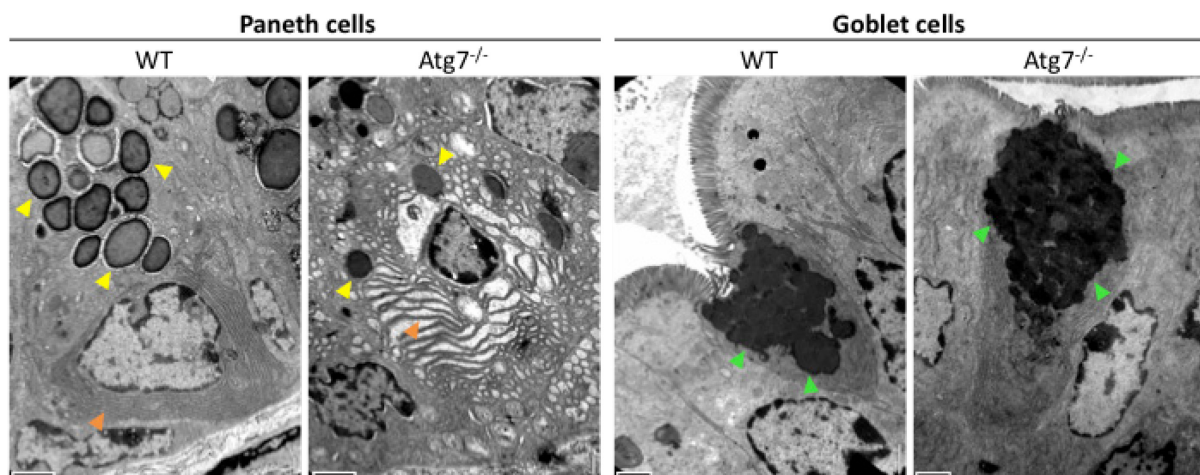


FIGURE 35. Effects of the loss of *Atg7* in secretory intestinal epithelial cells. Transmission electron microscopy images of Paneth cells and goblet cells in WT and *Atg7*^{-/-} mice. Yellow arrows indicate Paneth cell secretion granules, orange arrows indicate endoplasmic reticulum, green arrows indicate goblet cell mucin granules. Similar phenotypes have been described in mouse models invalidated for other autophagy genes, including *Atg16l1*, *Atg5*, or *Atg4b*. Scale: 1μm. Adapted from Trentesaux et al., 2017.

3.2 XENOPHAGY

Another form of antimicrobial defense, xenophagy, is also implicated in the homeostasis of the intestinal epithelium. A rapid induction of autophagy in intestinal epithelial cells was reported following infection of germ-free or conventionally raised mice with invasive pathogens like *Salmonella tiphimurium*, *Shigella flexneri*, or a specific strain of *Escherichia coli* associated with Crohn's disease (AIEC, adherent-invasive *Escherichia coli*), but also by opportunistically invasive commensal like *Enterococcus faecalis*. Epithelium-specific deletion of *Atg5*, *Atg7*, or *Atg16l1* abrogated this response (Benjamin et al., 2013; Chang et al., 2013; Conway et al., 2013; Mimouna et al., 2014). The lack of autophagic degradation of invasive pathogens by epithelial cells could also affect presentation of antigens to the adaptive immune system, further affecting organismal defenses. Of course, autophagy additionally plays an important role in cells of the innate or adaptive immune system, as discussed previously.

3.3 AUTOPHAGY, THE MICROBIOTA & INFLAMMATION

Thus, generally speaking, the loss of autophagy in the intestinal epithelium affects innate antimicrobial defenses. In addition, one study showed a direct implication for autophagy in enhancing epithelial tight junctions *in vitro* (Nighot et al., 2015). The combination of these factors could explain the increased permeability of the intestinal epithelium observed in autophagy-deficient mice (Lévy et al., 2015).

Two independent studies, including one from our group, recently showed that the deletion of *Atg7* in the murine intestinal epithelium led to alterations in the composition and localization of the intestinal microbiota. Bacteria were found in closer contact with the epithelium, within the crypt compartment of small intestine, and sometimes even in the lamina propria (Lévy et al., 2015). Furthermore, the *Firmicutes* phylum (more specifically the *Clostridiales* class) were overrepresented in both the ileic mucosa and feces of *Atg7*-deficient mice (Lévy et al., 2015; Tsuboi et al., 2015). This was further associated with the recruitment of specific immune populations (Lévy et al., 2015). Crohn's disease patients with the T300A polymorphism on *ATG16L1* also display a dysbiosis characterized by an overrepresentation of the *Enterobacteriaceae*, *Bacteroidaceae* and *Fusobacteriaceae* families (Sadabad et al., 2015).

Moreover, either expression of the hypomorphic form of *Atg16l1* in germ-free mice combined with chronic MNV infection (Cadwell et al., 2010) or the deletion of *Atg4b* (Cabrera et al., 2013) or *Atg7* (Tsuboi et al., 2015) in conventionally raised mice increased their sensitivity to DSS-induced colitis. In all three cases, antibiotic treatment rescued the aberrant response of autophagy-deficient mice to DSS treatment, revealing an important contribution of microbial alterations in this hypersensitivity.

It is interesting that both a partial but ubiquitous loss of autophagy (as is the case for *Atg16l1^{HM}* or *Atg4b*-knockout mice) and a complete but intestinal epithelium-specific loss of autophagy (in the case of *Atg7*) induce a similar phenotype. Cabrera et al. investigated the specific effects of *Atg4b* loss in hematopoietic cells and showed that it moderately contributes to the aggravated phenotype following DSS treatment; indeed, transplantation of *Atg4b*-deficient mice with wild-type bone marrow partially rescued their sensitivity, whereas transplantation of wild-type mice with *Atg4b*-deficient bone marrow induced a partial hypersensitivity to DSS (Cabrera et al., 2013). Along the same lines, mice lacking *Atg16l1* only in hematopoietic cells are also more susceptible to DSS-induced colitis (Saitoh et al., 2008). Using another mouse IBD model involving *Helicobacter hepaticus* infection combined with the blockade of immune regulatory circuits through anti-IL-10 receptor antibodies, Pott et al. showed that whereas loss of *Atg16l1* in myeloid cells only marginally affects the development of colitis, autophagy deficiency in intestinal epithelial cells severely exacerbates pathology (Pott et al., 2018). They further demonstrated that this was associated with TNF-induced apoptosis in the epithelium. Similarly, Burger et al. showed that the epithelium-specific or Paneth cell-specific deletion of *Atg5* increased susceptibility to *Toxoplasma gondii* in a tumor necrosis factor (TNF) -dependent manner (Burger et al., 2018). This highlights both a key role of Paneth cell-specific autophagy in modulating intestinal inflammation, and the importance of autophagy in protecting the epithelium in response to inflammatory signals. Interestingly, anti-TNF or anti-IFN γ treatment has proven effective both in reducing pathological symptoms in autophagy-deficient mouse models of colitis (Cadwell et al., 2010; Matsuzawa-Ishimoto et al., 2017; Burger et al., 2018; Pott et al., 2018) and to treat patients with IBD (Cohen and Sachar, 2017). It should be noted, however, that none of these mouse models spontaneously developed colitis in the absence of pro-inflammatory treatment.

3.4 AUTOPHAGY IN INTESTINAL PATHOLOGY

3.4.1 AUTOPHAGY IN CROHN'S DISEASE

Crohn's disease is an IBD that involves both genetic and environmental factors resulting in altered homeostasis of the intestinal microbiota, epithelium, and immune system. It is characterized by increased epithelial permeability, dysbiosis, defective antimicrobial peptide and mucus secretion, ER stress, increased production of pro-inflammatory cytokines (including IL-1 β , IL-6, IFN γ , and TNF α) and a T_H17-type immune response. Various susceptibility genes have been identified for Crohn's disease. *NOD2* polymorphisms were the first identified and remain the most significant to this day (Hugot et al., 2001; Ogura et al., 2001; Peter and Muddassar, 2001). As previously noted, *NOD2* is an intracellular receptor that recognizes bacterial surface MDP and activates pro-inflammatory signaling in response. In addition to its role in inflammation, *NOD2* has also been shown to directly induce autophagy (Cooney et al., 2010; Travassos et al., 2010). Genome wide association studies have also identified polymorphisms in several autophagy genes as risk alleles in Crohn's disease. These include *ATG16L1* (Hampe et al., 2007; Rioux et al., 2007; Glas et al., 2008), *IRGM* (Parkes et al., 2007; McCarroll et al., 2008; Prescott et al., 2010), *ULK1* (Henckaerts et al., 2011), *NDP52* (Ellinghaus et al., 2013), *LRRK2* (leucine-rich repeat kinase 2) (Barrett et al., 2008; Franke et al., 2010) and *PTPN2* (protein tyrosine phosphatase, non-receptor type 2) (Parkes et al., 2007).

As described above, knocking out or reducing expression of autophagy genes in mice mimicks several Crohn's disease phenotypes. Like *Atg* -knockout or -hypomorphic mice, mice knocked out for the *IRGM* orthologue *Irgm1* also display smaller granules within Paneth cells and decreased levels of antimicrobial peptides, as well as an increased susceptibility to DSS-induced colitis (Liu et al., 2013a) that is rescued by antibiotic treatment (Rogala et al., 2018). Expression of Crohn's disease variants of autophagy genes has a similar effect. The Crohn's disease-associated *ATG16L1*^{T300A} variant favors the cleavage of its protein product by Caspase-3 and 7 during cellular stress, resulting in lower *ATG16L1* protein levels (Lassen et al., 2014; Murthy et al., 2014). Mice expressing the *Atg16l1*^{T300A} allele have Paneth cell and xenophagy defects as well as increased secretion of pro-inflammatory IL-1 β by macrophages (Lassen et al., 2014). Likewise, *IRGM* regulates the induction of xenophagy, and Crohn's disease-associated *IRGM* variants lead to defects in xenophagy in myeloid cells (Singh et al., 2006; Intemann et al., 2009; Brest et al., 2011; Chauhan et al., 2015). Three Crohn's disease risk factors – *NOD2*, *ATG16L1*, and *IRGM* – may in fact be acting together in the same pathway, as they were shown to interact to initiate the autophagic handling of intracellular pathogens (Cooney et al., 2010; Travassos et al., 2010; Plantinga et al., 2011; Chauhan et al., 2015). Thus, defects in autophagy likely play an important role in the pathology of Crohn's disease patients carrying these risk alleles.

3.4.2 AUTOPHAGY IN COLORECTAL CANCER

Recently, autophagy has also been recognized as an important factor in CRC. Increased expression of several autophagy markers (*GABARAP*, *ULK1*, *LC3*, *ATG5*, *ATG7* or *BECLIN1*) is frequently found in both human CRC tissues and cell lines, and has been linked with poor

prognosis. Autophagy has also been shown to limit the effects of certain chemotherapeutic treatments on CRC cell lines (Weichert et al., 2004; Guo et al., 2011a; Park et al., 2013; Lévy et al., 2015; Zou et al., 2015; Schmitz et al., 2016) and xenografted CRC cells (Sasaki et al., 2012). Moreover, autophagy also allows the survival of CRC cell lines to starvation conditions similar to those found within the tumor mass.

Two recent studies have supported an essential role of autophagy in CRC development and progression using genetically engineered mouse models. Contrary to the dogma, deletion of *Atg7* in intestinal epithelial cells prevents the development of intestinal tumors induced by *Apc* loss (Lévy et al., 2015). As described above, loss of *Atg7* in the intestinal epithelium results in Paneth and goblet cell secretion defects, increased intestinal permeability, and alterations in the composition of the microbiota as well as its proximity to the epithelium. These microbial alterations are associated with the induction of an anti-tumoral immune response notably involving cytotoxic CD8⁺ T cell infiltration in the normal mucosa. Interestingly, either antibiotic treatment or antibody-mediated CD8⁺ T cell depletion abrogates the anti-tumoral effect of autophagy inhibition in this model.

Furthermore, the lack of autophagy within the few developing *Apc*-deficient tumors impairs their growth (Lévy et al., 2015). In the tumor cells, the inhibition of autophagy results in activation of AMPK and p53, reduced expression of glycolytic enzymes, and ultimately in cell cycle arrest. Similar results were obtained with mouse models where *Atg5* is deleted in intestinal epithelial cells following tumor induction by combined AOM and DSS treatments (Sakitani et al., 2015). In this model, autophagy-deficient tumor cells undergo ER stress and p53 activation resulting in tumor cell death and a reduction in tumor size. Thus, as in lung, pancreatic, or breast cancer, CRC cells rely on autophagy for their proliferation and survival.

Along lines of a microbiota-associated effect of autophagy, CRC patients homozygous for the *ATG16L1*^{T300A} allele have better long-term survival rates than patients with the T300 allele (Grimm et al., 2016), and this is presumably due to an increase in type I IFN production through mitochondrial antiviral signaling. Moreover, a recent study identified a role of microbiota-induced autophagy in CRC resistance to therapy. Specifically, *Fusobacterium nucleatum*, previously associated with CRC (Castellarin et al., 2011; Kostic et al., 2012; Warren et al., 2013), was shown to improve CRC cell survival to 5-FU or oxaliplatin by upregulating autophagy (Yu et al., 2017). Mechanistically, *Fusobacterium nucleatum* activates autophagy through a selective downregulation of miR-18a and miR-4802, which results in the upregulation of ULK1 and ATG7 both at the mRNA and protein levels. Moreover, the same study found this particular species to be abundant in patients with tumor recurrence after chemotherapy.

Overall, these studies highlight a key role of autophagy in the initiation, progression, and therapeutic resistance of intestinal tumors, often in tight functional cooperation with the intestinal microbiota and immune microenvironment.

RESULTS

RESEARCH CONTEXT

The WNT/ β -catenin pathway acts as a central driver of ISC homeostasis and of CRC initiation and progression. In order to identify new players in crypt homeostasis and intestinal tumorigenesis, our group developed several murine models carrying *Apc* mutations which mimic the different stages of human CRC (Colnot et al., 2004; Andreu et al., 2005). These models allowed the demonstration of a physiological role of this pathway in proliferation and differentiation within the crypt (Andreu et al., 2005, 2008), and in the maintenance of ISC (Durand et al., 2012). Our group recently described an induction of autophagy following the pathological activation of WNT/ β -catenin signaling in human CRC and in the intestinal tumors of *Apc*-mutant mice (Lévy et al., 2015). In fact, functional autophagy in the intestinal epithelium proved to be essential for tumor initiation and tumor growth in this model. Autophagy also plays a role in the homeostasis of the intestinal epithelium by regulating secretory cell function and antimicrobial defense (Cadwell et al., 2008, 2009, 2010; Patel et al., 2013; Lévy et al., 2015; Bel et al., 2017). However, in spite of its established role in intestinal tumor initiation and in the homeostasis of various other tissue stem cells (Boya et al., 2018), the role of autophagy in homeostatic function of ISC had not been investigated.

Therefore, the work of my thesis aimed to understand the role of autophagy in the maintenance and function of homeostatic ISC. To do so, we used various murine models developed in the lab:

- VillinCre^{ERT2}Atg7^{fl/fl} mice, which allow the tamoxifen-inducible deletion of the key autophagy gene *Atg7* specifically in the intestinal epithelium. These mice were used to study the effect of autophagy inhibition in the homeostatic intestinal epithelium.
- Lgr5-EGFP-Cre^{ERT2}Atg7^{fl/fl} mice, which allow the tamoxifen-inducible deletion of *Atg7* specifically in Lgr5⁺ ISC and, consequently, throughout all intestinal epithelial cells generated from these ISC. It should be noted that the Lgr5-EGFP-Cre^{ERT2} is expressed in a mosaic manner throughout the intestinal epithelium, so only about one third of intestinal crypts express EGFP and the Cre recombinase in their Lgr5⁺ ISC. This model was used to sort WT and *Atg7*-depleted ISC and their early TA progenitors, which no longer express *Lgr5* but have remaining EGFP protein in their cytoplasm. This allowed us to analyze the effects of autophagy inhibition in ISC and TA progenitors.
- VillinCre^{ERT2}Atg7^{fl/fl}Tp53^{fl/fl} mice, which allow the simultaneous deletion of both *Atg7* and *Tp53* in the intestinal epithelium upon tamoxifen treatment. These mice were used to investigate the role of p53 in the autophagy-deficient intestinal epithelium.

In addition to these mouse models, our work relied heavily on primary culture of intestinal organoids.

The principal findings of our work revealed that:

- The inhibition of autophagy throughout the intestinal epithelium results in the p53-dependent apoptosis of ISC specifically.
- Autophagy plays a unique role in ISC as opposed to the rest of the epithelium by protecting them from oxidative stress and DNA damages.
- Although the death of autophagy-deficient ISC is independent of the production of growth factors, including Wnt ligands, by Paneth cells, it does depend on the altered interactions with the microbiota resulting largely from Paneth cell defects in antimicrobial defense.
- p53-mediated apoptosis in ISC following the loss of *Atg7* is essential to prevent tumor initiation.

This work will be presented in the following manuscript, which we are currently preparing for submission.

AUTOPHAGY PROTECTS INTESTINAL STEM CELL INTEGRITY IN RESPONSE TO INTRINSIC AND ENVIRONMENTAL STRESS

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INTRODUCTION

The intestinal epithelium is highly organized as a hierarchy that originates from a pool of proliferative stem cells, distinguished by their expression of the leucine-rich repeat containing G-protein-coupled receptor 5 (Lgr5). These Lgr5-positive intestinal stem cells (Lgr5⁺ISC) reside at the bottom of the intestinal crypts of Leiberkuhn and are in direct contact with Paneth cells, which notably contribute to their niche by secreting Wnt ligands. From there, Lgr5⁺ISC give rise to transit amplifying (TA) progenitors that move towards the crypt-villus junction and differentiate into the 4 main differentiated cell types of the epithelium: enterocytes, Paneth cells, goblet cells and enteroendocrine cells (Barker, 2014). Thus, Lgr5⁺ISC are essential for the regeneration of epithelial cells that are turned over every 3 to 5 days at homeostasis or lost upon injury. Indeed, Lgr5⁺ISC are crucial for epithelial repair following cytotoxic stress (Metcalf et al., 2014). Moreover, Lgr5⁺ISC have also been shown to be the cell of origin for colorectal cancer (Barker et al., 2009). To achieve lifelong intestinal homeostasis, the functional and genomic integrity of Lgr5⁺ISC needs to be appropriately maintained against various stresses: infectious, physical or chemical. They may therefore be equipped with unique mechanisms to ensure their protection and survival. While the signaling pathways responsible for the proliferation and self-renewal of Lgr5⁺ISC are well known, the mechanisms controlling their integrity and resilience in the face of cytotoxic stress are far less understood.

Autophagy is an evolutionarily conserved pathway through which cellular materials are targeted to the lysosomes for degradation. At basal levels, this cellular process allows the elimination of damaged components that threaten cell integrity, but it also acts as a major adaptive stress response with functions ranging from metabolic sustenance in starving cells to warding off microbial attacks (Mizushima, 2018). Autophagy is also important for tumor development in many tissues, including the intestine. We previously showed that autophagic activity is enhanced during colorectal cancer development (Lévy et al., 2015). Using mice lacking the essential autophagy gene *Atg7* specifically in intestinal epithelial cells, we showed that autophagy is required both for tumor initiation and for the metabolism of intestinal tumor cells. We expanded these results by showing that the inhibition of autophagy affects the mucosal microenvironment by shaping an anti-tumoral immune response linked to a change in the composition of the microbiota.

Here, we show that Lgr5⁺ISC are particularly sensitive to the inhibition of autophagy following the deletion of *Atg7*. In contrast to differentiated cells and to TA progenitors, autophagy-deficient Lgr5⁺ISC show defective antioxidant and DNA repair responses, resulting in the accumulation of cytotoxic damages and the induction of p53-mediated apoptosis. Blocking p53 prevents the death of autophagy-deficient Lgr5⁺ISC and favors tumor onset. These results indicate that autophagy and p53 act in conjunction as protective mechanisms essential to Lgr5⁺ISC integrity. We further show that Paneth cell defects associated with the inhibition of autophagy do not affect their niche function but contribute to the accumulation of damages and apoptosis of Lgr5⁺ISC through a defective antimicrobial defense, revealing a close link between the microbiota, autophagy, and Lgr5⁺ISC integrity.

RESULTS

Loss of *Atg7* induces apoptosis of Lgr5⁺ISC

To analyze the role of autophagy in intestinal epithelial cell homeostasis, we generated mice that express a tamoxifen-inducible Cre recombinase driven by the *Villin* promoter and two floxed alleles of *Atg7*. By western blot analysis, we validated the loss of ATG7 protein expression in whole intestinal tissue lysates from tamoxifen-treated VillinCre^{ERT2}Atg7^{fl/fl} mice (hereafter referred to as Atg7^{-/-}) as compared to Atg7^{fl/fl} mice lacking the Cre recombinase transgene (hereafter referred to as WT) (supplementary figure 1A). As expected, we showed that the loss of ATG7 results in an inhibition of autophagy as indicated by the low abundance of the autophagosome-bound form of LC3 (LC3-II) in relation to its cytoplasmic form (LC3-I) (supplementary figure 1A). Furthermore, the accumulation of p62 and Ubiquitin-containing aggregates in both the differentiated villi and proliferative crypts (figure 1a, supplementary 1A) indicates that the inhibition of autophagy is effective throughout the intestinal epithelium. Despite the rapid turnover of the epithelium, this phenotype is maintained over time, confirming the loss of *Atg7* in the ISC compartment.

Deletion of *Atg7* in all intestinal epithelial cells led to the appearance of TUNEL-positive cells particularly at the base of the Atg7^{-/-} crypts, associated with accumulation of nuclear p53 and cleaved Caspase-3 (figure 1B,C). TUNEL-positive cells co-localize with Lgr5⁺ISC marker *Olfm4* and are mutually exclusive with Paneth cell product Lysozyme (figure 1D), indicating that Lgr5⁺ISC, and not Paneth cells, undergo apoptosis following the inhibition of autophagy.

Lgr5⁺ISC death, although sustained over time, is not detrimental to epithelial regeneration as a pool of Lgr5⁺ISC is maintained over time and Atg7^{-/-} mice have a lifespan equivalent to controls. Interestingly, we found an increase in BrdU incorporation in the ATG7-deficient crypts along with an expanded expression of ISC markers (supplementary figure 1B,C) suggesting that increased proliferation of surviving ISC, early progenitors or another ISC pool could account for the replenishment of Lgr5⁺ISC.

We next tested the capacity of isolated crypts from tamoxifen-treated WT and Atg7^{-/-} mice to develop intestinal organoids *ex vivo*. Autophagy-deficient organoids showed significantly reduced survival compared to WT organoids, revealing a stem cell defect (figure 1E). Consistent with the *in vivo* phenotype, we found TUNEL-positive cells in the crypts of Atg7^{-/-} organoids (figure 1F).

Atg7-deficient Lgr5⁺ISC apoptosis is dependent on p53

We next investigated the molecular events resulting in Lgr5⁺ISC death following the inhibition of autophagy. We and others previously reported defects in secretory lineage, particularly in Paneth cells, in autophagy-deficient mice (Cadwell et al., 2008; Lévy et al., 2015; Bel et al., 2017). Paneth cells neighbor Lgr5⁺ISC and secrete both protective antimicrobial peptides and growth factors involved in the stem cell niche. Consistent with previously published data, Atg7^{-/-} mice exhibit decreased expression of antimicrobial peptides and abnormal Lysozyme distribution within Paneth cells (supplementary figure 2A,B). In contrast, the expression of Paneth cell niche factors including Wnt and Notch ligands was not diminished following *Atg7* deletion (figure 2A). Moreover, the addition of exogenous Wnt ligand to the culture medium did not rescue *Atg7*-deficient organoid survival (figure 2B). Therefore, the death of autophagy-deficient Lgr5⁺ISC is likely independent of the Paneth cell niche function.

In contrast, the addition of the p53 inhibitor Pifithrin to the organoid culture medium rescued the survival defect of autophagy-deficient organoids (figure 2C), suggesting that the death of autophagy-deficient organoids is dependent on p53. To confirm the role of p53 in autophagy-deficient Lgr5⁺ISC death *in vivo*, we crossed VillinCre^{ERT2}Atg7^{fl/fl} mice with Tp53^{fl/fl} mice, allowing the simultaneous loss of both ATG7 and p53 proteins in the resulting progeny upon tamoxifen injection (hereafter referred to as Atg7^{-/-}p53^{-/-} mice), as confirmed by western blot and immunohistochemistry (figure 2D,E). We found significantly fewer TUNEL-positive cells in Atg7^{-/-}p53^{-/-} compared to Atg7^{-/-} crypts (figure 2E), confirming that the apoptosis of autophagy-deficient Lgr5⁺ISC is dependent on p53. Accordingly, organoids from Atg7^{-/-}p53^{-/-} crypts showed improved survival rates compared to organoids from Atg7^{-/-} crypts (figure 2F).

Loss of autophagy prevents efficient DNA damage repair in Lgr5⁺ISC

To gain insight into the role of autophagy in Lgr5⁺ISC, we crossed the Lgr5-EGFP-Cre^{ERT2} knock-in mice (referred to as Lgr5WT) with Atg7^{fl/fl} mice. In the resulting Lgr5-EGFP-Cre^{ERT2}Atg7^{fl/fl} mice, *Atg7* was deleted selectively in Lgr5⁺ISC upon tamoxifen injection, and consequently in all intestinal epithelial cells over time, thus generating Lgr5Atg7^{-/-} mice. We first validated the efficient loss of *Atg7* by RT-qPCR analysis as well as the consequent inhibition of autophagy by p62 accumulation in the EGFP-positive crypts of these mice (supplementary figure 3A,B). This model allows for the sorting of Lgr5⁺ISC cells and their early TA progenitors from both Lgr5WT and Lgr5Atg7^{-/-} crypts based on EGFP expression (EGFP^{High} for Lgr5⁺ISC cells and EGFP^{Low} for TA cells) (supplementary figure 3A). We confirmed these populations by RT-qPCR analysis on sorted cells (supplementary figure 3B). Microarray expression profiling of Lgr5⁺ISC from Lgr5Atg7^{-/-} and Lgr5WT crypts revealed 2334 significantly altered genes in this population while 825 genes were significantly deregulated in TA progenitors. Of these, only 46 genes were commonly deregulated between the ISC and TA signatures (supplementary figure 3C), revealing a drastically different effect of the loss of *Atg7* in these two populations. We therefore focused our analysis on signatures specific to Lgr5⁺ISC that may explain their particular sensitivity to the inhibition of autophagy.

Top canonical pathways identified by Ingenuity pathway analysis (IPA) revealed an effect of the loss of *Atg7* on the DNA damage response in Lgr5⁺ISC, including pathways like *ATM signaling*, *BRCA1 in DNA damage response*, and *CHK proteins in cell cycle checkpoint control* (figure 3A, yellow arrowheads). Of note, these pathways were not altered in TA cells from Lgr5Atg7^{-/-} mice compared to those from Lgr5WT mice (supplementary figure 3D). Likewise, Gene Set Enrichment Analysis (GSEA) revealed an enrichment of *DNA repair* and *G2/M checkpoint* genes in WT Lgr5⁺ISC compared to their Atg7^{-/-} counterparts (figure 3B). Analysis of relevant DNA repair genes from these signatures by RT-qPCR confirmed that autophagy-deficient Lgr5⁺ISC show reduced expression of DNA repair genes compared to their WT counterparts (figure 3C).

We next looked at DNA damages *in situ* in WT and Atg7^{-/-} mice. At basal levels, we found frequent zones in the Atg7^{-/-} epithelium with γ H2AX foci, indicating unrepaired double-stranded DNA breaks, specifically in the ISC compartment of the crypt (figure 3D). Elevated levels of DNA damages in autophagy-deficient Lgr5⁺ISC could be a result of inefficient DNA repair and contribute to their impaired survival. To directly assess the effect of autophagy on the DNA damage response pathways put in place to safeguard genomic integrity, we examined the effect of γ -irradiation (10G γ), an efficient way to induce a high degree of DNA damage, on WT and Atg7^{-/-} mice. Within 6 hours of irradiation, we found similar nuclear foci of γ H2AX in

the TA progenitors and differentiated cells of both WT and *Atg7^{-/-}* mice. However, *Lgr5⁺ISC* show distinct sensitivity depending on the status of autophagy: *Lgr5⁺ISC* from WT mice showed fewer γ H2AX foci than other epithelial cells suggesting more efficient repair in autophagy-proficient cells. In contrast, autophagy-deficient *Lgr5⁺ISC* show elevated levels of γ H2AX (figure 3E) associated with increased apoptosis (figure 3F), indicating a compromised capacity to repair DNA damages produced by ionizing radiation. Altogether our data suggest that autophagy protects *Lgr5⁺ISC* from lethal DNA damage by promoting DNA repair.

Defective antioxidant response renders autophagy-deficient *Lgr5⁺ISC* more sensitive to apoptosis

Intriguingly, by GSEA, we identified an enrichment in signatures linked to reactive oxygen species (ROS) detoxification, including NRF2 target genes, in *Lgr5⁺ISC* from *Lgr5^{WT}* mice compared to their counterparts from *Lgr5^{Atg7^{-/-}}* mice (figure 4A). This result is surprising since the inhibition of autophagy was previously shown to drive NRF2 stabilization and transcriptional activity in a p62-dependent manner (Komatsu et al., 2010). In line with this mechanism, along with the accumulation of p62 we observe an increase in NRF2 protein levels (figure 4B) and an upregulation of its antioxidant target genes by RT-qPCR analysis (figure 4C) in whole intestinal tissue from *Atg7^{-/-}* mice. However, in autophagy-deficient *Lgr5⁺ISC*, RT-qPCR analysis confirmed the predicted downregulation of NRF2 target genes compared to their WT counterparts, whereas this is not the case for TA progenitors (figure 4D, supplementary figure 4A). The opposing NRF2 responses of these populations may explain the high sensitivity of *Lgr5⁺ISC* following the inhibition of autophagy.

In light of the divergent responses between *Lgr5⁺ISC* and the rest of the epithelial tissue, we examined ROS directly in *Lgr5⁺ISC* using the cytoplasmic ROS-responsive fluorescent probe CellROX™. By flow cytometry, we detected a significant increase in CellROX mean fluorescence intensity in autophagy-deficient *Lgr5⁺ISC* compared to their WT counterparts, whereas autophagy-deficient TA progenitors have comparable ROS levels to WT TA progenitors (figure 4E). Likewise, we detected much higher CellROX fluorescence at the base of *Atg7^{-/-}* organoid crypts than in WT organoid crypts (supplementary figure 4B), indicating greater levels of ROS in the *Lgr5⁺ISC* compartment of autophagy-deficient crypts. To examine the contribution of the antioxidant response and ROS accumulation to autophagy-deficient ISC death, we treated *Atg7^{-/-}* organoids with N-acetyl Cysteine (NAC), a precursor of glutathione and a direct ROS scavenger, or sulforaphane, an activator of Nrf2 signaling. Both NAC and sulforaphane treatments tempered out CellROX intensity in organoid crypts (supplementary figure 4B) and partially rescued autophagy-deficient organoid survival (supplementary figure 4C), suggesting that ROS accumulation contributes to the death of *Lgr5⁺ISC* in *Atg7^{-/-}* mice. To verify this hypothesis *in vivo*, we added NAC to the drinking water of *Atg7^{-/-}* and WT mice. As expected, NAC treatment significantly reduced the number of TUNEL-positive cells in *Atg7^{-/-}* crypts (figure 4F). The inhibition of autophagy therefore leads to an unexpected downregulation of the antioxidant response in *Lgr5⁺ISC*, rendering them more prone to ROS accumulation and apoptosis.

We also looked at potential sources of ROS in the autophagy-deficient epithelium. In addition to its link with the antioxidant response, autophagy can directly regulate ROS production through the selective degradation of mitochondria, a major cellular source of ROS, in a process termed mitophagy. By transmission electron microscopy, we observed abnormally large, swollen mitochondria throughout the epithelium of *Atg7^{-/-}* mice, including in *Lgr5⁺ISC* (supplementary figure 4D). Surprisingly, we found no difference in the number of mitochondria following the inhibition of autophagy in either whole intestinal

tissue by quantitative PCR of mitochondrial DNA (supplementary figure 4E) or in sorted Lgr5⁺ISC and TA progenitors using the fluorescent probe MitoTracker™ (supplementary figure 4F). The high proliferation of Lgr5⁺ISC and TA progenitors as well as the short half-life of epithelial cells could explain the lack of accumulation of mitochondria despite the inhibition of their degradation pathway. Nevertheless, we found a significant increase in mitochondrial ROS production in autophagy-deficient Lgr5⁺ISC by flow cytometry using the fluorescent probe MitoSOX™ (figure 4E). In contrast, levels of mitochondrial ROS were similar in autophagy-deficient TA progenitors to those in WT TA progenitors. Mitochondria can therefore act as a cell-intrinsic source of ROS in autophagy-deficient Lgr5⁺ISC and subsequently disrupt their maintenance.

Disruption of the gut microbiota following loss of Atg7 contributes to the death of Lgr5⁺ISC

We previously showed that the faulty antimicrobial defenses following the loss of autophagy throughout the intestinal epithelium led to altered contact with the microbiota and a disruption of the resident gut bacterial communities (Lévy et al., 2015). To study the potential involvement of the altered gut microbiota on Lgr5⁺ISC survival, we explored the effect of orally-administrated antibiotics on Atg7^{-/-} mice. Interestingly, broad-spectrum antibiotic treatment significantly reduced the number of TUNEL-positive cells in the Atg7^{-/-} epithelium (figure 5A), suggesting an involvement of the microbiota in the perturbations of ISC homeostasis. Consistently, antibiotic-treated Atg7^{-/-} mice showed fewer γ H2AX-positive zones and lacked the global induction of antioxidant response genes (figure 5B,C), pointing to less accumulation of cytotoxic stresses throughout the autophagy-deficient epithelium in the absence of the microbiota.

We used organoid culture to further distinguish the epithelium-intrinsic from the microenvironment-dependent effects of autophagy inhibition in ISC. Although organoids derived from the crypts of tamoxifen-treated Atg7^{-/-} mice maintain Lgr5⁺ISC defects (figure 1E), neither the removal of Atg7 *ex-vivo* by the addition of 4-hydroxy-tamoxifen (4OHT) to crypt culture medium nor treatment with the autophagy inhibitor chloroquine induced a defect in organoid survival despite an efficient inhibition of autophagic flux (figure 5D,E). Altogether, this suggests that the intrinsic effect of autophagy inhibition in Lgr5⁺ISC is not itself sufficient to hinder organoid survival but that exposure to the gut microenvironment influences their survival. We therefore tested the direct impact of the microbiota by adding different purified microbial compounds on organoids derived from VillinCre^{ERT2}Atg7^{fl/fl} mice and treated *ex vivo* with 4OHT. Among the tested microbe-associated molecular patterns (MAMPs), we found that only muramyl dipeptide (MDP) induced the death of autophagy-deficient but not WT organoids (figure 5F). Altogether, our data suggest a role for autophagy in the cytoprotection and maintenance of Lgr5⁺ISC in response to specific microbial signals.

Loss of p53 in autophagy-deficient Lgr5⁺ISC is sufficient to initiate intestinal neoplasia

Thus far, our data reveals a protective role for autophagy in the maintenance of Lgr5⁺ISC in response to DNA damage, oxidative stress and microbial signals. We therefore reasoned that in the context of autophagy deficiency, the induction of apoptosis by p53 could be essential to remove damaged Lgr5⁺ISC and preserve the integrity of the active stem cell pool. To test this hypothesis, we examined the long-term effects of the simultaneous loss of ATG7 and p53 in intestinal epithelial cells by analyzing Atg7^{-/-}p53^{-/-} mice 12 months after tamoxifen injection. At this age, Atg7^{-/-} mice do not develop spontaneous intestinal tumors. As previously reported, p53 deletion in intestinal epithelial cells is not sufficient to initiate intestinal neoplasias either within this time frame (Schwitalla et al., 2013; Chanrion et al., 2014). In contrast, 10 out of

11 *Atg7*^{-/-}*p53*^{-/-} mice developed adenomas within 12 months (figure 6A). These tumors harbored numerous γ H2AX foci (figure 6B). In addition, we found a massive accumulation of γ H2AX staining in the non-tumoral intestinal epithelial cells of *Atg7*^{-/-}*p53*^{-/-} mice that, unlike γ H2AX staining in *Atg7*^{-/-} mice, was not restricted to the crypt bottom but rather propagated up the entire crypts (figure 6B). Altogether, we showed that autophagy and p53 act in conjunction as protective mechanisms essential to preserve the genomic integrity of Lgr5⁺ISC.

DISCUSSION

Lgr5⁺ISC are essential for the homeostatic maintenance of the intestinal epithelium (Barker, 2014), for the regeneration of the epithelium upon injury (Metcalf et al., 2014) and have been described as the cell-of-origin for intestinal cancer (Barker et al., 2009). Understanding the molecular processes controlling their maintenance, proliferation, self-renewal and integrity is crucial to determine the drivers and effectors of intestinal homeostasis and to develop effective strategies to detect, prevent and treat cancer. Here, we made significant advances in understanding how alterations of autophagy may influence Lgr5⁺ISC dynamics and tumor development depending on p53 status.

We show that the loss of *Atg7* inhibits autophagy throughout the intestinal epithelium, yet results in the p53-dependent apoptosis of Lgr5⁺ISC specifically. We further demonstrate a requirement for autophagy in the maintenance and protection of Lgr5⁺ISC against various intrinsic and environmental stresses. First, the loss of *Atg7* results in a transcriptional downregulation of several genes encoding essential DNA repair factors. Furthermore, our data exposed significant differences in the response of autophagy-deficient Lgr5⁺ISC to cytotoxic stress, particularly in their DNA-repair capacity and susceptibility to apoptosis. The fact that Lgr5⁺ISC are particularly sensitive to the inhibition of autophagy compared TA cells may be due to their greater dependence on specific DNA repair pathways. Consistent with previously published Lgr5⁺ISC signatures (Merlos-Suárez et al., 2011; Muñoz et al., 2012), we found several genes encoding DNA repair factors to be enriched in Lgr5⁺ISC compared to TA progenitors (*data not shown*). This is consistent with previous reports that Lgr5⁺ISC repair irradiation-induced DNA damages more efficiently than TA progenitors (Hua et al., 2012). However, this is not the case in autophagy-deficient Lgr5⁺ISC. Our observations therefore suggest that the DNA damage repair pathways put in place to maintain genomic integrity and maximize survival in Lgr5⁺ISC may be directly regulated by autophagy. Various *in vitro* reports support this notion, highlighting a molecular link between the inhibition of autophagy and defects in DNA damage repair (Chen et al., 2015; Liu et al., 2015; Hewitt et al., 2016; Wang et al., 2017). Furthermore, a recent study in *Drosophila melanogaster* showed increased levels of DNA damage in ISC upon RNAi-mediated silencing of several individual *Atg* genes (Nagy et al., 2018). Thus, the role of autophagy in supporting the DNA damage response in Lgr5⁺ISC may be conserved through evolution.

We further show that whereas TA progenitors and differentiated cells are appropriately protected against oxidative stress and can activate an NRF2-mediated response following the inhibition of autophagy, Lgr5⁺ISC specifically accumulate both mitochondrial superoxide and cytoplasmic ROS due to a defective antioxidant response, and that this contributes to their impaired survival. Inhibition of autophagy has been described to affect the redox state of various other adult stem cells, including hematopoietic stem cells (Mortensen et al., 2011; Watson et al., 2015; Ho et al., 2017), neural stem cells (Wang et al., 2016), and satellite cells (García-Prat et al., 2016). The requirement for autophagy to regulate ROS therefore appears

to be a common feature of adult stem cells, although the functional consequences of deregulating autophagy vary between tissues.

A recent report similarly showed that loss of another key autophagy gene, *Atg5*, in the intestinal epithelium led to increased ROS levels resulting in impaired ISC maintenance (Asano et al., 2017). However, in contrast to our findings, Asano et al. do not describe increased apoptosis in the ISC compartment of the *Atg5*-deficient epithelium but rather indicate reduced Lgr5⁺ISC numbers. A possible explanation for these discrepancies may be the distinct genetic models used to inhibit autophagy: in our study, ablation of *Atg7* is induced in adult mice after tamoxifen injection (using a VillinCre^{ERT2} transgene) whereas Asano et al. used a model where the deletion of *Atg5* is constitutive throughout embryonic development (using the VillinCre transgene). It is therefore possible that autophagy plays additional roles in Lgr5⁺ISC during development.

In addition, autophagy-deficient Paneth cells show secretory defects consistent with those reported in the literature (Cadwell et al., 2008; Lévy et al., 2015; Bel et al., 2017) resulting in a defective antimicrobial defense. We show that whereas the death of autophagy-deficient Lgr5⁺ISC is independent of the Paneth cell niche function, it is dependent on interactions with the microbiota. Several studies point to a role of the microbiota in both intestinal homeostasis and epithelial response following damage (McLaughlin et al., 1964; Onoue et al., 1981; Sommer and Bäckhed, 2013; Nigro et al., 2014; Sommer et al., 2015; Yu et al., 2015). Our results show that treating *Atg7*^{-/-} mice with wide-spectrum antibiotics significantly improves the survival of autophagy-deficient Lgr5⁺ISC and reduces signs of DNA damage and oxidative stress in the autophagy-deficient epithelium. The microbiota may therefore act on *Atg7*^{-/-} Lgr5⁺ISC by driving ROS accumulation and DNA damages. Interestingly, we found that the inhibition of autophagy affects the survival of organoids only if *Atg7* loss occurred *in vivo* prior to culture. This suggests that microenvironmental signals in autophagy-deficient mice induce lasting deleterious effects in Lgr5⁺ISC, contributing to their death. Recent studies have described direct crosstalks between the gut microbiota and Lgr5⁺ISC. Pattern recognition receptors that sense MAMPs are expressed by ISC and affect their proliferation and survival *in vivo* and *ex vivo* in organoid culture (Neal et al., 2012; Nigro et al., 2014), although the downstream effectors of this remain unclear. We tested the effects of several MAMPs on the survival of autophagy-deficient organoids. Of these, only MDP showed a differential effect on *Atg7*-depleted compared to WT organoid survival. Interestingly, MDP has been shown to exert cytoprotective effects on Lgr5⁺ISC mediated by its intracellular receptor NOD2 (Nigro et al., 2014), which directly interacts with the autophagy machinery (Cooney et al., 2010; Travassos et al., 2010; Chauhan et al., 2015). Autophagy may therefore play a role in the protection of Lgr5⁺ISC in response to specific microbial signals. Nevertheless, we cannot exclude additional effects of other microenvironmental signals driven by the presence of the microbiota, such as microbial metabolites or inflammatory signals, on the survival of autophagy-deficient Lgr5⁺ISC.

Fortunately, despite the loss of Lgr5⁺ISC following the inhibition of autophagy, the pool of Lgr5⁺ISC is continuously replenished and intestinal homeostasis is maintained over time. This reflects, as previously reported with acute damages to Lgr5⁺ISC, a high degree of plasticity in the intestinal crypt, where surviving Lgr5⁺ISC, reserve stem cells and/or committed progenitors are able to replenish the Lgr5⁺ISC pool and ensure epithelial homeostasis. Moreover, as inhibiting autophagy is not ultimately deleterious to the maintenance of the Lgr5⁺ISC pool and epithelium, it could safely be considered in therapeutic approaches.

Finally, we show that the p53-mediated elimination of damaged Lgr5⁺ISC is essential to preserve the integrity of the autophagy-deficient epithelium, as the combined ablation of Atg7 and p53 dramatically increases DNA damage throughout the epithelium and triggers the development of adenomas. This comes in stark contrast to the effect of the loss of Atg7 alone on tumor initiation (Lévy et al., 2015) and reveals an important interplay between autophagy and p53 in Lgr5⁺ISC integrity.

Overall, given the rapid and lifelong proliferation of Lgr5⁺ISC, it is becoming increasingly clear that these cells require unique protective mechanisms to ensure their function. We propose autophagy as a gatekeeper of Lgr5⁺ISC integrity that minimizes intrinsic and environmental stress and enhances DNA repair to promote Lgr5⁺ISC maintenance and prevent tumor development.

METHODS

Mouse models and treatments

Mice carrying floxed alleles of the *Atg7* gene (provided by M. Komatsu, Niigata University, Japan; Komatsu et al., 2005) and mice carrying floxed alleles of the *Tp53* gene (provided by A. Berns, Netherlands Cancer Institute, The Netherlands; Jonkers et al., 2001) or both were bred with VillinCre^{ERT2} mice (provided by S. Robine, Institut Curie, France; Marjou et al., 2004) or Lgr5-EGFP-IRES-Cre^{ERT2} (provided by H. Clevers, Hubrecht Institute, The Netherlands; Barker et al., 2007). All experiments were carried out on C57Bl/6 background mice. Mice were housed in conventional conditions. Tamoxifen injections were performed on mice 2 to 3 months old. Both male and female mice were used in the studies. For tamoxifen treatment, mice were injected intraperitoneally with 1 mg tamoxifen and their diet was supplemented with tamoxifen 5 days per month. Unless indicated otherwise, mice were sacrificed one month after tamoxifen treatment. For proliferation studies, mice were injected with BrdU (2.5 mg, Sigma-Aldrich) 1.5 hours before sacrifice. For irradiation studies, mice were exposed to a ¹³⁷Cs source of γ -irradiation with a dose rate of 1.47 Gy/min for a final dose of 10 Gy and sacrificed 6 hours later. For NAC treatment, 12.5 mg/ml NAC was to the drinking water (for an estimated intake of 200 mg/kg/day) and pH was adjusted. Water was changed every other day for a month following tamoxifen treatment. For antibiotic treatment, a combination of 1 g/l ampicillin, 1g/l neomycin, 1g/l metronidazole, and 0.5 g/l vancomycin (all from Sigma-aldrich) was added to the drinking water of the mice and changed every other day for a month following tamoxifen treatment.

Histology, immunostainings and in situ hybridization

Immediately after the mouse was sacrificed, its entire gastrointestinal tract was removed, splayed open along its length and rolled up from the proximal to distal end to form a 'Swiss roll'. Tissues were fixed by incubation in 4% paraformaldehyde overnight at 4°C and were embedded in paraffin wax. Hematoxylin and eosin staining was carried out on 3 μ m paraffin sections. Immunohistochemistry was performed as previous described (Lévy et al., 2015). For immunohistochemistry, 5 μ m sections were treated with 3% hydrogen peroxide for 15 min at room temperature. For both immunohistochemistry and immunofluorescence, antigens were retrieved by boiling for 15 min in citrate buffer (10 mM, pH 6) or for 40 min in Tris-EDTA buffer (100mM Tris, 12.6 mM, pH 9 for γ H2AX in a microwave pressure cooker (EZ retriever, Biogenex). For BrdU staining, DNA was denatured in 2,5N HCl for 30 min. Sections were incubated in blocking solution (2% goat serum, 1% BSA in TBST) for 20 min at room temperature. For mouse primary antibodies, Vector's M.O.M. kit was used. The sections were then incubated overnight at 4°C with primary antibodies diluted in

blocking solution. Primary antibodies targeted against the following proteins were used: p62 (Enzo, BML-PW9860-0100, 1:200), Ubiquitin (MBL, MK 11-3, 1:200), cleaved Caspase-3 (Cell Signaling, 9661S, 1:200), p53 (Leica, NCL-p53-CM5p, 1:200), Lysozyme (Dako, EC.3.2.1.17, 1:200), BrdU (Abcam, ab6326, 1:500), Olfm4 (Cell Signaling, CS 39141S, 1:400), γ H2AX (Millipore, 05-636, 1:300), GFP (Abcam, ab13970, 1:200). Specific binding was detected with a biotinylated secondary antibody and ABC reagent (Vector) for immunohistochemistry, and the signal was developed with DAB (Vector). Hematoxylin was used as a nuclear counter-stain. For immunofluorescence, Alexa fluor-coupled secondary antibodies (ThermoFisher Scientific) were used, Hoechst was used as a nuclear counter-stain, and slides were mounted in Vectashield fluorescence mounting medium (Vector). Slides were then imaged either on a wide-field Olympus BX63F microscope or a Yokogawa CSU-X1 Spinning Disk (Yokogawa, Tokyo, Japan) coupled with a DMI6000B Leica microscope (Leica microsystems GmbH, Wetzlar, Germany). Acquisitions were made with MetaMorph 7 software. Apoptosis was analysed by TUNEL assay kit, according to the manufacturer's instructions (Calbiochem, QIA33 for visible staining, Promega, G3250 for fluorescent labeling). In situ hybridization were performed as described previously (Gregorieff and Clevers, 2015). cRNA probes were generated from plasmids containing the *Olfm4* cDNA sequences (gift from H. Clevers, Hubrecht Institute for Developmental Biology and Stem Cell Research and University Medical Centre Utrecht, Utrecht, Netherlands).

Crypt isolation and organoid culture

Crypt isolation and crypt culture were performed as previously described (Sato et al., 2009). Briefly, intestines were opened longitudinally, washed with cold PBS, and incubated in PBS with 15mM EDTA for 25 minutes on ice. The tissue was then removed from EDTA and vigorously vortexed in multiple fractions to release crypts. Fractions enriched for crypts were passed through a 70- μ m cell strainer (BD Bioscience) to remove residual villi. For organoid culture, isolated crypts were counted and pelleted. 500 crypts were mixed with 50 μ l growth factor reduced matrigel (Corning) and plated on 24-well plates with 500 μ l advanced DMEM/F12 (Invitrogen) containing the growth factors: EGF (Peprotech); R-spondin 1 (R&D Systems); and noggin (Peprotech); and supplemented with N2 and B27 (Invitrogen). Organoid culture medium was changed every 2-3 days. In some experiments, media were supplemented with 100 ng/mL human Wnt3a (Peprotech), 2 μ M Pifithrin (Sigma-aldrich), 500 μ M NAC (Sigma-aldrich), 250 nM sulforaphane (Sigma-aldrich), 500nM 4OHT (Sigma-aldrich), 500nM chloroquine (Sigma-aldrich), 50 μ g/ml MDP, 100 μ g/ml LPS, 10 ng/ml Flagellin, 500ng/ml PolyIC, or 10 μ g/ml LTA (all from InvivoGen).

Whole-mount staining and live imaging on organoids

Prior to whole-mount staining, 10 μ M BrdU was added to the culture medium for 16 hours to label proliferative crypt cell. For staining, medium was washed in PBS and organoids were fixed in 4% paraformaldehyde for 30 min at room temperature. Then, DNA was denatured in 2N HCl for 25 minutes, non-specific antigens were blocked in 5% goat serum, 1% BSA, and 0,3% TritonX-100 in PBS before incubating organoids overnight at 4°C with primary antibodies diluted in the blocking solution. Organoids were then washed in PBS, incubated overnight at room temperature with fluorescent secondary antibodies and Hoescht, washed, and suspended in Vectashield fluorescence mounting medium (Vector), then flattened between a slide and coverslip for imaging using using a Yokogawa CSU-X1 Spinning Disk (Yokogawa, Tokyo, Japan) coupled with a DMI6000B Leica microscope (Leica microsystems GmbH, Wetzlar,

Germany). For CellROX staining on live organoids, 10 μ M CellROX™ deep red reagent (ThermoFisher Scientific) was added to the culture medium and organoids were incubated for 30 min at 37°C, washed in PBS and imaged directly on an inverted Zeiss Axiovert 200M microscope. Hoechst 33342 (ThermoFisher Scientific) was used to mark the nuclei of all cells. Acquisitions were made with MetaMorph 7 software.

Cell sorting and flow cytometry

Isolated crypts (as described above) were dissociated in Dispase buffer containing 0.3U/ml Dispase (Corning), 0.8U/ml DNase (Ambion), 10 μ M Y-27632 (Enzo) at 37°C during 30 min. Dissociated cells were passed through 40 μ m cell strainer (BD Bioscience) centrifuged 5 min at 700g and washed with PBS. Cells were stained with APC-conjugated anti-CD24 antibody (clone M1/69, BioLegend) and PE-Epcam (clone G8.8 BioLegend) and for 20 min at 4°C. For RNA extraction, cells were sorted on a BD FACSAria III cell sorter (BD Biosciences) and collected directly in lysis buffer. For analysis of reactive oxygen species, cells were incubated with either 5 μ M CellROX™ deep red, 5 μ M MitoSOX™ red, or 250 nM MitoTracker™ deep red reagent (all from ThermoFisher Scientific) for 20 min at 37°C then washed in PBS before analysis on a BD LSRFortessa™ flow cytometer (BD Biosciences) using the BD FACSDIVA™ software (BD Biosciences). Viable epithelial single cells were gated by forward scatter, side scatter and pulse-width parameter, and negative staining for propidium iodide (ThermoFisher Scientific) or Live/Dead™ Blue Dead Cell Stain Kit (ThermoFisher Scientific).

Quantitative PCR and microarray analysis

Total RNA from cells and tissues was isolated with Trizol reagent (Invitrogen), according to the manufacturer's protocol. For RT-qPCR, total RNA was reverse-transcribed with the Maxima First-Strand cDNA Synthesis Kit (ThermoFisher Scientific). For microarray and RT-qPCR experiments on sorted Lgr5+ISC and TA cells, cells were sorted directly into lysis buffer and extracted using the RNeasy Micro Kit according to the manufacturer's instructions (Qiagen). After validation of the RNA quality with Bioanalyzer 2100 (using Agilent RNA6000 nano chip kit), 500 pg of total RNA is reverse transcribed following the Ovation Pico System V2 (Nugen). The resulting double strand cDNA is used for amplification based on SPIA technology. For mitochondrial DNA dosage, DNA was extracted from whole intestinal tissue. qPCR was carried out with a LightCycler 480 System, with the Luminaris Color HiGreen Kit (ThermoFisher Scientific). Results are expressed relative to 18s rRNA for all RT-qPCR. Atg7^{fl/fl} and mt-Rnr1, used to measure mitochondrial DNA, are expressed relative to Rbm15, used to dose nuclear DNA. All primers are listed in [supplementary table 1](#).

For microarray experiments, amplified cDNA from sorted cells was purified according to the Nugen protocol, then 3.6 μ g of Sens Target DNA are fragmented and biotin labelled using the Encore Biotin Module kit (Nugen). After control of fragmentation using the Bioanalyzer 2100, cDNA is then hybridized to GeneChip® Mouse Gene 2.0 ST (Affymetrix) at 45°C for 17 hours. Chips are then washed on the fluidic station FS450 according to the manufacturer's protocols (Affymetrix) and scanned using the GCS3000 7G. The scanned images are then analyzed with Expression Console software (Affymetrix) to obtain raw data (cel files) and metrics for Quality Controls. Data were normalized using RMA algorithm in Bioconductor with the custom CDF vs 21 ([Dai et al., 2005](#)). Statistical analysis were carried out using Partek® GS 6.6. First, variations in gene expression were analyzed using unsupervised hierarchical clustering and PCA to assess data from technical biases and outlier samples. We applied a two-way ANOVA between groups by

genotype and cell type. Then, we used unadjusted p-value and fold changes to filter and select differentially expressed gene. Differentially expressed genes with a p-value<0.05 and fold-change>1.2 were selected as significant.

Western blotting

Protein extracts were prepared from intestinal tissue or organoids by lysis in laemmli buffer. Protein extracts were then analysed by western blotting as described previously (Peignon et al., 2011). Antibodies against the following proteins were used: LC3 (Sigma, L7543, 1:1,000), p62 (Enzo, BML-PW9860 1:1,500), ATG7 (Cell Signaling, 2631, 1:1,000), p53 (Leica, NCL-p53-CM5p, 1:200), NRF2 (Cell Signaling, 12721S, 1:400), γ -tubulin (Sigma-aldrich, T6557).

Electron microscopy

2 mm tissue pieces were fixed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer and kept at 4°C. After several thorough washes in 0.1 M phosphate buffer, the tissues were post-fixed in 1% osmium tetroxide and washed in water before staining with uranyl acetate. After inclusion in epoxy resin, 300-400 nm semithin sections were first realized to control the good orientation of the samples; then 80-90 nm sections were cut on a Reichert Ultracut E ultramicrotome. The ultrathin sections were transferred onto 150-mesh copper grids before staining with uranyl acetate and lead citrate. The sections were then viewed under a JEOL 1011 transmission electron microscope with a GATAN Erlangshen CCD (charge-coupled device) camera.

Statistical analysis.

Data are expressed as mean \pm s.d. Differences were analyzed by unpaired two-tailed Student's t -tests. p-values<0.05 were considered significant. Detailed n values for each panel in the figures are stated in the corresponding legends. A minimum of n= 3 animals was systematically analyzed.

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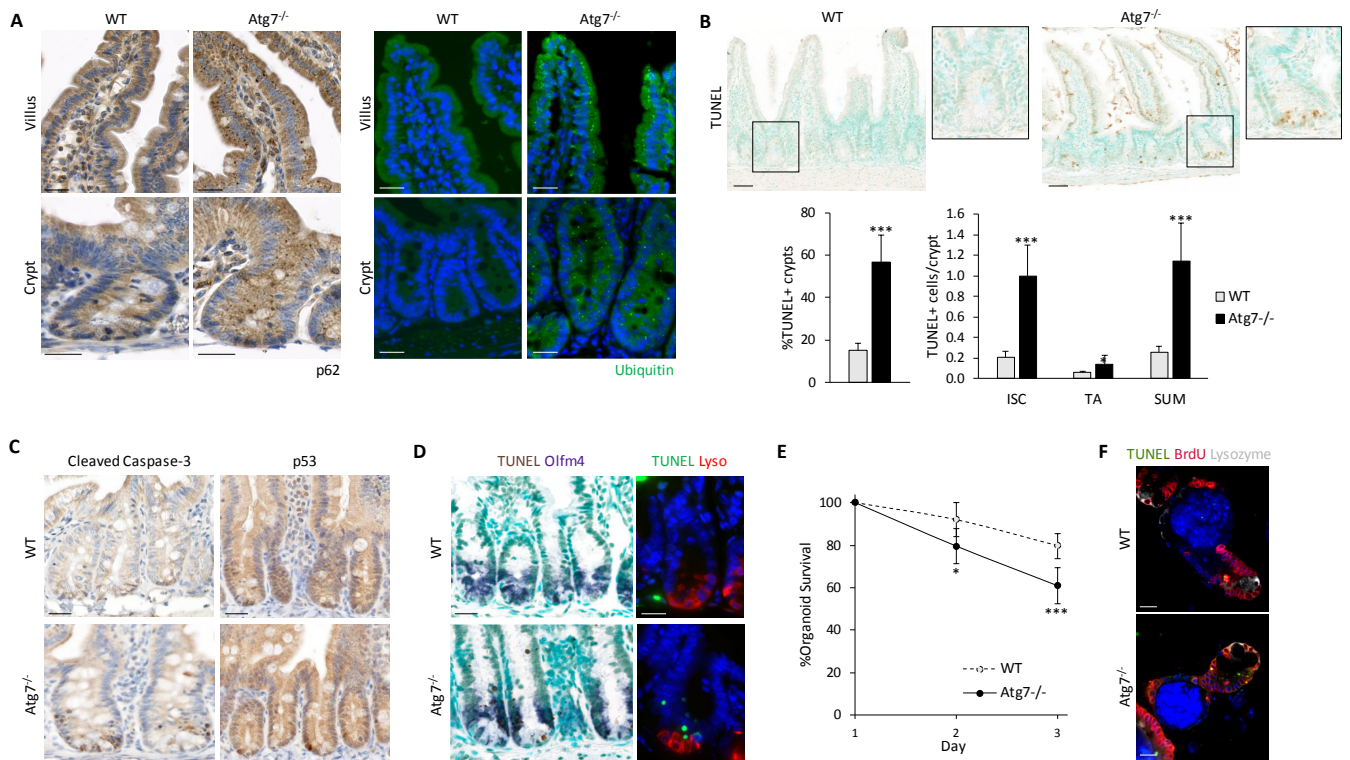


Figure 1. Atg7 deletion leads to the inhibition of autophagy throughout the intestinal epithelium and apoptosis of Lgr5⁺ISC.

- (A)** Representative staining for p62 (left panel) and Ubiquitin (right panel) in crypts and villi of WT and Atg7^{-/-} tissue sections. Hematoxylin is used as a nuclear counter-stain for p62 and Hoechst is used as a counter-stain for Ubiquitin.
- (A)** Representative TUNEL staining on WT and Atg7^{-/-} tissue sections. Methyl green is used as a nuclear counter-stain. Quantification of the percentage of TUNEL-positive crypts and average number of TUNEL-positive cells per crypt over 50 consecutive whole crypts in 6 WT and 11 Atg7^{-/-} mice. Data are represented as mean ± s.d.
- (B)** Representative cleaved Caspase-3 and p53 staining on tissue sections from WT and Atg7^{-/-} intestines. Hematoxylin is used as a nuclear counter-stain.
- (C)** Left panel: Representative TUNEL staining combined with in situ hybridization for Olfm4. Methyl green is used as a nuclear counter-stain. Right panel: Representative TUNEL staining combined with Lysozyme staining. Hoechst is used as a nuclear counter-stain.
- (D)** Percent survival from day 1 of organoids from the crypts of WT and Atg7^{-/-} mice (n= 9 mice for WT and 7 mice for Atg7^{-/-}). Data are represented as mean ± s.d.
- (E)** Representative whole mount staining of organoids grown from the crypts of WT and Atg7^{-/-} mice, 16 hours after addition of BrdU to the culture medium of organoids. TUNEL staining marks apoptotic cells. Lysozyme staining marks Paneth cells, BrdU staining marks crypt cells. Hoechst is used as a nuclear counter-stain.

All scale bars: 25 μm. Significant differences: * p<0.05, **p<0.01, ***p<0.005.

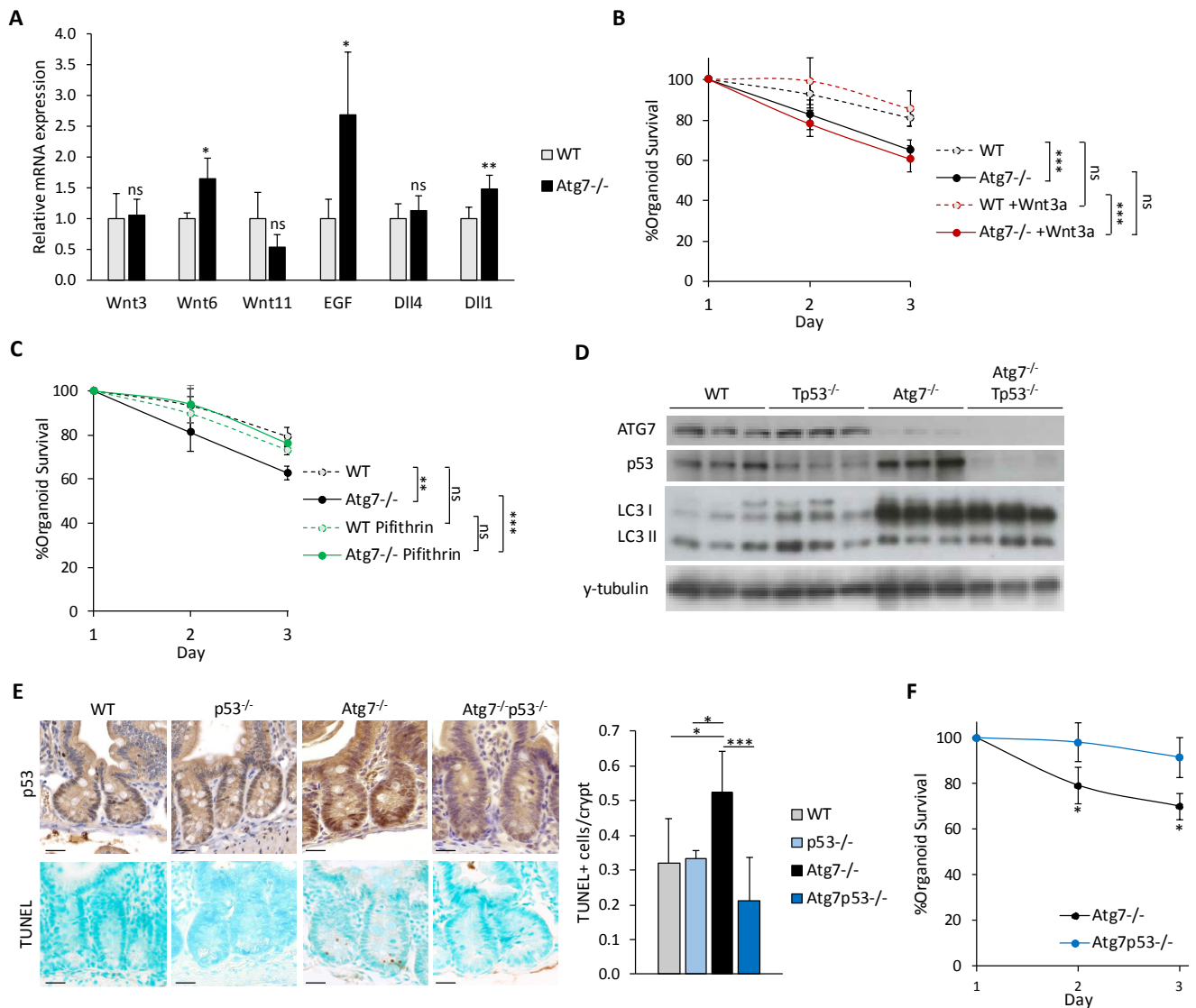


Figure 2. Autophagy-deficient Lgr5⁺ISC apoptosis is independent of the Paneth cell niche but dependent on p53.

- (A)** Relative mRNA expression levels of genes encoding growth factors produced by Paneth cells assessed by RT-qPCR analysis of whole intestinal tissue lysates of 4 WT and 5 Atg7^{-/-} mice. Data are represented as mean ± s.d.
- (B)** Percent survival from day 1 of organoids from the crypts of WT and Atg7^{-/-} mice in the presence or absence of exogenous Wnt3a in the culture medium (n= 5 mice for WT and 7 mice for Atg7^{-/-}). Significant differences are shown in the legend for day 3.
- (C)** Percent survival from day 1 of organoids from the crypts of WT and Atg7^{-/-} mice in the presence or absence of Pifithrin in the culture medium (n= 3 mice of each genotype). Significant differences are shown in the legend for day 3.
- (D)** Western blotting for ATG7, p53, and LC3b on whole intestinal tissue lysates from WT, p53^{-/-}, Atg7^{-/-}, and Atg7^{-/-}p53^{-/-} mice. γ-tubulin serves as a loading control. 3 individual mice of each genotype are shown.
- (E)** Representative p53 and TUNEL staining in the crypts of WT, p53^{-/-}, Atg7^{-/-}, and Atg7^{-/-}p53^{-/-} mice. Hematoxylin and methyl green are used as a nuclear counter-stains for p53 and TUNEL stainings, respectively. Scale bars: 25 μm. Quantification of the average number of TUNEL-positive cells per crypt over 50 consecutive whole crypts in 7 WT, 3 p53, 5 Atg7^{-/-} and 8 Atg7^{-/-}p53^{-/-} mice. Data are represented as mean ± s.d.
- (F)** Percent survival from day 1 of organoids from the crypts of Atg7^{-/-} or Atg7^{-/-}p53^{-/-} mice (n=3 mice of each genotype).

Significant differences: * p<0.05, **p<0.01, ***p<0.005, ns= not significant.

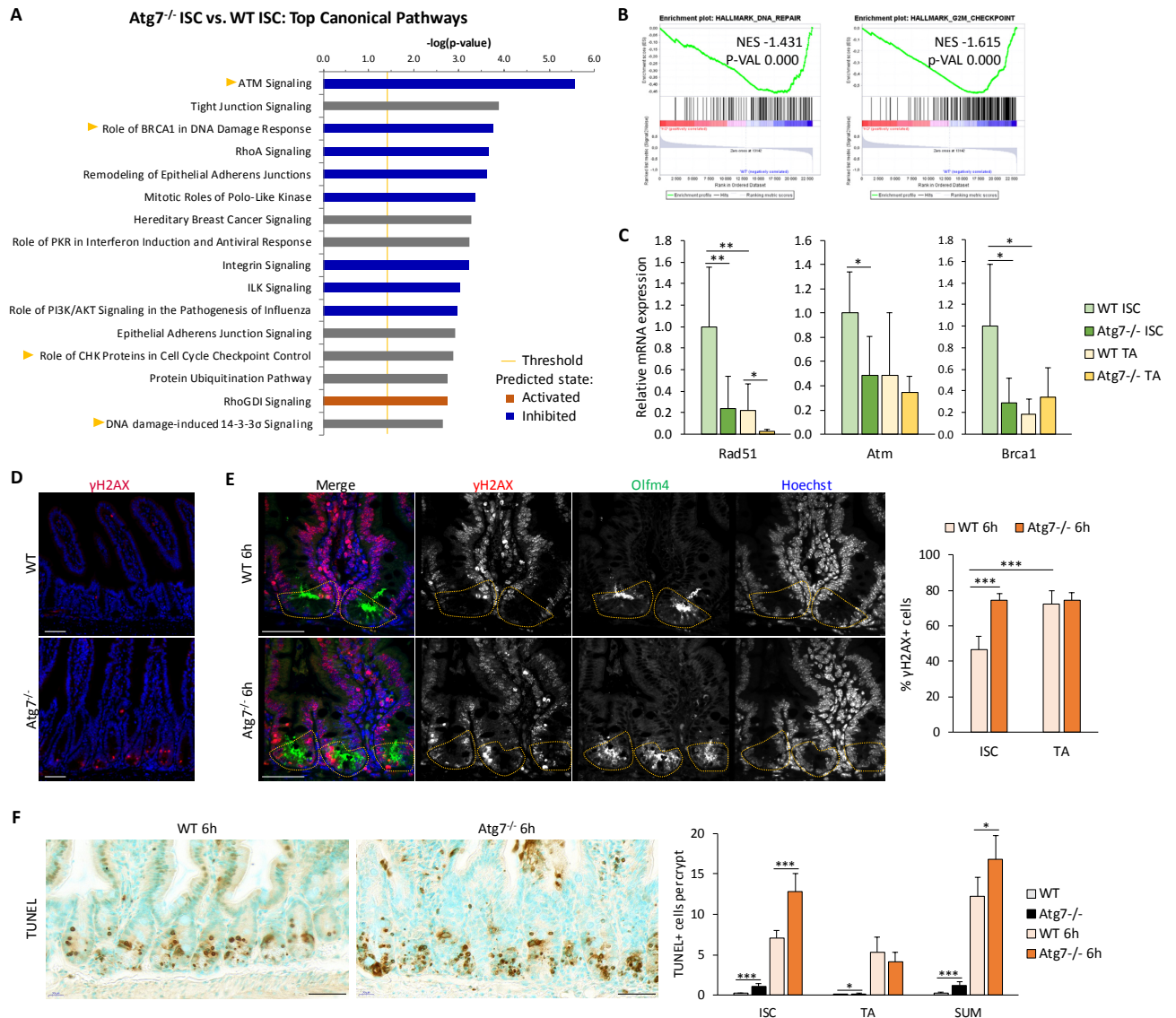


Figure 3. Loss of autophagy disrupts DNA damage repair in Lgr5⁺ISC.

- (A)** Top canonical pathways predicted by Ingenuity Pathway Analysis as significantly deregulated in the transcriptomic signature of sorted Lgr5⁺ISC from the crypts of Lgr5Atg7^{-/-} versus Lgr5WT mice.
- (B)** Enrichment plots generated by gene set enrichment analysis for hallmark DNA repair and G2M checkpoint gene sets on transcriptomic data from Lgr5⁺ISC from the crypts of Lgr5Atg7^{-/-} versus Lgr5WT mice. NES: normalized enrichment score. P-VAL: p-value.
- (C)** Relative mRNA expression levels of genes encoding DNA damage repair factors assessed by RT-qPCR analysis of sorted Lgr5⁺ISC or TA progenitors from Lgr5WT and Lgr5Atg7^{-/-} mice. Data are represented as mean ± s.d. (n= 6 mice per condition).
- (D)** γH2AX staining showing a lack of epithelial staining in WT crypts and a representative positive zone in the Atg7^{-/-} epithelium. Hoechst is used as a nuclear counter-stain.
- (E)** Representative z-projection (from 20 stacks spanning over 6 μm to include whole nuclei) of combined γH2AX and Olfm4 staining in the crypts of WT and Atg7^{-/-} mice 6 hours after 10 Gy whole-body irradiation. Hoechst is used as a nuclear counter-stain. Quantification of the percentage of γH2AX-positive cells was performed on at least 10 randomly selected whole crypts per mouse (n= 4 mice of each genotype). Cells with >4 γH2AX foci in their nucleus were considered as γH2AX-positive. Olfm4⁺ cells were considered as ISC and Olfm4⁻ cells above them and below the crypt-villus junction were considered as TA cells. Data are represented as mean ± s.d.
- (F)** Representative TUNEL staining tissue sections from WT and Atg7^{-/-} mice 6 hours after 10 Gy whole-body irradiation. Methyl green is used as a nuclear counter-stain. Quantification of the average number of TUNEL-positive cells per crypt over 50 consecutive whole crypts in 6 control WT mice, 11 control Atg7^{-/-} mice and 4 irradiated mice of each genotype. Data are represented as mean ± s.d.

All scale bars: 50 μm. Significant differences: * p<0.05, **p<0.01, ***p<0.005.

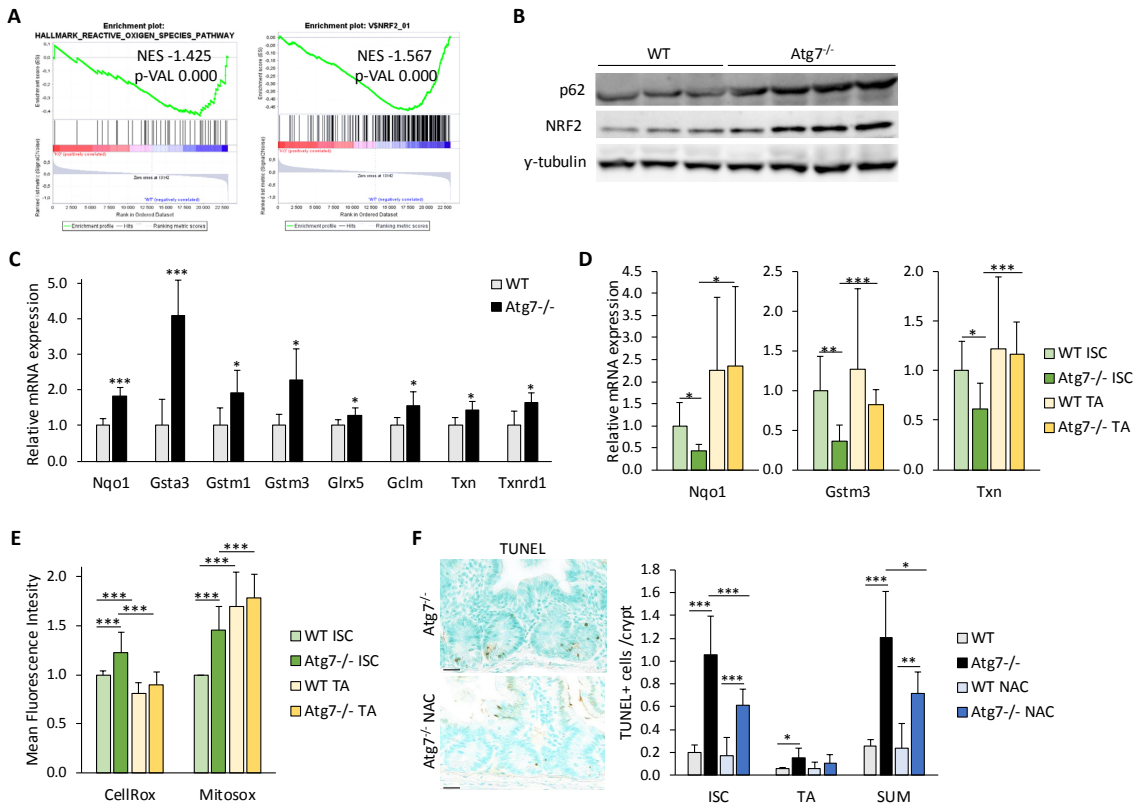


Figure 4. Defective antioxidant responses and ROS accumulation contribute to the death of autophagy-deficient *Lgr5*⁺ISC.

- (A)** Enrichment plots generated by gene set enrichment analysis for hallmark reactive oxygen species pathway and NRF2 targets gene sets on transcriptomic data from sorted *Lgr5*⁺ISC of *Lgr5Atg7*^{-/-} compared to *Lgr5*WT mice. NES: normalized enrichment score. P-VAL: p-value.
- (B)** Western blotting for p62 and NRF2 in whole intestinal tissue lysates from WT and *Atg7*^{-/-} mice. γ -tubulin serves as a loading control. 3 WT and 4 *Atg7*^{-/-} mice are shown.
- (C)** Relative mRNA expression levels of NRF2 target genes encoding antioxidant response proteins assessed by RT-qPCR analysis of whole intestinal tissue lysates of 4 WT and 5 *Atg7*^{-/-} mice. Data are represented as mean \pm s.d.
- (D)** Relative mRNA expression levels of NRF2 target genes encoding antioxidant response proteins assessed by RT-qPCR analysis of sorted *Lgr5*⁺ISC or TA progenitors from *Lgr5*WT and *Lgr5Atg7*^{-/-} mice. Data are represented as mean \pm s.d. (n= 6 mice per condition).
- (E)** Mean CellROXTM or MitoSOXTM fluorescence intensity of sorted *Lgr5*⁺ISC or TA progenitors from *Lgr5*WT and *Lgr5Atg7*^{-/-} mice. Data are represented as mean \pm s.d. (n= 13 *Lgr5*WT and 14 *Lgr5Atg7*^{-/-} mice for CellROX analysis, 4 *Lgr5*WT and 6 *Lgr5Atg7*^{-/-} mice for MitoSOX analysis).
- (F)** Representative TUNEL staining on tissue sections of *Atg7*^{-/-} mice treated with water or NAC. Methyl green is used as a nuclear counter-stain. Scale bars: 25 μ m. Quantification of the percentage of TUNEL-positive crypts and average number of TUNEL-positive cells per crypt over 50 consecutive whole crypts in 6 control WT mice, 11 control *Atg7*^{-/-} mice and 4 NAC-treated mice of each genotype. Data are represented as mean \pm s.d.

Significant differences: * p<0.05, **p<0.01, ***p<0.005.

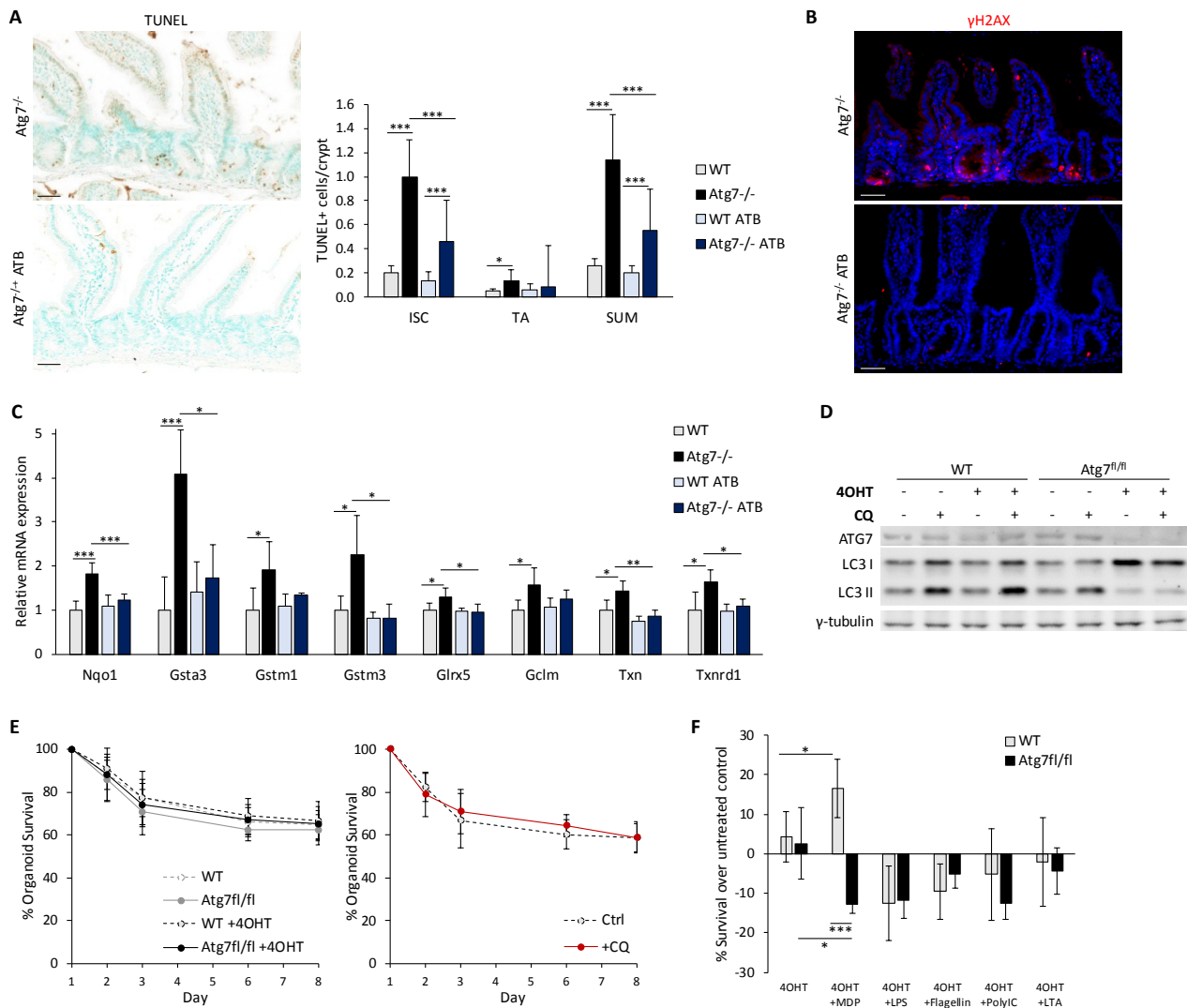


Figure 5. Interactions with the microbiota contribute to the apoptosis of autophagy-deficient Lgr5⁺ISC.

- (A)** Representative TUNEL staining on tissue sections of *Atg7^{-/-}* mice treated with water or wide-spectrum antibiotics (ATB). Methyl green is used as a nuclear counter-stain. Scale bars: 50 μ m. Quantification of the percentage of TUNEL-positive crypts and average number of TUNEL-positive cells per crypt over 50 consecutive whole crypts in 6 control WT mice, 11 control *Atg7^{-/-}* mice, 8 WT and 7 *Atg7^{-/-}* ATB-treated. Data are represented as mean \pm s.d.
- (B)** Representative γ H2AX staining on tissue sections of *Atg7^{-/-}* mice treated with water or wide-spectrum antibiotics (ATB). Hoechst is used as a nuclear counter-stain.
- (C)** Relative mRNA expression levels of NRF2 target genes encoding antioxidant response proteins assessed by RT-qPCR analysis of whole intestinal tissue lysates of 4 control WT, 5 control *Atg7^{-/-}*, 5 ATB-treated WT and 3 ATB-treated *Atg7^{-/-}* mice. Data are represented as mean \pm s.d.
- (D)** Western blotting for ATG7 and LC3b on protein extracts from day 4 WT or *Atg7^{fl/fl}* organoids in the presence or absence of 4OHT and/or CQ in the culture medium. γ -tubulin serves as a loading control.
- (E)** Percent survival from day 1 of WT or *Atg7^{fl/fl}* organoids in the presence or absence of 4OHT (left panel) or chloroquine (CQ, right panel) in the culture medium. (n=6 mice of each genotype).
- (F)** Percent change in survival compared to untreated controls of WT or *Atg7^{fl/fl}* organoids in the presence of 4OHT and in the presence or absence of muramyl dipeptide (MDP), lipopolisaccharide (LPS), flagellin, polyinosinic:polycytidylic acid (PolyIC) or lipoteichoic acid (LTA) in the culture medium. (n=6 mice of each genotype).

All scale bars: 50 μ m. Significant differences: * p < 0.05, ** p < 0.01, *** p < 0.005.

Figure 6

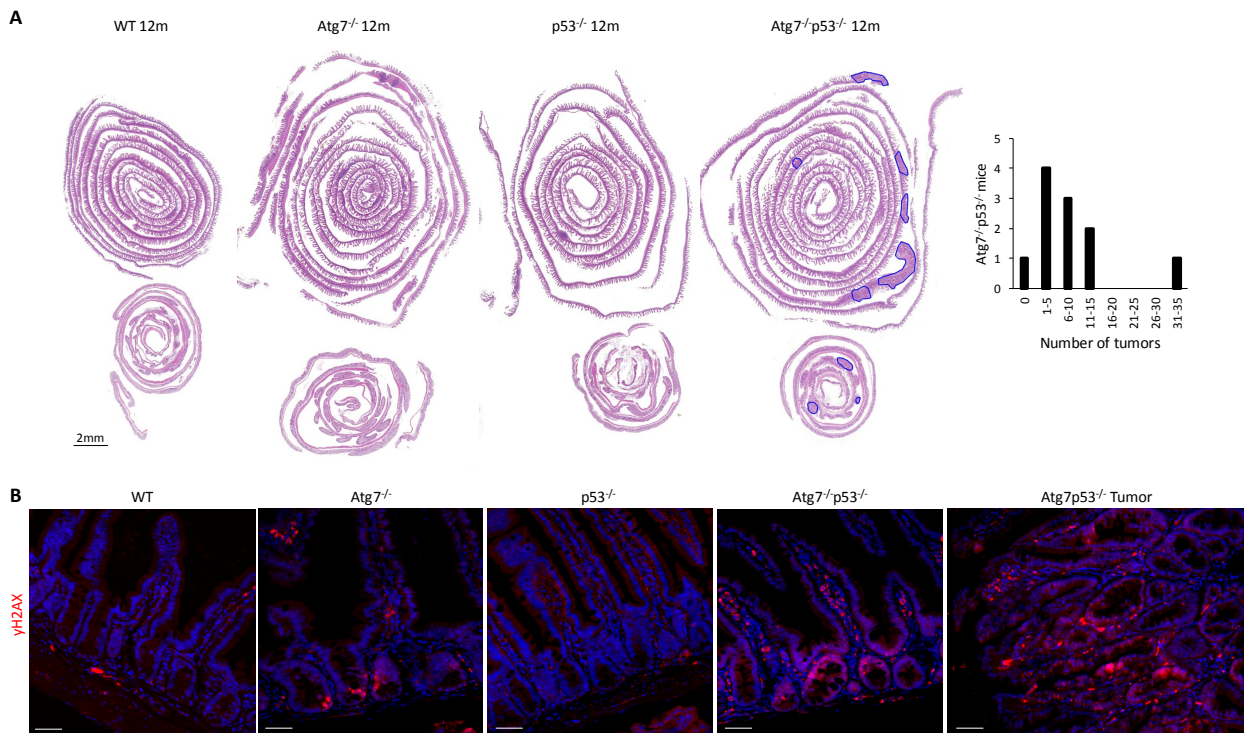
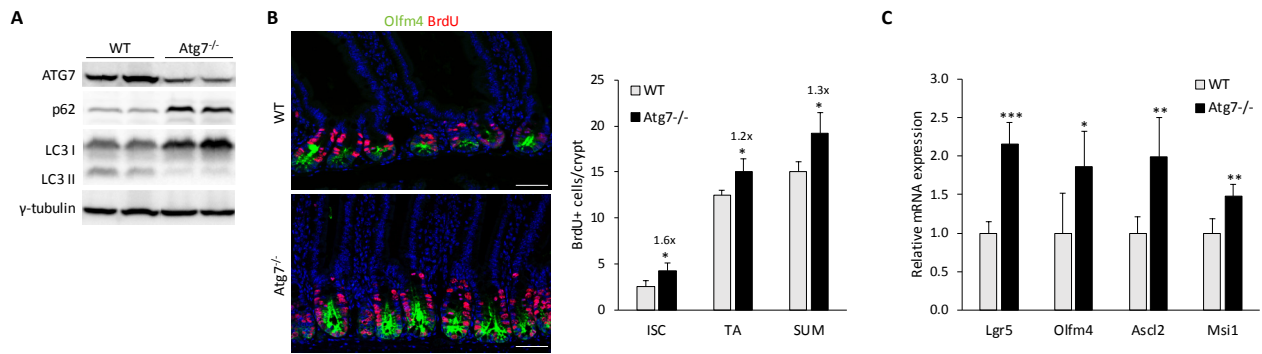


Figure 6. p53 blocks tumor initiation in the autophagy-deficient intestinal epithelium.

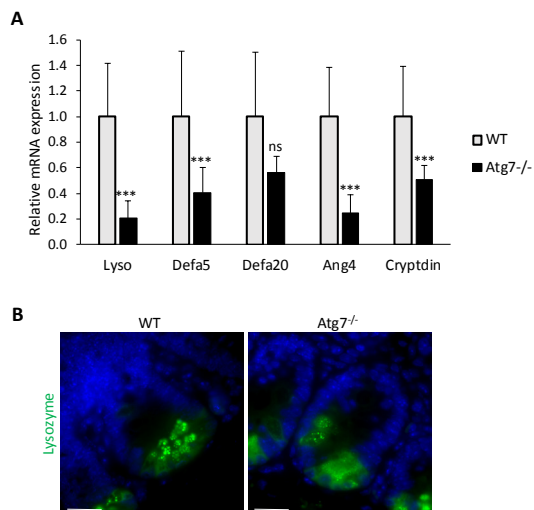
- (A)** Representative hematoxylin and eosin staining tissue sections of small intestinal and colonic swiss rolls from WT, Atg7^{-/-}, p53^{-/-} and Atg7^{-/-}p53^{-/-} mice sacrificed 12 months after tamoxifen treatment. Tumors are circled in blue. Scale bar: 2 mm. Right panel: Histogram of tumor counts in 11 Atg7^{-/-}p53^{-/-} mice.
- (B)** Representative γ H2AX staining on tissue sections of WT, Atg7^{-/-}, p53^{-/-} and Atg7^{-/-}p53^{-/-} mice sacrificed 12 months after tamoxifen treatment. Hoechst is used as a nuclear counter-stain. Scale bars: 50 μ m.



Supplementary Figure 1

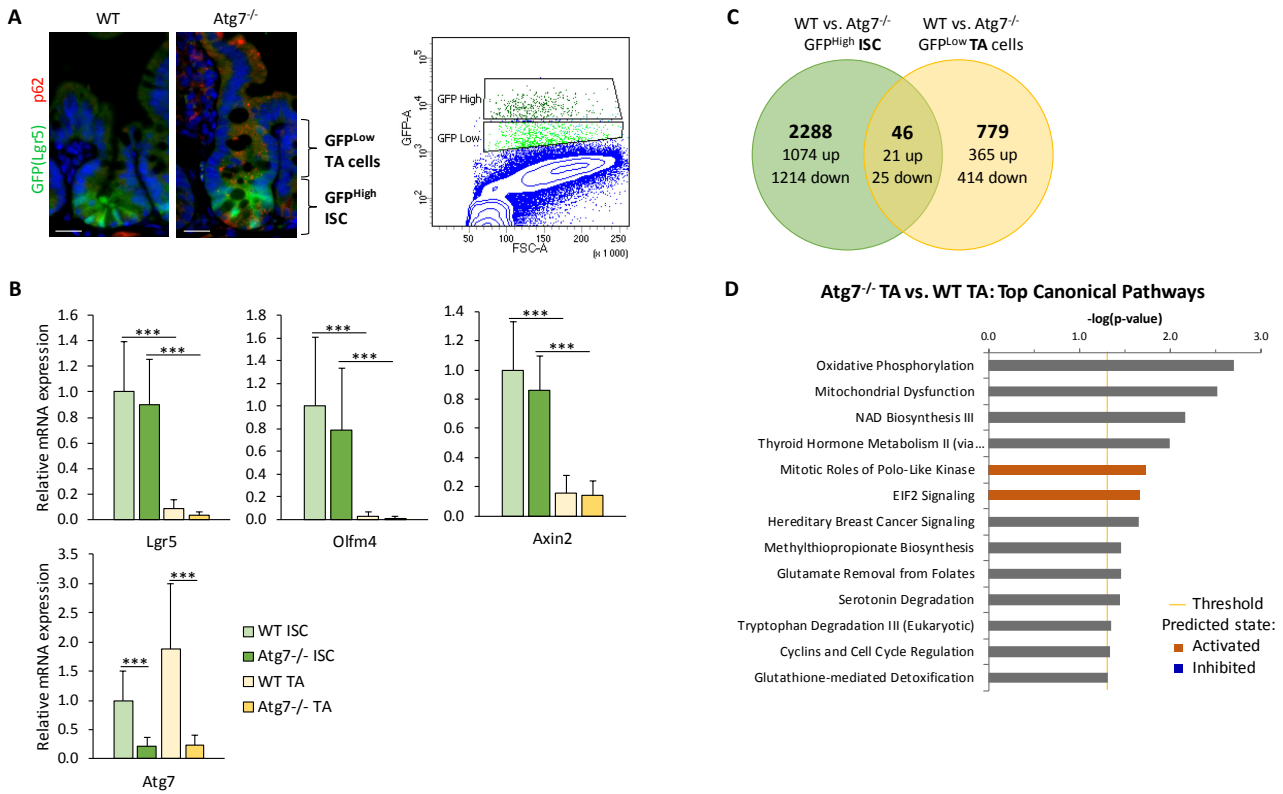
- (A)** Western blotting for ATG7, p62, LC3b in whole intestinal tissue lysates from WT and Atg7^{-/-} mice. γ -tubulin serves as a loading control. 2 mice of each genotype are shown.
- (B)** Representative Olfm4 and BrdU co-staining on tissue sections from WT and Atg7^{-/-} intestines. Hoechst is used as a nuclear counter-stain. Scale bars: 50 μ m. Quantification of the average number of BrdU-positive cells per crypt over 50 consecutive whole crypts in 3 WT and 4 Atg7^{-/-} mice. Data are represented as mean \pm s.d.
- (C)** Relative mRNA expression levels of genes encoding growth factors produced by Paneth cells assessed by RT-qPCR analysis of whole intestinal tissue lysates of 4 WT and 5 Atg7^{-/-} mice. Data are represented as mean \pm s.d.

Significant differences: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.



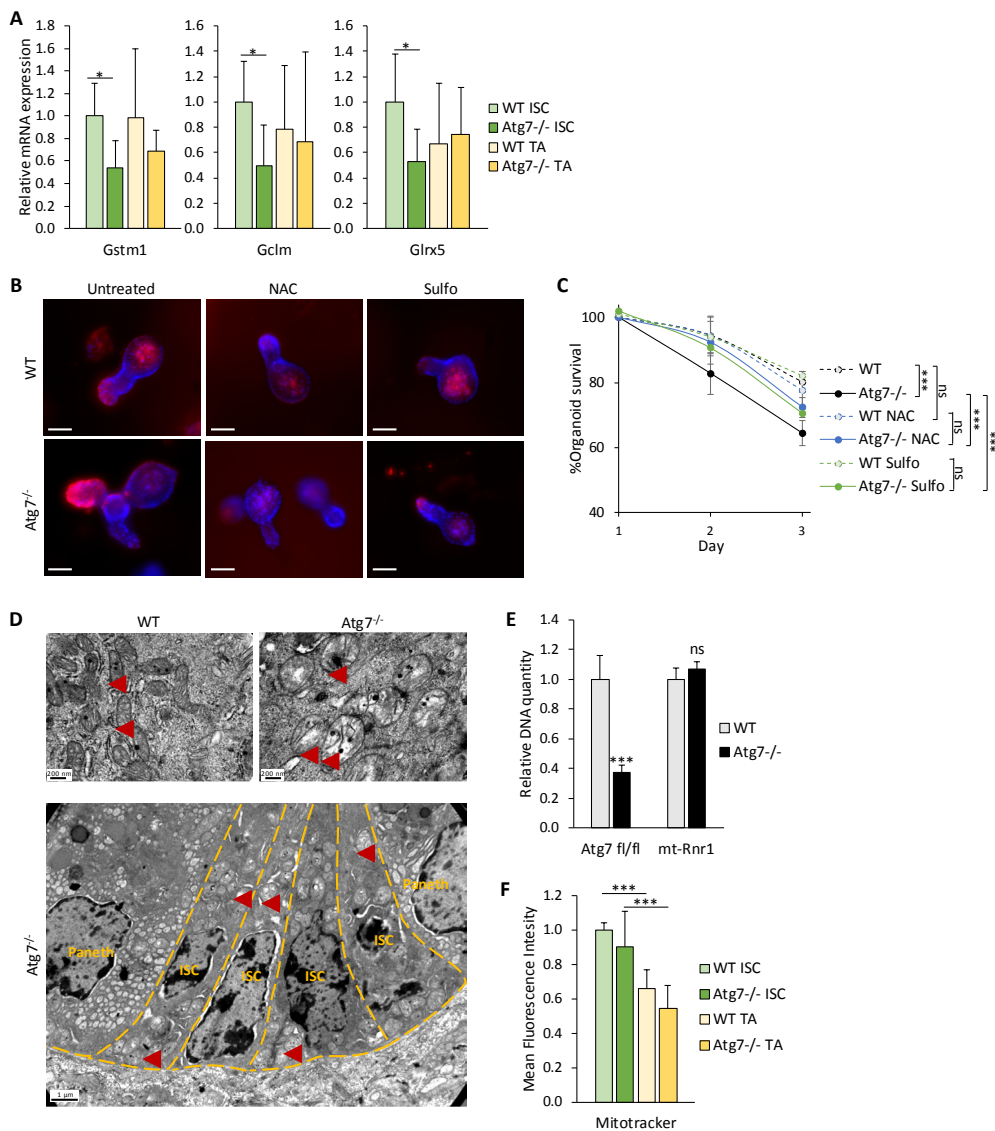
Supplementary Figure 2

- (A)** Relative mRNA expression levels of genes encoding antimicrobial peptides produced by Paneth cells assessed by RT-qPCR analysis of whole intestinal tissue lysates of 4 WT and 5 Atg7^{-/-} mice. Data are represented as mean ± s.d. Significant differences: * p<0.05, **p<0.01, ***p<0.005.
- (B)** Representative Lysozyme staining on tissue sections from WT and Atg7^{-/-} intestines. Hoechst is used as a nuclear counter-stain. Scale bars: 25 μm.



Supplementary Figure 3

- (A)** Left panel: Representative GFP and p62 co-staining in tissue sections of *Lgr5*^{WT} and *Lgr5*^{Atg7^{-/-}} mice. Hoechst is used as a nuclear counter-stain. Scale bars: 25 μ m. Right panel: representative sorting gates for GFP^{High} *Lgr5*⁺ISC and GFP^{Low} TA cell populations.
- (B)** Relative mRNA expression levels confirming higher expression of *Lgr5*⁺ISC markers in sorted *Lgr5*⁺ISC, higher expression of differentiation markers in assessed by RT-qPCR analysis of sorted *Lgr5*⁺ISC or TA progenitors from *Lgr5*^{WT} and *Lgr5*^{Atg7^{-/-}} mice. Data are represented as mean \pm s.d. (n= 6 mice per condition). Significant differences: * p<0.05, **p<0.01, ***p<0.005.
- (C)** Summary of significantly deregulated genes in sorted *Lgr5*⁺ISC or TA progenitors from *Lgr5*^{Atg7^{-/-}} versus *Lgr5*^{WT} mice.
- (D)** Top canonical pathways predicted by Ingenuity Pathway Analysis as significantly deregulated in the transcriptomic signature of sorted TA progenitors from *Lgr5*^{Atg7^{-/-}} compared to *Lgr5*^{WT} crypts.



Supplementary Figure 4

- (A)** Relative mRNA expression levels of NRF2 target genes encoding antioxidant response proteins assessed by RT-qPCR analysis of sorted Lgr5⁺ISC or TA progenitors from Lgr5^{WT} and Lgr5^{Atg7^{-/-}} mice. Data are represented as mean ± s.d. (n= 6 mice per condition).
- (B)** Representative CellroxB staining on live organoids from the crypts of WT and Atg7^{-/-} mice after 3 days in culture in the absence or presence of NAC or sulforaphane (Sulfo) in the culture medium. Hoechst is used as a nuclear counter-stain. Scale bars: 50 μm.
- (C)** Percent survival from day 1 of organoids from the crypts of WT and Atg7^{-/-} mice in the absence or presence of NAC or sulforaphane (Sulfo) in the culture medium. (n= 5 mice for each genotype). Significant differences are shown in the legend for day 3.
- (D)** Transmission electron microscopy images of intestinal epithelium sections from WT and Atg7^{-/-} mice showing mitochondria, indicated by red arrowheads. Bottom panel shows the bottom of an Atg7^{-/-} crypt, with ISC traced out in yellow and swollen mitochondria in their cytoplasm indicated by red arrowheads. Scale bars: 200nm for top panels, 1 μm for bottom panel.
- (E)** Relative DNA quantity assessed by qPCR analysis of whole intestinal tissue DNA extracts of 4 WT and 4 Atg7^{-/-} mice. Primers for the floxed allele of *Atg7* were used to assess deletion of the gene, and *mt-Rnr1* was used to assess mitochondrial DNA content. Both were normalized to nuclear *Rbm15* DNA content.
- (F)** Mean Mitosox fluorescence intensity of sorted Lgr5⁺ISC or TA progenitors from Lgr5^{WT} and Lgr5^{Atg7^{-/-}} mice. Data are represented as mean ± s.d. (n= 4 Lgr5^{WT} and 6 Lgr5^{Atg7^{-/-}} mice).

Significant differences: * p<0.05, **p<0.01, ***p<0.005, ns= not significant.

Supplementary table 1

Gene	F primer	R primer
18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
Ang4	CCCAGTTGGAGGAAAGCTG	CGTAGGAATTTTCGTACCTTTCA
Aslc2	CTACTCGTCGGAGGAAAG	ACTAGACAGCATGGGTAAG
Atg7 (mRNA)	CAGCAGTGATGACCGCATGA	CAAATGCCAGGCTGACAGGA
Atm	TGCAGATTTATATCCATCATCCAC	TTTCATGGATTCATAAGCACCTT
Axin2	GATTCCCCTTTGACCAGGTGG	CCATTACAAGCAAACCAGAAGT
Brca1	TTCACCAACATGCCCAAAG	AGTCCTTCACCACGGAAG
Cryptdin1	CCAGATCTCTCAACGATTCCTCTT	TTGGAGACCCAGAAGGCACTT
Defa20	CCAGGGGAAGAGGACCAG	TGCAGCGACGTTTTCTACA
Defa5	CAGGCTGATCCTATCCACAAA	GGCTCCAAAGGAGATAGACA
Dll1	GGTTTTCTGTTGCGAGGTCATC	CCCATCCGATTTCCCTTCG
Dll4	TTCCAGGCAACCTTCTCCGA	ACTGCCGCTATTCTTGTCCC
EGF	TCCTGGACAAAACGGCTCTTC	CCTGATAAGACGGACGGAGC
Gclm	TGACTCACAATGACCCGAAA	TCAATGTCAGGGATGCTTTCT
Glrx5	CGGGCAGGTGCTTTTACTT	AGTGGTTGTACGTTACCAGAG
Gsta3	GCCTACTTGAGGTACAGCACAAT	AAAGTAATGAAGGACTGGCTTCC
Gstm1	GCAGCTCATGCTCTGTTA	TTTCTCAGGGATGGTCTTCAA
Gstm3	TGAAGGCCATCCCTGAGAAA	CTTGGGAGGAAGCGGCTACT
Lgr5	CCTTGCCCTGAACAAAATA	ATTTCTTTCCAGGGAGTGG
Lyso	GGTCTACAATCGTTGTGAGTTGG	CTCCGCAGTTCCGAATATACT
Msi1	GATGCCTTCATGCTGGGTAT	TAGGTGTAACCAGGGGCAAG
Nqo1	AGCGTTCGGTATTACGATCC	AGTACAATCAGGGCTCTTCTCG
Olmf4	GCCACTTTCCAATTTTAC	GAGCCTCTTCTCATAAC
Rad51	AAAAACCCATTGGAGGGAAC	CCCCTTTCCTTTTCAGG
Reg4	TCCGGAAGCTAAGAAACTGG	TGGGATCCATTTCCATATGAC
Txn	TGAAGCTGATCGAGAGCAAG	AGAAGTCCACCACGACAAGC
Txnrd1	CCACATTCACACACGTTTCT	TTTTGTACACCCGACTCCTCT
Wnt11	CTGCATGAAGAATGAGAAGGTG	ACTGCCGTTGGAAGTCTTGT
Wnt3	CTCGCTGGCTACCCAATTT	GAGGCCAGAGATGTGTACTGC
Wnt6	GTGCAACTGCACAACAACG	GGAACGGAGGCAGCTTCT
Atg7 ^{fl/fl} (DNA)	GCTGTGGAGCTGATGGTCTC	GCAGGGTAAGACCGG TCAAG
Rbm15	GGACACTTTTCTTGGGCAAC	AGTTTGGCCCTGTGAGACAT
mt-Rnr1	AGGAGCCTGTTCTATAATCGATAAA	GATGGCGGTATATAGGCTGAA

DISCUSSION

Lgr5⁺ ISC are indispensable for the homeostatic maintenance of the intestinal epithelium and have been described as the cell-of-origin for intestinal cancer. It is therefore crucial to better understand the molecular mechanisms controlling the integrity and survival of these cells, and how they affect either self-renewal and regenerative expansion in homeostasis or proliferation and resistance to treatment in CRC.

1. AUTOPHAGY AS A PROTECTIVE STRESS RESPONSE IN INTESTINAL STEM CELLS

Our data indicate a key role for autophagy in the survival of ISC in response to stress. Indeed, the inhibition of autophagy throughout the intestinal epithelium upon *Atg7* deletion results in the p53-mediated apoptosis of ISC whereas the survival of other intestinal epithelial cells – including TA progenitor cells – remains unaffected. We further show that the apoptosis of autophagy-deficient ISC is a result of a defective response to both intrinsic and environmental stress.

1.1 AUTOPHAGY PROTECTS INTESTINAL STEM CELLS FROM OXIDATIVE STRESS

First, autophagy protects ISC against the accumulation of ROS by controlling antioxidant responses and mitochondrial homeostasis.

Contrary to our expectations and what has been found in other tissues and adult stem cells (Mortensen et al., 2011; Wang et al., 2013; García-Prat et al., 2016; Ho et al., 2017), the inhibition of autophagy did not affect mitochondrial load in the intestinal epithelium nor in isolated Lgr5⁺ISC. This could be due to several factors: (1) the short lifetime of intestinal epithelial cells, which may not allow time for mitochondrial accumulation, (2) the rapid division and consequent dilution of cytoplasmic components in proliferative crypt cells, or (3) the presence of compensatory mechanisms, such as a decreased synthesis of mitochondrial components, that may counteract the lack of mitochondrial degradation through autophagy.

Regardless of mitochondrial numbers, we found that mitochondria throughout the autophagy-deficient epithelium appeared swollen and irregular by transmission electron microscopy, including in ISC, suggesting that mitochondrial function may be affected by the loss of autophagy. Despite this epithelium-wide effect, mitochondrial ROS levels were only significantly increased in autophagy-deficient ISC, which likely contributes to the increased levels of cytoplasmic ROS in these cells. On the contrary, mitochondrial superoxide levels and cytoplasmic ROS levels were unaffected by the loss of *Atg7* in TA cells. This could be explained by the ISC-specific down regulation of antioxidant gene expression in the autophagy-deficient tissue. In fact, antioxidant treatment (NAC) or forced activation of antioxidant responses (sulforaphane) improves the survival of autophagy-deficient organoids and of ISC *in vivo*. To parallel these results, we plan to investigate the effects of inducing further ROS either in organoids or *in vivo* by treating with H₂O₂, Paraquat or Rotenone (which both mimic mitochondrial superoxide production), or ROS-inducing chemotherapeutic drugs like Doxorubicin.

Interestingly, despite higher mitochondrial ROS levels, cytoplasmic ROS are lower in TA cells than ISC, regardless of autophagy status. Several explanations can again be put forward to

explain this: (1) ROS levels may be regulated in an autophagy-independent manner in these differentiating progenitors, (2) TA cells may have a greater intrinsic capacity to neutralize ROS than ISC, or (3) the unique ISC program could render them more susceptible to variations in redox state, such as that induced by the inhibition of autophagy and consequent mitochondrial defects.

In support of the latter explanation, a similar repression of NRF2 in response to oxidative challenge and inflammation was described in *Drosophila* ISC, in contrast to the observed activation of NRF2 in differentiated cells (Hochmuth et al., 2011). In this report, NRF2 repression is shown to be required to stimulate ISC proliferation and regeneration after a challenge, whereas active NRF2 maintains low ROS levels and limits proliferation rates in resting ISC. A similar mechanism could be at play in mammalian ISC, where ROS levels have also been shown to drive proliferative responses (Jones et al., 2013; Leoni et al., 2013; Myant et al., 2013). The need to maintain high ROS levels to induce regenerative proliferation in the ISC compartment could therefore explain why ISC, unlike other cells of the epithelium, do not induce but rather repress antioxidant responses following the inhibition of autophagy. However, elevated ROS levels beyond a certain threshold result in oxidative stress and apoptosis (see **FIGURE 17D**). ISC may therefore rely on autophagy to maintain appropriate levels of ROS and to control the adverse effects of elevated ROS in response to pro-proliferative stimuli.

1.2 AUTOPHAGY PROTECTS INTESTINAL STEM CELLS FROM DNA DAMAGE

Second, autophagy protects ISC against DNA damages by regulating DNA repair. We show that the autophagy-deficient epithelium accumulates unrepaired DSBs specifically in the ISC compartment at basal levels. Furthermore, our data exposes significant differences in the response of autophagy-deficient ISC to IR, particularly in their DNA-repair capacity and susceptibility to apoptosis. Thus, autophagy is required to preserve the genomic integrity and maximize the survival of ISC following cytotoxic stress. A recently published study in *Drosophila melanogaster* showed increased DNA damage in ISC upon RNAi-mediated silencing of several individual *Atg* genes, in this case resulting in cell cycle arrest and consequent loss of ISC (Nagy et al., 2018). Thus, the protective role of autophagy against DNA damage in ISC may be conserved through evolution. Likewise, a recent study by Asano et al. showed more important loss of ISC in *Atg5*-depleted murine intestinal crypts compared to WT crypts upon IR, and showed that this could be partially rescued by pre-treating mice with the antioxidant NAC (Asano et al., 2017). Indeed, in our model, the increased levels of ROS in autophagy-deficient ISC may directly contribute to DNA damage as mice treated with NAC had considerably fewer γ H2AX-positive zones throughout their epithelium (*data not shown*). However, we additionally show that DNA repair mechanisms are also affected by the loss of autophagy in ISC, suggesting that autophagy not only helps prevent DNA damages in ISC, but also contributes to their repair.

The fact that ISC are particularly sensitive to the inhibition of autophagy compared to TA cells may be due to their greater dependence on specific DNA repair pathways. Several ISC signatures have identified genes involved in the DDR as significantly enriched in ISC compared to their direct TA progenitors – including genes encoding HR proteins RAD50, BRCA2, PALB2, CHK1, and

RAD51c, BER protein MSH2, and NER proteins DDB2 and XPC (Merlos-Suárez et al., 2011; Muñoz et al., 2012). The fact that ISC express higher levels of DNA repair factors suggests they may be better equipped to efficiently repair DNA damages. Indeed, a recent study demonstrated that following IR, DSBs (as indicated by γ H2AX foci) accumulate throughout the epithelium but are more rapidly resolved in ISC than in TA or differentiated cells (Hua et al., 2012). Further analysis of irradiation-induced foci formation by DDR factors involved in HR and NHEJ suggested that both ISC and TA progenitors use NHEJ effectively, but that ISC are more efficient in high-fidelity HR. Thus, ISC favor a more rapid and less error-prone DDR than their progenitors, which likely acts to preserve ISC integrity and survival. Supporting the importance of an efficient DSB repair in ISC, ATM-deficient mice were found to be more sensitive to IR and undergo gastrointestinal syndrome at lower doses (Barlow et al., 1996). Moreover, the loss of ATM favors the development and growth of adenomas in *Apc*^{+/ Δ} mice (Kwon et al., 2008). Similarly, the gastrointestinal tract of *Rad21* heterozygous mice is hypersensitive to IR compared to mice expressing both alleles (Xu et al., 2010), revealing the importance of gene dosage of DDR factors in the intestinal epithelial response to IR.

Consistent with published ISC signatures, we found higher expression of DNA repair genes in ISC compared to TA cells isolated from *Lgr5*WT mice in our transcriptomic analysis (*data not shown*), and RT-qPCR analysis on selected genes encoding DDR factors support these observation, reinforcing the idea that ISC have greater DNA repair capacities than their direct TA progenitors. Our data is also consistent with the observation by Hua et al. that WT ISC have a relatively high capacity for resolution of DNA damages following irradiation compared to their progenitors (Hua et al., 2012), although this no longer appears to be the case following the loss of autophagy.

Several of the down-regulated DNA repair factors in our *Atg7*-null ISC signature are associated with DSB repair and more specifically with HR, including *Rad51*, *Atm*, and *Brca1*. This is consistent with various *in vitro* reports highlighting a molecular link between the inhibition of autophagy and HR defects (Chen et al., 2015; Liu et al., 2015; Hewitt et al., 2016; Wang et al., 2016e). Overall, although the exact mechanisms remain to be elucidated, our data hints at a role for autophagy in promoting efficient DNA repair in ISC, perhaps by favoring high-fidelity HR. We hope to further elucidate this by analyzing the recruitment of different DNA repair factors to IR-induced DNA damages in both WT and *Atg7*-null ISC and by studying the contribution of individual DDR pathways in the response of both WT and autophagy-deficient organoids upon genotoxic stress.

Interestingly, while the inhibition of autophagy impairs ISC resistance to genotoxic injury, the opposite may also be true: Short-term fasting, known to induce autophagy, has been shown to protect the small intestinal epithelium from lethal DNA damage induced by etoposide treatment (Tinkum et al., 2015). Fasting improves both the survival and DNA repair capacity of ISC during etoposide treatment– as shown by the transcriptomic upregulation of several DNA repair genes and accelerated resolution of γ H2AX foci. In contrast to ISC, fasting had no protective effect on

TA cells. Although the contribution of autophagy to these observations was not investigated in this study, it would certainly be of great interest.

1.3 AUTOPHAGY PROTECTS INTESTINAL STEM CELLS FROM MICROENVIRONMENTAL STRESS

Third, autophagy protects ISC against extrinsic stress linked with the microbiota. Our findings reveal a complex interplay between the gut microbiota, autophagy and ISC maintenance.

Inhibition of autophagy in Paneth cells does not affect the expression of the Wnt ligands essential to ISC maintenance, nor does it perturb their role as the dedicated niche for organoid growth. However, our data and previous studies have shown that autophagy in the intestinal epithelium is essential to limit bacterial infection as it controls the secretion of AMPs from Paneth cells. Our group previously reported that autophagy deficiency in the intestinal epithelium affects epithelial contact with the gut microbiota and contributes to dysbiosis (Lévy et al., 2015). Our results expand these findings by showing that autophagy plays an additional protective role in bacteria-ISC crosstalks.

Several studies point to a role of the microbiota in both intestinal homeostasis and in epithelial response following damage and suggest that the gut microbiota could be an essential component of the stem cell niche. In germ free conditions, intestinal crypts are less deep, contain fewer proliferative ISC and both their transcriptomic and DNA methylation profile is altered (Sommer and Bäckhed, 2013; Sommer et al., 2015; Yu et al., 2015). Germ-free conditions or antibiotics treatment also dampen radiation-induced apoptosis and favor crypt survival. In fact, these animals survive longer after lethal doses of radiation and the minimum dose to induce a gastrointestinal syndrome is higher than in conventionally raised animals (McLaughlin et al., 1964; Matsuzawat, 1965; Onoue et al., 1981). Moreover, it is becoming increasingly evident that not only the presence but also the diversity and composition of the microbiota are important for these effects on crypt homeostasis (Preidis et al., 2012; Jones et al., 2013, 2015).

Our results show that treating autophagy-deficient mice with wide-spectrum antibiotics significantly reduces the number of apoptotic Lgr5⁺ISC following the inhibition of autophagy. In addition to improving ISC survival, antibiotic treatment also reduced signs of DNA damage and oxidative stress in the autophagy-deficient epithelium, suggesting that the microbiota may act on Atg7^{-/-} ISC by driving ROS accumulation and DNA damages. In line with this, commensal bacteria have been shown to drive NOX1-mediated ROS production and consequent proliferation in both *Drosophila* and mammalian ISC (Jones et al., 2013).

Interestingly, we found that the inhibition of autophagy affects the survival of organoids only if Atg7 deletion occurred *in vivo* prior to culture. This suggests that the microenvironmental changes in autophagy-deficient mice induce lasting effects in Lgr5⁺ISC, contributing to their death. The mechanisms of such a microenvironmental imprint on isolated crypts cultured *ex vivo* poses intriguing new questions. One possible mechanism is the establishment of microenvironment-induced epigenetic adaptations following the loss of autophagy *in vivo* that are maintained *ex vivo*. This idea is supported by recent data showing that the microbiota can affect Lgr5⁺ISC homeostasis through epigenetic regulation (Yu et al., 2015).

The mechanisms through which the intestinal microbiota affects ISC homeostasis are still unclear. The microbiota may affect crypt cells indirectly, for example by activating inflammatory pathways in epithelial or mesenchymal cells of the intestinal mucosa that will in turn signal to ISC (Gong et al., 2016a; Saha et al., 2016). The microbiota may also be directly sensed by ISC either through MAMPs or microbiota-derived metabolites. TLRs and NLRs, which sense MAMPs, were shown to be expressed by ISC and to affect their proliferation and survival (Neal et al., 2012; Nigro et al., 2014), although the downstream effectors of this remain unclear. We tested the effects of several individual bacterial MAMPs on WT and ex-vivo autophagy-depleted organoids. LPS was previously shown to induce apoptosis of ISC both *in vivo* and in organoids via TLR4 (Neal et al., 2012). These effects were found to be independent of MyD88 signaling and TNF α , but dependent on activation of the p53-upregulated modulator of apoptosis (PUMA). We show that LPS similarly affects survival of WT and autophagy-deficient organoids, suggesting that its effects are independent of autophagy. Likewise, we found that the TLR5 agonist Flagellin, the TLR2 agonist LTA, and the TLR3 agonist PolyIC had similar effects on both WT and autophagy-deficient organoids. Of the tested MAMPs, only MDP showed a differential effect on organoid survival. Interestingly, MDP has been shown to exert cytoprotective effects on Lgr5⁺ISC mediated by NOD2 signaling (Nigro et al., 2014). However, the mechanism remained unclear since more than 30 cellular proteins interact with NOD2 directly and regulate its functional activity. Among these, NOD2 has been shown to direct autophagy by recruiting components of the autophagy machinery (Cooney et al., 2010; Travassos et al., 2010; Chauhan et al., 2015). Consistent with the reported cytoprotective effect of MDP in ISC, treatment of WT organoids with MDP improved their survival. On the contrary, MDP treatment reduced the survival of autophagy-deficient organoids, suggesting that autophagy is required for the cytoprotective effects of MDP. Thus, autophagy plays a key role in ISC protection in response to specific microbial signals.

Still, we cannot exclude an effect of other microenvironmental signals driven by the presence of the microbiota, such as metabolites or inflammatory signals, on the survival of autophagy-deficient ISC. In fact, taking into account recent studies showing that the loss of autophagy increases the sensitivity of intestinal epithelial cells to pro-inflammatory cytokines (Burger et al., 2018; Pott et al., 2018), it is possible that microbiota-induced production of these cytokines also contribute to the death of Lgr5⁺ ISC. This could be easily verified using organoids.

2. OTHER MODELS OF ATG DEPLETION IN THE INTESTINAL EPITHELIUM

A recent study showed a similar role for autophagy in ISC maintenance using a mouse model of *Atg5* deletion in the intestinal epithelium (VillinCreAtg5^{fl/fl}) (Asano et al., 2017). However, in contrast to our findings, this study does not describe apoptosis in the ISC compartment of the *Atg5*-deficient epithelium but rather indicates reduced numbers of ISC. Moreover, they describe these phenotypes to be dependent solely on cell-intrinsic mechanisms, specifically on the accumulation of ROS in autophagy-deficient ISC, whereas we show an important effect of microenvironmental alterations on ISC death. A possible explanation for these discrepancies may be the distinct genetic models used to inhibit autophagy: In our study, ablation of *Atg7* is

induced in adult mice after tamoxifen injection (using a VillinCre^{ERT2} transgene) whereas Asano et al. used a model where the deletion of *Atg5* is constitutive (using the VillinCre transgene). Villin expression begins early in embryonic development whereas mature intestinal crypts develop post-natally (Maunoury et al., 1988; Spence et al., 2011). It is therefore possible that autophagy plays an additional role in the regulation of ISC numbers during development. Likewise, nearly all previous studies on the effects of autophagy in the intestinal epithelium – which focused primarily on Paneth cells, goblet cells and inflammation – have relied on germline loss of *Atg* genes. These studies either did not investigate an effect of autophagy in ISCs or did not observe any differences in the ISC compartment. Along with the possible differential effects of germline versus inducible loss of autophagy genes, other possible explanations for the differences between our study and previous studies include:

- Unique functions of *Atg7*, although several of the aforementioned studies also used loss of *Atg7* as a model.
- As we found that the presence of the microbiota affects the death of autophagy-deficient ISC, differences in housing facilities may also play a part.
- Similarly, as the effect of autophagy inhibition is predominantly evident in response to stress, basal conditions may not have been sufficient to observe an effect on ISC.
- In the case of *Atg16l1*^{HM} mice, the partial loss ATG16L1 may allow low but sufficient levels of autophagy to promote ISC survival.

Overall, whereas germline autophagy-deficient models may be more appropriate to study the development of IBD in patients with autophagy-associated risk alleles, the inducible ablation of autophagy in adult mice is a more appropriate approach to study the effects of therapeutic autophagy inhibition, as in cancer treatment.

3. REPLENISHMENT OF THE AUTOPHAGY-DEFICIENT INTESTINAL STEM CELL POOL

Fortunately, despite injury to ISC induced by the loss of autophagy, the pool of Lgr5⁺ISC is continuously replenished and intestinal homeostasis is maintained over time. This reflects, as previously reported with acute damages to Lgr5⁺ISC, a high degree of plasticity within small intestinal crypts where either remaining Lgr5⁺ISC, reserve pools of ISC and/or committed progenitors are able to ensure epithelial intestinal homeostasis. Indeed, we observe increased proliferation both in the ISC and TA compartments of the *Atg7*-deficient crypts, without any apparent changes in villus height or migration speed along the crypt-villus axis (*data not shown*). This suggests that either remaining ISC or early progenitors, which we found to be more resistant to the inhibition of autophagy, could contribute to the replenishment of the Lgr5⁺ISC pool. Moreover, although we did not observe any differences in the mRNA expression levels of +4SC markers *Bmi1*, *HopX*, or *Lrig1* (*data not shown*), we cannot exclude the possibility that the loss of autophagy-deficient Lgr5⁺ISC results in the activation of these reserve stem cells to replenish the active stem cell pool. Interestingly, we were able to observe crypt fission events in the epithelium of *Atg7*^{-/-} mice, further supporting a homeostatic regenerative response to the loss of autophagy-deficient ISC. All in all, the fact that inhibiting autophagy is not ultimately

deleterious to the maintenance of the intestinal epithelium suggests that it could safely be considered in therapy.

4. AUTOPHAGY & P53 IN INTESTINAL STEM CELL MAINTENANCE AND TUMOR INITIATION

We show that the loss of *Atg7* results in the p53-dependent apoptosis of ISC. Both ROS and DNA damages have been linked to p53 activation. P53 plays an important role in the DNA damage response, and is typically activated downstream of DDR factors to induce cell cycle arrest and allow damage to be repaired before progression to M phase. Persisting damage and p53 activation, however, can also result in apoptosis to eliminate irreparably damaged cells. ROS can indirectly activate p53 by inducing DNA damages but also by acting on other signaling pathways, including p38 MAPK, which we found to be activated in autophagy-deficient crypts (*data not shown*). Lastly, p53 can also be directly oxidized on its cysteine residues, which enhances its DNA-binding and transcriptional activity. Thus, both oxidative stress and DNA damages in autophagy-deficient ISC can induce p53-mediated cell death as a way to eliminate damaged ISC and preserve the genomic integrity of the ISC pool.

Importantly, we show that the p53-mediated elimination of damaged ISC is essential to preserve the integrity of the *Atg7*-deficient epithelium, as the combined ablation of *Atg7* and *Tp53* dramatically increases DNA damage throughout the epithelium and triggers the development of adenomas. Although loss of p53 alone does not result in the formation of adenomas until at least 14 months (Schwitalla et al., 2013a; Chanrion et al., 2014) and *Atg7* deletion alone does not favor tumor development, the combined loss of these two factors results in the formation of several tumors with a high penetrance within 12 months. We are currently carrying out experiments to see if tumors can be detected in the double-knockout epithelium at even earlier time points. The activation of p53 therefore appears to counteract the adverse effects of autophagy inhibition by eliminating damaged and potential tumor-initiating ISC. Looking at things from a different perspective, the loss of autophagy could act as the pro-mutagenic factor required to initiate tumorigenesis in the p53-deficient intestinal epithelium. Furthermore, our group previously showed that the deletion of *Atg7* prevented tumor initiation in *Apc^{+/-}* mice (Lévy et al., 2015) and our more recent data indicates that this is not the case in the absence of p53 (*data not shown*); hence the effect of autophagy inhibition on tumor initiation appears to be dependent on the p53 status of the epithelium. Further analysis is required to determine the combined effect of *Atg7* and *Tp53* deletion on the proliferation and survival of tumor cells.

Our group previously showed that the effects of autophagy on tumor initiation were in part dependent on microenvironmental alterations, including changes in the proximity and composition of the gut microbiota as well as the induction of an anti-tumoral immune response (Lévy et al., 2015). Our present study identifies an additional mechanism through which the inhibition of autophagy blocks tumor initiation: by enhancing the sensitivity of damaged, potential tumor-initiating ISC to p53-mediated apoptosis. We hypothesize that these two mechanisms may in fact be linked. The presence of the microbiota is required for the death ISC, the induction of an anti-tumoral immune response, as well as the inhibition tumor initiation in *Atg7^{-/-}* mice. One possibility is that the microbiota-dependent death of autophagy-deficient ISC,

in addition to blocking the accumulation of damages in the stem cell compartment, helps prime the anti-tumoral response described in our previous study. Another possibility is that the cytotoxic immune response induced by alterations of the microbiota contributes to the death of ISC. It would therefore be interesting to characterize the immune response in *Atg7*^{-/-}*p53*^{-/-} mice and analyze the effects of immune signaling on the survival of autophagy-deficient ISC to test our hypothesis.

5. COLON STEM CELLS & AUTOPHAGY

Although mouse models of intestinal tumorigenesis develop tumors throughout the intestinal tract, human cancer typically develops more distally, in the colon and rectum. At basal levels, the inhibition of autophagy does not appear to induce the death of ISC in the colon. This is consistent with the established notion that colonic crypts are more resistant to stress and p53-induced apoptosis than small intestinal crypts (Merritt et al., 1994). The higher resistance of colonic stem cells has been attributed both to higher expression of the anti-apoptotic protein Bcl-2 (Merritt AJ et al., 1995) and more efficient DNA repair (Hua et al., 2017). Nevertheless, the effects of autophagy on tumorigenesis are also true in the colon. As our group previously showed, the loss of *Atg7* prevents the onset and growth of tumors in both the small intestine and colon of *Apc*^{+/ Δ} mice (Lévy et al., 2015). Moreover, the simultaneous deletion of both *Atg7* and *Tp53* induced the development of colonic polyps within a year. Several studies have described an increased sensitivity of the autophagy-deficient colonic epithelium to inflammation (Cadwell et al., 2010; Cabrera et al., 2013; Liu et al., 2013a; Tsuboi et al., 2015) and we confirmed this in our model as well (*data not shown*). Whether autophagy also affects DNA repair, oxidative stress, or interactions with the microbiota in colonic ISC remains to be determined but would be of great interest in the context of its role in tumorigenesis.

6. AUTOPHAGY & TUMOR PROGRESSION

Lgr5⁺ISC are thought to drive not only the homeostatic renewal of the epithelium (Barker et al., 2007; Barker, 2014) and tumor initiation (Barker et al., 2009) but also the continued growth of intestinal tumors (Schepers et al., 2012). In light of the important role of autophagy in ISC homeostasis and in tumor initiation, its role in intestinal tumor progression should not be disregarded. Autophagy has emerged as an essential driver of tumor growth in several tissues, including the lung (Guo et al., 2013; Strohecker et al., 2013; Rao et al., 2014), pancreas (Rosenfeldt et al., 2013; Yang et al., 2014), and, recently, in the intestine (Lévy et al., 2015; Sakitani et al., 2015). Our group previously showed an activation of autophagy at various stages of human CRC (Lévy et al., 2015). In addition, the deletion of *Atg7* coupled to the monoallelic loss of *Apc* in intestinal epithelial cells not only inhibited tumor initiation but also led to metabolic stress, AMPK activation and p53-mediated cell cycle arrest in the few developing tumors. These results indicate that autophagy does play an important role in intestinal tumor growth. However, in this model, autophagy is lost prior to the development of tumors, which may not necessarily reflect the effects autophagy inhibition as a therapeutic strategy in established tumors.

To study the effect of autophagy in tumor progression independently of its effects on tumor initiation, we are currently using a model established in our lab carrying a germline mutation on one allele of the *Apc* gene (*Apc*^{+/ Δ 14}), resulting in the spontaneous development of tumors throughout the intestinal tract (Colnot et al., 2004). We crossed these mice with VillinCre^{ERT2}*Atg7*^{fl/fl} mice to allow the tamoxifen-inducible deletion of *Atg7* in already-established tumors. In preliminary experiments, we treated these mice with tamoxifen at 3 months of age – when tumors are already present – and sacrificed them at 4.5 months (tumor load generally becomes lethal between 4.5 and 5 months of age in these mice). Contrary to our expectations, we found that the inhibition of autophagy in *Apc*^{+/ Δ 14} mice at this timing did not affect tumor load. In a model of colitis-associated tumorigenesis, Sakitani et al. similarly found that either the deletion of *Atg5* or CQ treatment did not significantly affect tumor load. It did, however, impair tumor growth due to stress-induced p53-mediated apoptosis (Sakitani et al., 2015). Further analysis is still required to determine the effects of *Atg7* deletion in tumor cells in our model.

Autophagy has also been shown to contribute to tumor cell resistance to therapy in various cell types, including human CRC cells. Notably, induction of autophagy was reported upon 5-FU treatment in human CRC cell lines, and the inhibition of autophagy potentiated the 5-FU-induced apoptosis of colon cancer cells *in vitro* in xenografts (Li et al., 2009b, 2010). Further studies confirmed the beneficial anti-tumoral effects of autophagy inhibition in combination with chemotherapy both *in vitro* and using xenografts (Sasaki et al., 2010, 2012; Choi et al., 2012; Paillas et al., 2012; Selvakumaran et al., 2013; Schonewolf et al., 2014; Tan et al., 2015). Just as the inhibition of autophagy affects the DNA repair capacities of ISC, it may therefore also sensitize tumor cells to DNA damage induced by chemotherapy. We are currently testing this hypothesis *in vivo* by combining the deletion of *Atg7* with 5-FU treatment, both starting at 3 months of age, in *Apc*^{+/ Δ 14} mice. Our preliminary data shows that whereas *Atg7* deletion alone or 5-FU treatment alone do not significantly affect tumor load in this context, the combination of the two reduced the number of tumors. We are currently looking into the effects of autophagy inhibition, 5-FU treatment, and the combination of the two on the proliferation, accumulation of DNA damages and survival of tumor cells in these mice. Although our preliminary results merit further investigation, they indicate that the inhibition of autophagy could enhance the anti-tumoral effects of chemotherapy in established tumors by reducing their DNA repair and proliferative capacity. These results could provide *in vivo* support for a beneficial effect of combining classical chemotherapy with autophagy inhibition as treatment for colorectal cancer. Two phase 1/2 clinical trials are currently under way to test the combination of HCQ with chemotherapy to treat advanced CRC, along with two additional trials evaluating the combination of HCQ and the histone deacetylase inhibitor vorinostat (from www.clinicaltrials.gov).

These results additionally suggest that autophagy mediates resistance to stress not only in ISC but also in intestinal tumor cells. ISC markers are expressed in CRC stem cells (Merlos-Suárez et al., 2011) and the organization of intestinal tumors mimics that of the homeostatic epithelium with stem-like cells driving tumor growth (Schepers et al., 2012). Therefore, just as autophagy

inhibition affects ISC specifically in the homeostatic crypt, it may specifically render stem-like cells within the tumor mass more sensitive to cytotoxic treatment. This will also be investigated.

Whether the dual effect of autophagy in tumor initiation based on p53 status also applies in the case of tumor growth and progression remains to be elucidated. Many of the *in vitro* studies combining autophagy inhibition with chemotherapy used p53-mutated CRC cell lines (including Caco2, HT29, DLD1 cell lines), suggesting that autophagy inhibition could still be of therapeutic value in p53-mutant CRC. Nevertheless, as p53 mutations are common in CRC, it would be important to consider the possibility of a differential effect of autophagy inhibition on tumor progression based on p53 status before using autophagy inhibitors in therapy.

Overall, we establish a role for autophagy in various aspects of ISC homeostasis: repair of DNA damage, ROS regulation, and maintenance in response to specific microbial signals. Upon loss of autophagy, the tumor suppressor p53 and apoptosis act as an additional layer of control to eliminate damaged cells from the functional ISC pool and thereby prevent tumor development. Autophagy can therefore be considered as a gatekeeper of ISC integrity and survival. Importantly, the inhibition of autophagy in a p53-proficient context is not detrimental to intestinal homeostasis whereas it could be a promising route to block intestinal tumor growth.

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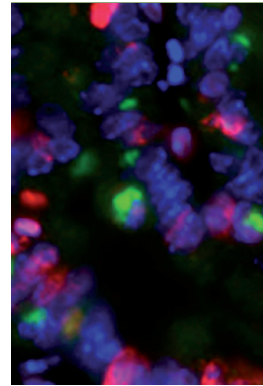
APPENDIX

► L'épithélium intestinal est la plus grande surface du corps exposée à l'environnement. L'homéostasie intestinale repose essentiellement sur l'intégrité des cellules épithéliales, processus complexe impliquant un équilibre avec la flore intestinale, le système immunitaire et les dépenses énergétiques liées au métabolisme. L'autophagie est au centre de ces fonctions et permet à l'épithélium de s'adapter à son environnement et aux différentes situations de stress en participant aux défenses antibactériennes, en contrôlant la composition de la flore intestinale, la réponse immunitaire et en participant à l'homéostasie énergétique. Une altération de ce mécanisme de protection est observée dans les maladies inflammatoires chroniques de l'intestin et le cancer colorectal. ◀

L'épithélium intestinal dont la fonction principale est d'assurer la digestion et l'absorption des nutriments doit se préserver contre les diverses menaces infectieuses et non infectieuses liées à l'ingestion. Différents stratagèmes lui permettent de se protéger et d'assurer son homéostasie. Le processus, incessant, d'autorenouvellement de son intégrité permet d'éviter l'accumulation de stress et de mutations [1]. L'épithélium assure également une fonction de barrière physique et chimique essentielle pour lutter contre les agressions de l'environnement. Les cellules épithéliales sécrétrices jouent un rôle fondamental dans cette fonction de protection grâce à la couche de mucus, fabriquée par les cellules caliciformes (*goblet cells*, en anglais), et aux molécules antimicrobiennes synthétisées notamment par les cellules de Paneth dans l'intestin grêle. De nombreuses cellules immunitaires résidentes de la muqueuse intestinale sondent les différents antigènes auxquels l'organisme est exposé et dirigent une réponse immunitaire adaptée : l'organisme tolère en effet les antigènes présentés par les microbes commensaux ; une réponse inflammatoire locale est en revanche induite contre les pathogènes afin d'empêcher une éventuelle

L'autophagie, l'homéostasie intestinale et ses pathologies

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infection systémique. Le maintien de cette homéostasie intestinale est assuré par un grand nombre de mécanismes cellulaires parmi lesquels la participation de l'autophagie apparaît de plus en plus évidente. Dans cette revue, nous avons choisi de détailler les fonctions essentielles de ce processus dans l'homéostasie intestinale, en particulier ses rôles spécifiques au sein des cellules épithéliales, afin de mieux comprendre son implication dans les pathologies inflammatoires et tumorales.

L'autophagie, un senseur de perturbations environnementales

Au-delà des fonctions connues de l'autophagie basale qui permettent la dégradation des agrégats protéiques et des organites altérés, plusieurs études réalisées au cours de cette dernière décennie ont mis en évidence des rôles qui s'avèrent spécifiques des types cellulaires. Stimulée en condition de stress, l'autophagie joue un rôle fondamental dans la cellule épithéliale intestinale. Elle participe à la défense antimicrobienne et à la composition de la flore. Elle permet également le maintien du métabolisme, en cas de rupture d'apport énergétique.

L'autophagie, un mécanisme de défense contre l'infection bactérienne

Régulation de la fonction sécrétrice des cellules épithéliales impliquées dans la défense antimicrobienne

L'épithélium constitue une barrière physique et chimique essentielle permettant de se défendre contre les agressions potentielles de l'environnement. La couche de mucus représente une barrière physique. Son épaisseur varie le long du tube digestif. Elle est maximale dans l'iléon

et le côlon où elle permet la rétention de molécules antimicrobiennes, comme les défensines et les IgA sécrétées, limitant ainsi la présence de bactéries au contact immédiat de l'épithélium. Les cellules de Paneth, situées à la base des cryptes intestinales au niveau de l'intestin grêle contribuent à la production de telles défensines. Elles contiennent des granules de sécrétion renfermant des peptides antimicrobiens et des enzymes comme le lysosome, les α -défensines (cryptdines), l'angio-génine-4 et RegIII γ (*regenerating islet-derived protein 3 gamma*). Bien que certains peptides antimicrobiens soient libérés de façon constitutive, la détection par les cellules de signaux d'origine bactérienne stimule également leur sécrétion. Les cellules de Paneth permettent ainsi de contrôler et limiter la colonisation de l'épithélium par la flore commensale et le protègent des pathogènes [2]. Plusieurs études ont révélé que les acteurs moléculaires de l'autophagie étaient impliqués dans les fonctions sécrétrices des cellules caliciformes et des cellules de Paneth (Figure 1). En effet, l'inactivation de gènes d'autophagie comme *Atg5* (*autophagy-related gene 5*), *Atg7*, *Atg16L1*, *Atg4b* ou l'expression d'un allèle hypomorphe¹ d'*Atg16L1* (*Atg16L1^{HM}*), chez la souris, conduit à une désorganisation des granules de sécrétion des cellules de Paneth et à un sévère défaut d'exocytose des peptides antimicrobiens [3]. Une expression de TNF α (*tumor necrosis factor alpha*), de leptine, d'adiponectine et une activation de la signalisation PPAR (*peroxisome proliferator-activated receptor*) sont de plus retrouvées dans les cellules de Paneth isolées des souris exprimant l'allèle *Atg16L1^{HM}* (souris *ATG16L1^{HM}*) [4]. Toutefois, il est important de noter que les défauts observés dans les cellules de Paneth déficientes en autophagie semblent dépendre de plusieurs facteurs. En particulier, les souris *ATG16L1^{HM}* élevées en animalerie exempte d'organismes pathogènes spécifiques, présentent des cellules de Paneth avec une morphologie normale. Leurs défauts de sécrétion peuvent cependant être rétablis par une infection virale chronique, par une souche de norovirus (MNVCR6, *murine norovirus strain CR6*) [5]. Contrairement aux cellules de Paneth, les granules de sécrétion des cellules caliciformes ne présentent pas de différences morphologiques majeures lorsque l'autophagie est inhibée. Ces cellules accumulent cependant leurs granules de mucines et présentent des défauts de sécrétion (Figure 1). De telles altérations ont été observées dans des modèles de souris invalidées pour différents gènes *Atg* (*Atg5*, *Atg16L1*, *Atg7*), soulignant ainsi l'implication de la machinerie autophagique dans le processus de sécrétion via une interaction directe avec les endosomes [6-9]. Ces différentes études mettent donc en évidence l'importance de l'autophagie dans les fonctions de la barrière épithéliale intestinale. Elles montrent aussi le rôle fondamental qu'exerce l'exocytose des peptides antimicrobiens et des mucines dans le maintien de l'homéostasie intestinale. Elles permettent d'envisager les mécanismes à l'origine des pathologies qui affectent les patients atteints de la maladie de Crohn qui présentent des polymorphismes touchant les gènes de l'autophagie.

¹ Un allèle est hypomorphe lorsque le produit du gène a la même fonction que le gène sauvage, mais qu'il est moins exprimé.

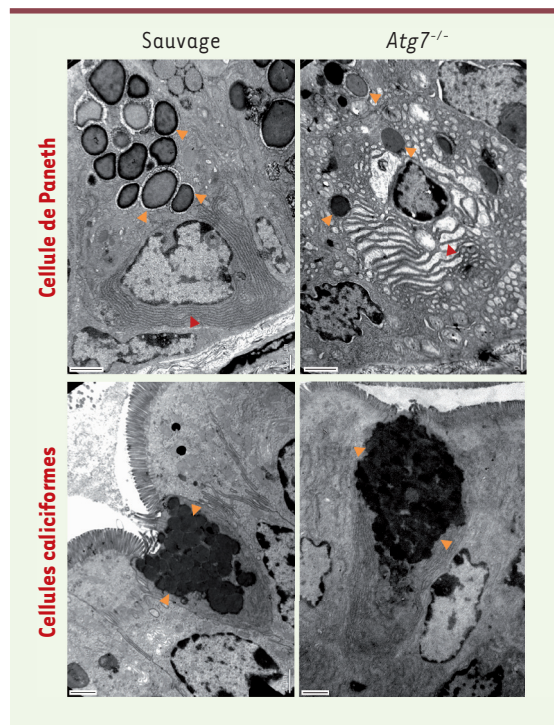


Figure 1. Effets de la perte de gènes de l'autophagie dans la fonction des cellules épithéliales sécrétrices. La sécrétion d'une couche de mucus par les cellules caliciformes et de molécules antimicrobiennes par les cellules de Paneth est essentielle pour le maintien de l'homéostasie intestinale. Ces images de microscopie électronique en transmission montrent les défauts liés à la sécrétion dans ces deux lignages épithéliaux après invalidation chez la souris du gène *Atg7*. On note en particulier une diminution du nombre de granules de sécrétion matures et un stress du réticulum endoplasmique dans les cellules de Paneth, ainsi que l'accumulation de granules non sécrétés dans les cellules caliciformes. Des phénotypes semblables sont retrouvés dans les modèles murins invalidés pour d'autres gènes de l'autophagie (*Atg16L1*, *Atg5*, *Atg4b*). Échelle : 1 μ m. Les granules de sécrétion (cellules de Paneth) et les vésicules contenant le mucus (cellules caliciformes) sont indiqués par des flèches jaunes (►). Le réticulum endoplasmique des cellules de Paneth est indiqué par des flèches rouges (►).

Autophagie, stress du réticulum endoplasmique et cellules de Paneth

Au-delà des défauts de sécrétion des cellules de Paneth, l'inhibition de l'autophagie dans ces cellules conduit à un stress du réticulum endoplasmique responsable de l'accumulation de protéines mal repliées non matures. La signalisation UPR (*unfolded protein response*) est alors activée afin de réduire la synthèse globale des protéines, d'induire celle des protéines

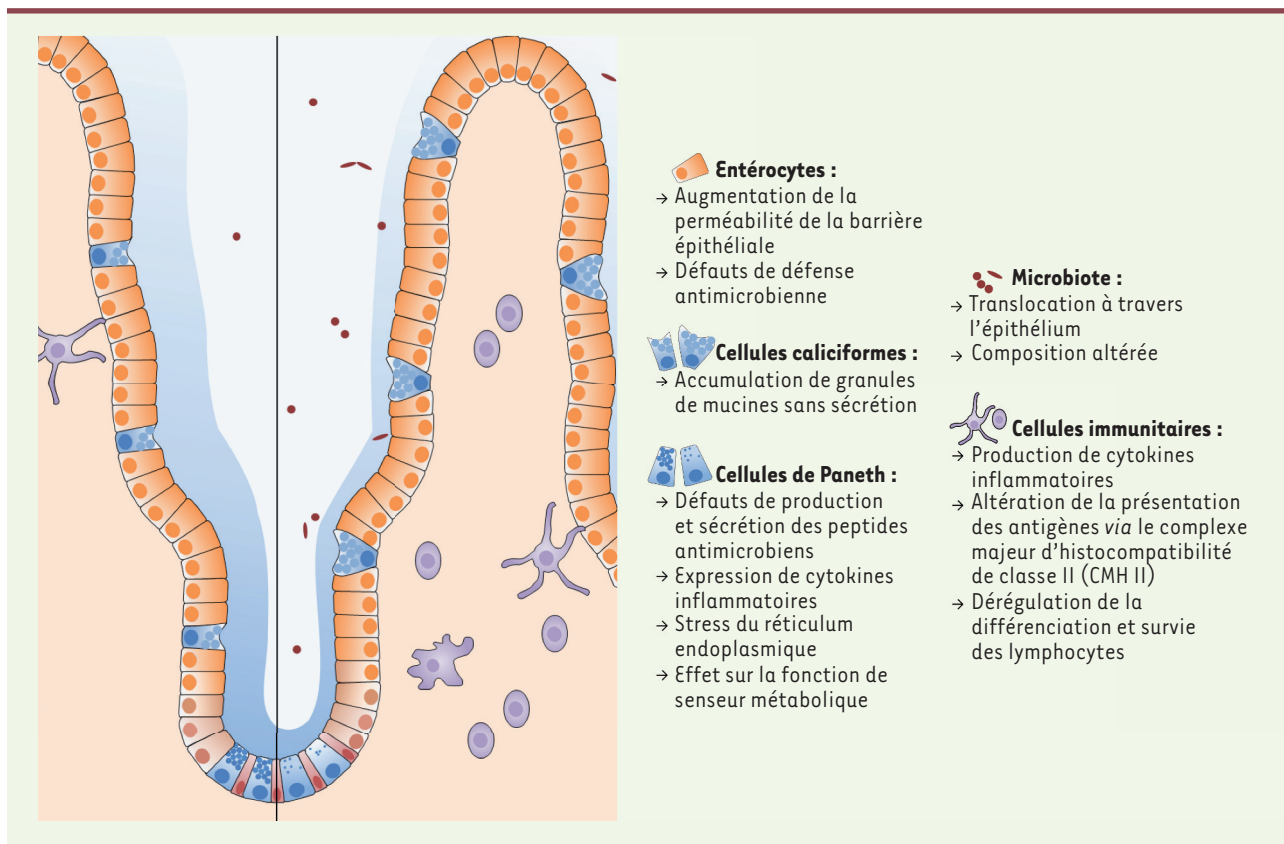


Figure 2. Conséquences de l'inhibition de l'autophagie dans l'épithélium intestinal. Un blocage de l'autophagie entraîne de nombreuses perturbations de l'homéostasie intestinale. Dans l'épithélium, on retrouve notamment des défauts dans les cellules caliciformes et les cellules de Paneth conduisant à une altération de la couche de mucus, un défaut de sécrétion de peptides antimicrobiens et une augmentation de la perméabilité de la barrière épithéliale. Associées à ces anomalies, on observe une translocation bactérienne à travers l'épithélium et une altération de la composition de la flore. Des défauts d'autophagie affectent également la fonction des cellules immunitaires avec plusieurs conséquences pro-inflammatoires.

chaperonnes impliquées dans le repliement des autres protéines, et d'augmenter l'activité protéosomale. Toutefois, si ce processus adaptatif échoue, un mécanisme d'apoptose est alors déclenché. L'implication de l'autophagie dans la réponse au stress du réticulum a été démontrée chez les souris déficientes pour le gène *XBPI* (*X-Box binding protein 1*) spécifiquement dans les cellules de Paneth, qui deviennent incapables d'activer la réponse UPR [10]. La délétion du gène *XBPI* conduit à une activation du processus autophagique et à des anomalies des cellules de Paneth à l'origine d'une iléite (inflammation de l'iléon). L'inactivation additionnelle d'un gène de l'autophagie (*Atg7* ou *Atg16L1*), chez les souris *XBPI*^{-/-}, conduit à une inflammation très sévère. Dans les cellules de Paneth, l'autophagie agit donc conjointement avec la réponse UPR, pour réguler l'homéostasie intestinale.

Implication de l'autophagie dans la composition de la flore intestinale et le contrôle de l'inflammation

La dérégulation de l'autophagie dans l'épithélium intestinal, à l'origine de la perte de capacité de production des défenses antimicrobiennes innées, conduit à des modifications de la flore intestinale.

Deux études indépendantes, dont la nôtre, ont en effet récemment démontré que, chez la souris, l'inactivation d'*Atg7* dans l'épithélium intestinal résultait en une altération de la composition de la flore intestinale (dysbiose), avec une plus forte prévalence du phylum *Firmicutes* et particulièrement de la classe des Clostridiales [9, 11], et un recrutement de différentes populations immunitaires [11]. Elle confère également une hyper-sensibilité à la colite induite par le dextran sulfate de sodium (DSS)² [9] qui est consécutive à la modification de la flore intestinale. Chez l'homme, l'iléon inflammé de patients atteints de la maladie de Crohn et porteurs du polymorphisme T300A du gène *Atg16L1*, présente également une dysbiose qui est marquée par une augmentation des *Enterobacteriaceae*, des *Bacteroidaceae* et des *Fusobacteriaceae* [12].

² L'utilisation du DSS représente un modèle de colite induite chimiquement communément utilisé.



La xénophagie

L'effet protecteur de l'autophagie vis-à-vis du microbiote intestinal est également renforcé par sa capacité à dégrader les bactéries présentes dans les cellules épithéliales intestinales et les macrophages. Ce processus sélectif de l'autophagie, appelé xénophagie, permet de cibler des bactéries intracellulaires, localisées dans le cytosol ou dans des vacuoles, et de limiter leur croissance. Dans la majorité des cas, des vésicules séquestrent les bactéries qui sont ensuite éliminées lors de la fusion avec les lysosomes. La dégradation des pathogènes permet ainsi de délivrer les antigènes microbiens aux molécules du complexe majeur d'histocompatibilité (CMH) de classe I et de classe II portées par les cellules présentatrices d'antigènes. L'autophagie participe ainsi directement à l'activation et au développement de la réponse lymphocytaire (lymphocytes de type Th[helper]1, Th2, Th17 et lymphocytes T cytotoxiques) permettant l'élimination des micro-organismes pathogènes. Une induction rapide de l'autophagie dans l'épithélium se produit lors d'infections de souris axéniques³ ou « conventionnelles » par des bactéries invasives pathogènes comme *Salmonella* Tiphimurium, *Shigella flexneri* ou des souches d'*Escherichia coli* particulières retrouvées associées à la maladie de Crohn, les AIEC (*adherent-invasive Escherichia coli*), mais également par des souches commensales comme *Enterococcus faecalis* [13-16]. Ces mêmes infections ne suscitent pas de réponse autophagique chez les souris dont les cellules épithéliales sont déficientes pour les gènes *Atg5*, *Atg16L1* ou *Atg7*. Ajoutées aux défauts de sécrétion des cellules de Paneth et des cellules caliciformes, ces anomalies permettent la dissémination bactérienne et une infection systémique.

Rôle de l'autophagie dans la régulation métabolique

La carence nutritionnelle représente un stress majeur à l'origine de l'activation de l'autophagie par sa capacité d'inhiber mTORC1 (*mammalian target of rapamycin complex 1*), d'activer l'AMPK (*AMP-activated protein kinase*) et de déacétyler des gènes clés de l'autophagie (*Atg5*, *Atg7* et *Atg8*), grâce à SIRT1 (*Sirtuin 1*). Des données expérimentales et des observations cliniques ont clairement démontré l'influence des nutriments et de leur disponibilité sur l'homéostasie intestinale. Au-delà de leur rôle dans l'immunité innée, les cellules de Paneth agissent comme senseur nutritionnel capable de stimuler la prolifération des cellules souches à la suite de l'inhibition de mTORC1 [17, 18]. Ainsi, la restriction calorique stimule la prolifération des cellules souches intestinales en affectant leur niche [17, 18]. Les cellules de Paneth interviennent également dans le maintien des cellules souches : elles sont ainsi nécessaires à la prolifération et la genèse d'organoïdes en culture à partir de cellules souches intestinales isolées [19, 20]. Notons également que la survie d'organoïdes issus de cellules souches sauvages et de cellules de Paneth déficientes en autophagie est altérée, confortant l'implication de l'autophagie dans la niche des cellules souches [7]. Étant donné l'importance de l'autophagie dans les processus de sécrétion des cellules de Paneth et de sa

régulation par mTORC1, il apparaît fondamental d'étudier son implication directe au cours de la régénération cellulaire associée à la restriction calorique.

La déplétion en acides aminés représente un autre contexte métabolique connu pour stimuler l'autophagie [21]. En effet, des travaux récents suggèrent l'implication de l'autophagie dans le contrôle de l'inflammation et de l'intégrité de l'épithélium intestinal dans le contexte de la carence en acides aminés [22]. Ainsi, l'inactivation du gène *GCN2* (*general controlled non-repressed 2*), codant une kinase sensible au déficit en acides aminés, dans les cellules épithéliales, conduit à une inflammation très sévère caractérisée par une augmentation des lymphocytes T CD4⁺ produisant de l'IL(interleukine)-17 (une réponse immunitaire souvent associée aux colites), ainsi qu'une perte cryptique dans le côlon, après administration de DSS. Un lien entre effets de *GCN2* sur l'homéostasie intestinale et autophagie a été suggéré. En effet, les souris invalidées pour les gènes *Atg16L1*, *Atg5* ou *Atg7* présentent également une sensibilité accrue au traitement par le DSS et révèlent des phénotypes qui sont similaires à ceux des souris *GCN2*^{-/-}. Ces résultats renforcent donc la notion d'un rôle clé de l'autophagie dans le contrôle de l'homéostasie intestinale selon la disponibilité en acides aminés.

Plusieurs études suggèrent que la restriction calorique et l'activation de l'autophagie pourraient contribuer à l'allongement de la vie de nombreuses espèces animales [23, 24]. Chez le nématode *Caenorhabditis elegans*, les vers invalidés pour le gène *eat-2*, prédisposés à manger moins, vivent plus longtemps [25]. Ce gain de longévité des vers mutants serait le résultat d'une activation de l'autophagie dans l'intestin et d'une préservation de la perméabilité intestinale, qui s'altère au cours du vieillissement. D'autres données obtenues chez *C. elegans* et chez la drosophile suggèrent également que le maintien de l'homéostasie intestinale serait un gage de longévité. L'augmentation de la perméabilité intestinale pourrait donc participer au vieillissement en affectant le métabolisme, la réponse inflammatoire et le microbiote [26-28]. Ces résultats ouvrent ainsi de nouvelles pistes intéressantes à explorer chez les mammifères afin de mieux comprendre les effets de l'autophagie intestinale sur la longévité.

Autophagie et pathologies intestinales

Au vu des fonctions essentielles assurées par l'autophagie, il n'est pas surprenant qu'une dérégulation de ce processus contribue à des pathologies intestinales, en particulier, inflammatoires et tumorales.

³ Se dit d'un animal prélevé stérilement par césarienne, élevé toute sa vie à l'abri de toute contamination microbienne et dont le tube digestif ne contient aucun microorganisme.

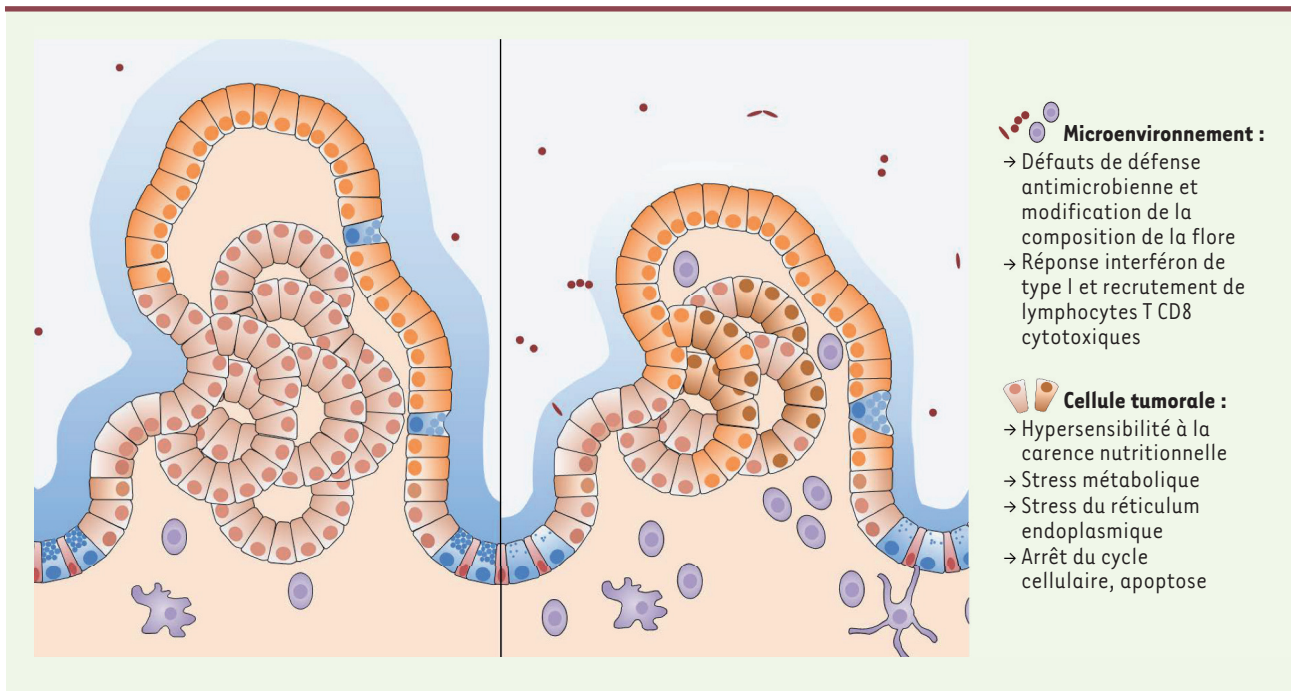


Figure 3. Effets anti-tumoraux de l'inhibition de l'autophagie dans l'épithélium intestinal. Chez la souris, l'inhibition de l'autophagie empêche l'initiation tumorale via ses effets sur la flore intestinale et sur le microenvironnement immunitaire. Elle bloque la progression tumorale en induisant plusieurs stress menant à l'arrêt du cycle cellulaire et à l'apoptose des cellules tumorales.

Autophagie et prédisposition à la maladie de Crohn

La maladie de Crohn (MC) est une maladie inflammatoire complexe reposant sur des facteurs génétiques et environnementaux responsables d'un déséquilibre entre microbiote, épithélium intestinal et système immunitaire. Elle se caractérise en particulier par une augmentation de la perméabilité épithéliale, une dysbiose, un défaut de sécrétion de peptides antimicrobiens et de mucus, un stress du réticulum endoplasmique, une augmentation de la production de cytokines pro-inflammatoires (IL-1 β , IL-6, IFN γ [interféron gamma], TNF α , etc.) et une réponse immunitaire de type Th17. Plusieurs gènes de susceptibilité ont été décrits pour la maladie de Crohn : *ATG16L1* et *IRGM* (*immunity-related GTPase M*), qui participent au processus autophagique ; *NOD2* (*nucleotide-binding oligomerization domain 2*), un récepteur de type NLR (*Nod-like receptor*) de l'immunité innée qui est également impliqué dans la régulation de l'autophagie. Un polymorphisme du gène *ATG16L1* a été associé à la maladie [29] : le variant T300A du gène (*ATG16L1*^{T300A}) favorise en effet le clivage de la protéine par les caspases-3 et -7, conduisant à une diminution de son expression [7, 30]. Dans les modèles murins exprimant l'allèle *ATG16L1*^{T300A}, un défaut des cellules de Paneth, de xénophagie et une sécrétion accrue de la cytokine pro-inflammatoire IL-1 β par les macrophages sont observés [7]. D'autres modifications responsables d'une perte de régulation de l'autophagie et entraînant un défaut de xénophagie [31] sont retrouvées chez les patients atteints de maladie de Crohn : une délétion en amont du gène *IRGM* et plusieurs polymorphismes non codants. Les polymorphismes du gène *NOD2* ont été les premiers à avoir été associés à la maladie. Ils restent à ce jour les facteurs de risques les

plus significatifs [32]. *NOD2* code un récepteur intracellulaire qui reconnaît le muramyl-dipeptide (MDP) présent à la surface de certaines bactéries. La détection du MDP par *NOD2* induit une activation de la voie NF κ B (*nuclear factor kappa B*) à l'origine d'une réponse immunitaire inflammatoire. Un « ménage à trois » faisant intervenir des interactions fonctionnelles entre *NOD2*, *ATG16L1* et *IRGM* a été décrit pour la prise en charge intracellulaire des pathogènes et l'activation par le MDP illustrant le rôle de l'autophagie dans ce processus [33-35]. Via l'autophagie, *NOD2* pourrait ainsi jouer un rôle essentiel dans le maintien de l'équilibre de la flore intestinale. Des études d'association ont également permis d'identifier d'autres polymorphismes associés à la maladie de Crohn dans des gènes d'autophagie, *ULK1* (*Unc-51-like autophagy-activating kinase 1*), *NDP52* (*nuclear domain 10 protein 52*), ainsi que dans des gènes pouvant affecter *LRRK2* (*leucine-rich repeat kinase 2*) et *PTPN2* (*protein tyrosine phosphatase, non-receptor type 2*). Toutefois, les conséquences fonctionnelles de ces polymorphismes dans la pathologie restent à démontrer.

Bien que l'autophagie apparaisse à l'interface des différentes fonctions biologiques altérées dans la maladie de Crohn, les polymorphismes des gènes impliqués dans l'autophagie sont des facteurs de risques et n'ont actuellement pas de lien causal direct [29]. Des modèles murins



exprimant les variants des gènes *IRGM* ou *ATG16L1* sont plus sensibles aux effets du DSS [4, 7, 36] ; ils ne développent cependant pas d'inflammation intestinale spontanée.

Autophagie et cancer colorectal

L'autophagie est apparue récemment comme un facteur favorisant le développement du cancer colorectal (CRC). Une augmentation de l'expression de plusieurs marqueurs de l'autophagie (*GABARAP* [*gamma-aminobutyric acid receptor-associated protein*], *ULK1*, *LC3*, *Atg5*, *Atg7* ou *Beclin-1*) fréquemment retrouvée dans des lignées et dans des tissus humains isolés de CRC, est généralement associée à un mauvais pronostic. L'autophagie limiterait également l'action de certaines chimiothérapies [11, 37-41]. Lors d'une carence nutritionnelle, mimant les contraintes liées aux défauts de vascularisation des tumeurs, l'activation de l'autophagie permet la survie des cellules coliques cancéreuses. Deux études récentes ont renforcé cette notion dans des modèles murins de cancérogenèse colique. L'altération de l'autophagie, induite par la délétion conditionnelle du gène *Atg7* dans les cellules épithéliales intestinales, affecte en effet la progression tumorale induite par la perte du gène suppresseur de tumeur *APC* (*adenomatous polyposis coli*) [11]. L'inhibition de l'autophagie, dans les cellules tumorales intestinales, est responsable d'un arrêt du cycle cellulaire, ce qui illustre l'addiction des cellules tumorales au processus d'autophagie, comme cela a été décrit pour les cancers mammaires, du poumon et du pancréas [42-44]. Les cellules tumorales intestinales n'exprimant pas le gène *Atg7* présentent une diminution d'expression des enzymes de la glycolyse, une activation de *p53*, et du senseur énergétique universel *AMPK*. Des données similaires ont été obtenues dans un modèle murin invalidé pour le gène *Atg5* dans les cellules épithéliales intestinales, et traité par le DSS et un agent carcinogène (AOM, azoxyméthane) [45]. Les cellules tumorales déficientes en autophagie présentent un stress du réticulum endoplasmique qui active la signalisation *UPR* qui est probablement inefficace, ce qui contribue à la mort des cellules cancéreuses. Il est donc vraisemblable que le rôle suppresseur de tumeur, ayant pour origine l'inhibition de l'autophagie dans l'intestin, illustre, là encore, l'implication fondamentale de l'autophagie dans le contrôle du métabolisme tumoral. L'autophagie permet aux cellules cancéreuses de s'adapter aux différentes situations de stress, de répondre aux nécessités énergétiques et en biomasse, pour permettre l'augmentation de leur division. La perte du gène *Atg7* dans la cellule épithéliale intestinale conduit à des modifications de la flore intestinale. L'utilisation d'une antibiothérapie suggère que ces modifications pourraient être responsables de la réponse immunitaire antitumorale. Les mécanismes restent cependant à définir. Ces données sont à relier à celles montrant que les patients homozygotes pour le variant *ATG16L1*^{T300A} présentent une activation de la signalisation interféron de type I et une meilleure survie [46]. L'autophagie apparaît donc être un partenaire à part entière dans le développement tumoral intestinal en agissant non seulement sur la cellule cancéreuse en contrôlant son métabolisme, mais également en intervenant sur le microenvironnement immunitaire et bactérien, deux alliés fondamentaux pour lutter contre le cancer.

Conclusion


Cinquante ans après la découverte de l'autophagie qui faisait suite à l'identification des lysosomes par Christian de Duve, la caractérisation des phénomènes biologiques qui lui sont associés permettent de mieux comprendre l'homéostasie intestinale, le développement tumoral et les maladies inflammatoires chroniques de l'intestin. Les données collectées ont ainsi permis d'illustrer l'importance de l'autophagie dans l'homéostasie de l'épithélium intestinal. Ce processus lui permet en effet de s'adapter aux nombreuses situations de stress auxquelles seul l'intestin est exposé, qu'elles soient intrinsèques, de par sa régénération incessante, ou liées à son microenvironnement. La dérégulation de l'autophagie a des effets qui sont spécifiques à certains types cellulaires présents au sein de cet épithélium. En particulier, des défauts touchant les cellules de Paneth ont été largement décrits. La sensibilité particulière de ces cellules pourrait être liée à leur longue durée de vie (environ 40 jours, les autres cellules épithéliales différenciées sont renouvelées tous les 3 à 4 jours), à leur production protéique élevée, ou à un rôle, non conventionnel, des protéines de l'autophagie spécifiques aux fonctions uniques de ces cellules.

L'inhibition de l'autophagie dans des modèles murins génétiquement modifiés suggère qu'une altération du processus, chez les patients à risque de développer des CRC, pourrait représenter une nouvelle stratégie thérapeutique. La mise en place d'une réponse immunitaire antitumorale, qui serait possible après inhibition de l'autophagie, pourrait accroître l'efficacité des traitements d'immunothérapie. Compte tenu de l'implication des cellules de Paneth dans la niche des cellules souches et du lien entre autophagie et cancer, il semble important, à présent, de déterminer si l'autophagie participe directement à la survie ou à l'activité des cellules souches intestinales. Son implication dans la maladie de Crohn apparaît plus complexe. L'identification d'autres facteurs génétiques ou environnementaux agissant de concert avec les polymorphismes déjà décrits pour leur rôle dans l'inflammation chronique est une étape fondamentale pour le développement de nouvelles cibles thérapeutiques et pour la prédiction des risques face à la maladie. ♦

SUMMARY

Contribution of autophagy to intestinal homeostasis and pathology

The intestinal epithelial cells are crucial mediators of intestinal homeostasis. The intestinal epithelium is the largest of the body's mucosal surfaces exposed to

the environment. Intestinal homeostasis is essentially based on the maintenance of intestinal epithelial cell integrity, a complex process involving a balance between the intestinal flora, the immune system and the energy expenses linked to metabolism. Autophagy appears to be central to these functions and allows the epithelium to adapt to its environment and different stress situations by participating in anti-bacterial defense, by controlling the composition of the intestinal flora and the immune response, and by participating in energy homeostasis. Alterations of this protective mechanism are involved in inflammatory bowel diseases and colorectal cancer. 

LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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TIRÉS À PART

B. Romagnolo

CHAPTER ONE

Intestinal Stem Cells and Their Defining Niche

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1. THE MAMMALIAN INTESTINAL EPITHELIUM

The adult mammalian intestinal epithelium consists of a single layer of cells folded upon itself creating cavities called crypts of Lieberkühn surrounded by connective mesenchyme. In the small intestine, the crypts are almost entirely composed of proliferative cells, with the exception of Paneth cells, a lineage first recognized for their ability to secrete antimicrobial peptides (AMP) that protect the crypt environment. As will be discussed later, these cells are also largely involved in the intestinal stem cell (ISC) niche.

Larger epithelial folds called villi protrude outward into the intestinal lumen and contain exclusively differentiated cells. These are primarily enterocytes, which make up over 80% of intestinal epithelial cells and whose function is to absorb dietary nutrients from the lumen. In addition, villi contain goblet cells that secrete a protective layer of mucus around the epithelium and enteroendocrine cells that produce hormones (Gordon, 1989). Three rarer and less-characterized cell types are also found within the differentiated epithelium: tuft cells (Gerbe et al., 2011), cup cells (Madara, 1982), and microfold (M) cells associated with lymphoid follicles called Peyer's patches (Kraehenbuhl & Neutra, 2000). Along the cephalocaudal axis of the small intestine, from the duodenum to the ileum, the length of the villi diminishes and the amount of secretory cells namely goblet cells and Paneth cells, increases (Cheng, 1974; Darmoul & Ouellette, 1996). This gradient accompanies a diminishing need for absorption and a considerable increase in microbe density (Savage, 1977). The colon, where stool is compacted for excretion, has a flat surface with no villi; the bottom third of colonic crypts contains proliferative cells while the upper crypt is differentiated. Microbe density is at its greatest in this segment of the intestine and, accordingly, the proportion of mucus-secreting goblet cells is considerably higher (Savage, 1977). Paneth cells, however, are notably absent in the colon.

The adult mouse intestine contains approximately 1.1 million crypts, with around two-thirds of the cells of each crypt dividing every 12 h, producing over 300 million new cells each day (Gordon, Schmidt, & Roth, 1992). Three to 16 (depending on the study) actively dividing ISC, called crypt basal columnar cells (CBC) reside at the crypt bottom alongside Paneth cells in the small intestine and related secretory cells in the colon (Altmann, 1983; Bjercknes & Cheng, 1981b; Potten & Loeffler, 1987; Sasaki et al., 2016; Sato, van Es, et al., 2011). CBC give rise both to new CBC and to progenitors called transit amplifying (TA) cells that will rapidly proliferate and progressively differentiate as they migrate up the crypt. TA daughters will either be absorptive lineage progenitors, giving rise to enterocytes, or secretory progenitors that will give rise to goblet, enteroendocrine, and Paneth cells (Bjercknes & Cheng, 1999; Schmidt, Wilkinson, & Ponder, 1985). In the small intestine, cells become terminally differentiated upon reaching the villus, then continue their columnar migration to the tip of the villus, where they are expelled and die in a process termed anoikis. Paneth cells, unlike the other progenitor lineages, terminally differentiate as they migrate down to the bottom of the crypt. The position of a Paneth cell is therefore linked to its maturity, with the oldest and more mature

Paneth cells found at the crypt base (Bjerknes & Cheng, 1981a). The entire process of proliferation, differentiation, and expulsion of intestinal epithelial cells takes only 4–5 days (Cheng & Leblond, 1974b; Wright & Irwin, 1982). The 6–8-week lifespan of Paneth cells further distinguishes them from other differentiated cell types of the intestinal epithelium (Ireland, Houghton, Howard, & Winton, 2005).

Epithelial patterning occurs not just along the crypt-villus axis, but also along the cephalocaudal axis. Fetal small intestinal isografts implanted subcutaneously develop into a histologically normal epithelium with a well-developed lamina propria, enteric nervous system, and smooth muscle layer. Strikingly, despite the absence of luminal contents, secretory goblet cells and Paneth cells are present, and the grafted tissue undergoes active peristalsis. Furthermore, the proportion and distribution of epithelial cell types as well as the gene expression profile of the isografts match those of the normal tissue segment it came from (Rubin, Roth, Birkenmeier, & Gordon, 1991; Rubin, Swietlicki, Roth, & Gordon, 1992). This suggests that ISC somehow retain a memory of their position-specific program. Region-specific gene programs are also conserved in cultured organoids from murine or human crypts (Middendorp et al., 2014), indicating that this “memory” is intrinsic to the epithelium. Strengthening this notion, small intestinal organoids reimplanted into colonic epithelium reconstitute a self-renewing epithelium with features of the small intestine (Fukuda, Mizutani, & Mochizuki, 2014).

2. ISC AND THEIR MARKERS

2.1 Historical Models of ISC

The identity of ISC has remained a subject of debate over the past decades. Two historical models of ISC have been originally proposed but direct evidence to illustrate their stemness was put forward only in the last decade. Pioneer work by Cheng, Bjerknes, and Leblond using electron microscopy, ³H-thymidine incorporation, and clonal mutagenesis demonstrated the presence of CBC, undifferentiated proliferative cells capable of giving rise to the different intestinal epithelial lineages (and therefore said to be multipotent), at positions 1–4 from the bottom of the intestinal crypt (Bjerknes & Cheng, 1999, 2002; Cheng & Leblond, 1974a). At the same time, works led by Potten recognized cells at the fourth position from the crypt bottom (+4) as capable of long-term ³H-thymidine label retention

(Potten, Hume, Reid, & Cairns, 1978), an established property of tissue stem cells. Without specific molecular markers to isolate them, it remained unclear whether the mitotically active CBC or the relatively quiescent label-retaining cells (LRC) ought to be considered as the bona fide ISC (Fig. 1).

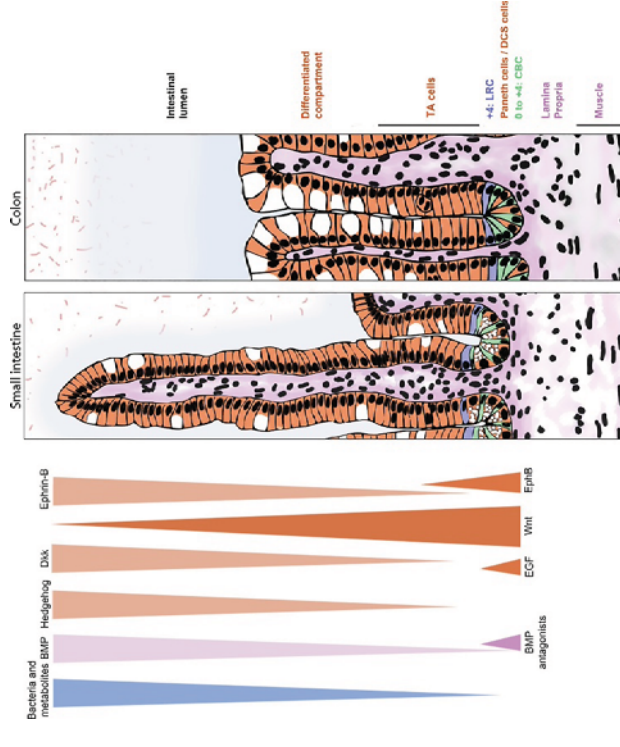


Fig. 1 Morphology of the small intestinal and colonic epithelium and niche signals involved in the regulation intestinal stem cells (ISC). The intestinal epithelium consists of a single layer of cells divided into two compartments: the differentiated compartment (villi in the small intestine, crypt tops in the colon) primarily contain absorptive enterocytes and secretory goblet cells while the proliferative crypts harbor transit amplifying (TA) cells that progressively differentiate as they migrate up the crypt, secretory niche cells (Paneth cells in the small intestine, deep crypt secretory (DCS) cells in the colon), and ISC. Two types of ISC have been proposed: crypt basal columnar cells (CBC) reside in positions 0 to +4 from the crypt bottom, intercalated between Paneth or DCS cells in the small intestine and colon, respectively. CBC are actively cycling and classically recognized by their marker Lgr5. More quiescent, label-retaining cells (LRC) at the +4 position expressing markers like Bmi1 have been proposed as reserve ISC. Several signals—most notably canonical Wnt signaling—regulate ISC proliferation, differentiation, and response to microenvironmental changes. These signals come from epithelial cells, particularly Paneth or DCS cells, from mesenchymal cells and extracellular matrix components present in the lamina propria, as well as from luminal nutrients, bacteria, and their metabolites. All these components and signals constitute the ISC niche.

2.2 Markers for CBC Cells

The Clevers lab and collaborators identified Lgr5 as the first marker for CBC. *Lgr5* encodes a leucine-rich repeat-containing G protein-coupled transmembrane receptor first identified as a Wnt/ β -catenin target gene. Although Lgr5 labeling has proven difficult due to its low expression levels, the development of a mouse model in which an EGFP and tamoxifen-inducible Cre-recombinase cassette was knocked in at the *Lgr5* locus (Lgr5-EGFP-CreER^{T2}) allowed characterization of the Lgr5⁺ cells. In the adult small intestine, Lgr5⁺ cells are located at the base of the crypt, intermingled with Paneth cells. They are rapidly cycling since within 24h almost all Lgr5⁺ cells of a crypt undergo mitosis. In the colon, Lgr5⁺ cells are also found at the crypt bottom interspersed with secretory cells but have slower cycling kinetics than in the small intestine. The generation of *in vivo* lineage tracing from Lgr5⁺ cells using an Lgr5-EGFP-CreER^{T2}/Rosa26lacZ mouse model indicated that Lgr5⁺ cells transmit lacZ staining to their progeny and repopulate the entire epithelium during homeostasis. Lgr5⁺ cells are long lived and contribute to tissue renewal over the entire lifetime of the mouse. Therefore, Lgr5 is a reliable marker of CBC (Barker et al., 2007), and Lgr5-EGFP-CreER^{T2} mice a valuable tool to understand their regulation. The ultimate proof of Lgr5⁺ CBC as ISC is the capacity of single isolated Lgr5⁺ cells cultured *in vitro* to generate structured intestinal organoids that contain the four major differentiated lineages of the intestinal epithelium and that self-renew, as shown by continuous passages (Sato et al., 2009). A CBC stem cell signature using gene expression and proteome profiling has been determined using FACS-purified Lgr5⁺ cells from the Lgr5-EGFP-CreER^{T2} model. A comparison between Lgr5⁺ CBC and their progeny sorted by different levels of EGFP (high in CBC and low in their progeny) provided a comprehensive Lgr5⁺ ISC signature of approximately 500 genes (Muñoz et al., 2012). The signature contains many Wnt/ β -catenin modulators and target genes such as *Lgr5*, *Sox9*, *Ascl2* (achaete-scute homolog 2), *EphA2*, *Troy/Tyrfsf19*, *Axin2*, *Znfx43* (zinc and ring finger 43) (Hao et al., 2012), and *Rnf3* (ring finger 3) (Koo et al., 2012). This and subsequent Lgr5⁺ cell signatures (Muñoz et al., 2012; van der Flier, van Gijn, et al., 2009) along with candidate-approach studies also provided several other verified CBC markers. Among the new markers identified, some have been confirmed as ISC markers by lineage tracing: *Smoc2* (SPARC-related modular calcium binding 2), encoding a BMP (bone morphogenic protein) signaling inhibitor (Muñoz et al., 2012), *Musashi-1*, encoding an RNA-binding protein (Potten et al., 2003), *Promitin-1* (Zhu et al., 2009)

and notably *Olfm4*, which is highly expressed compared to other mentioned CBC markers and allows easy detection of CBC (Schuijers, Van Der Flier, Van Es, & Clevers, 2014; van der Flier, Haegebarth, Stange, van de Wetering, & Clevers, 2009). *Ascl2*, a basic helix-loop-helix transcription factor, was identified as a master regulator of CBC maintenance. The conditional deletion of *Ascl2* results in the loss of CBC and its overexpression promotes drastic expansion of the ISC compartment (Reed et al., 2012; Schuijers et al., 2015; van der Flier, van Gijn, et al., 2009).

The identification of these CBC markers has allowed better characterization of ISC during homeostasis but also in disorders like colorectal cancer (CRC). In light of the high number of proliferative cells in the intestine and comparatively low occurrence of spontaneous intestinal tumors, it has long been proposed that only mutagenic hits accumulated in ISC could result in tumor development. Although it has proven difficult to ascertain which cell types bear the initial tumor-initiating mutations in humans, recent studies in mice suggest that CBC are likely to be the CRC cell of origin (Barker et al., 2009). Direct evidence has been obtained by the aberrant activation of Wnt signaling upon the inactivation of tumor suppressor *Apc* in Lgr5⁺ CBC, leading to a rapid onset of adenomas. In contrast, deletion of *Apc* in more differentiated cells results only in ectopic proliferative foci which rarely progress to microadenomas. In addition, lineage tracing of Lgr5⁺ adenoma cells revealed that they fuel the growth of adenoma by generating all the cell types present in the adenoma indicating that they act as multipotent stem cells of the adenoma (Schepers et al., 2012). Many CBC markers including *Lgr5*, *Musashi-1*, *Ascl2*, *Olfm4*, and *EphA2* are expressed both in human CRC and in genetically induced murine adenomas (Fan et al., 2010; Jang et al., 2016; Jubb et al., 2006; Li et al., 2015; Potten et al., 2003).

2.3 Markers for +4 Stem Cells

The first +4 stem cell (+4SC) marker investigated by lineage tracing was *Bmi1*, a polycomb-repressing complex member essential in hematopoietic and neural stem cell self-renewal. In intestinal homeostasis, *Bmi1* is expressed at the +4 position in a minority of small intestinal crypts (around 10%) and particularly in the duodenum. By *in vivo* lineage tracing using a *Bmi1*-CreER^{T2}/Rosa26lacZ mouse model and organoid culture experiments with sorted cells, *Bmi1*⁺ cells were shown to self-renew and give rise to the different epithelial lineages but with much slower cycling kinetics compared to Lgr5⁺ CBC (Sangiorgi & Capecchi, 2008; Yan, Chia, & Li, 2012).

In addition, ablation of $Bmi1^+$ cells using targeted expression of diphtheria toxin (DT) caused crypt loss consistent with stem cell defect.

Ablation of the $Lgr5^+$ CBC using targeted expression of the DT receptor (DTR) caused an increase of $Bmi1^+$ cells which can regenerate the $Lgr5^+$ stem cell population and maintain intestinal homeostasis (Tian et al., 2011). Thus, these relatively quiescent +4SC, reminiscent of the LRC described by Potten, have been considered as reserve stem cells in case of damage to the active CBC stem cells. In support of the mobilization of the +4SC following injury, such as high dose of radiation (up to 12 Gy), $Lgr5^+$ cells are rapidly lost whereas $Bmi1^+$ cells expand and allow epithelial recovery through replenishment of $Lgr5^+$ cells (Yan et al., 2012). In fact, $Bmi1$ has been shown to be involved in DNA damage response signaling; it may therefore play a direct role in the resistance of reserve stem cells to irradiation (Ginjala et al., 2011; Ismail, Andrin, McDonald, & Hendzel, 2010; Pan, Peng, Hungs, & Lin, 2011).

However, $Bmi1$ -expressing cells are only present in a small proportion of crypts in the proximal small intestine and are not responsible for the post-injury regeneration throughout the distal small intestine or colon in these contexts (Tian et al., 2011; Yan et al., 2012). Other markers must therefore be used to identify the reserve stem cells in these regions. Coincidentally, *Tert* (telomerase reverse transcriptase) also labels single cells at the +4 position that are slowly cycling, label retaining, resistant to radiation, and capable of regenerating the epithelium and $Lgr5^+$ cells following injury (Breault et al., 2008; Montgomery et al., 2011). Other reported markers of +4SC include *Hopx* (homeodomain only) (Takeda et al., 2011), *Lrig1* (leucine-rich repeats and immunoglobulin-like domains 1) (Powell et al., 2012), and, debatably, *Dcamk1* (doublecortin-like kinase 1) (May et al., 2008, 2009). Remarkably, cells expressing these markers can give rise to CBC and, conversely, $Lgr5^+$ cells can give rise to +4SC (Takeda et al., 2011).

2.4 Current View of ISC

The mutually exclusive nature of these two proposed stem cell populations, CBC and +4SC, has been extensively challenged in the literature in the past few years and appears less straightforward than initially thought. Single molecule in situ hybridization illustrates the restricted expression of certain markers like *Lgr5*, *Msi1*, *Ascl2*, *Olfm4*, and *EphB3* at the crypt bottom, but also that markers like *Prominin-1* and *Lrig1* have a broader pattern of expression along the crypt axis including both the majority of $Lgr5^+$ and

TA cells (Itzkovitz et al., 2012). Likewise, Muñoz et al. clearly demonstrate that +4SC markers *Bmi1*, *Tert*, and *Hopx* are enriched in $Lgr5^+$ CBC. This has been supported by several approaches both at the mRNA (Grün et al., 2015; Muñoz et al., 2012; van der Flier, van Gijn, et al., 2009) and protein levels (Scheepers, Vries, van den Born, van de Wetering, & Clevers, 2011; Wong et al., 2012), indicating that these markers may not be specific to quiescent stem cells and that the exact relationship between CBC and +4SC still needs to be further defined.

2.5 Progenitors as Potential ISC

The plasticity of progenitor cells represents another source of potential ISC. One study demonstrated that secretory progenitors expressing Delta-like (Dll) 1, a Notch ligand, can revert to multipotent stem cells. Using a Dll1-GFP-CreER^{T2}/Rosa26lacZ mouse model, lineage tracing indicated that secretory progenitors can revert to $Lgr5^+$ ISC upon ablation of CBC by irradiation and form $Lgr5^+$ ISC-containing organoids ex vivo in the presence of Wnt signals (van Es, Haegerbarth, et al., 2012; van Es, Sato, et al., 2012). Similarly, Buczacki and colleagues used an elegant model to mark only LRC by using a fluorescent-labeled histone H2B reporter construct (H2B-YFP) (Buczacki et al., 2013). They demonstrated that during homeostasis LRC express +4SC markers including *Lrig1*, *Tert*, and *Hopx*, but also *Lgr5* and markers of early Paneth cells and enteroendocrine cells. Using an ingenious lineage tracing approach, they demonstrated that during homeostasis LRC remain quiescent and express markers of Paneth cells. However, upon epithelial injury induced by irradiation or chemotherapy treatment, LRC proliferate, and act as ISC to give rise to all differentiated cell lineages. This study therefore indicates that committed secretory precursors are a subpopulation of $Lgr5^+$ cells which can act as a potential ISC pool upon injury. More recently, enterocyte progenitors have been shown to provide an alternative source of potential ISC. Using lineage tracing based on intestinal alkaline phosphatase (Alpi), a marker for progenitors and differentiated enterocytes, Tetteh et al. demonstrated that upon eradication of $Lgr5^+$ cells through DT treatment, the proliferating crypt Alpi⁺ cells and not differentiated villus Alpi⁺ cells were able to dedifferentiate and to produce all differentiated cell types, thereby acting as an alternative source of ISC (Tetteh et al., 2016). Two new studies from the groups of Kuo and Shivdasani categorize *Bmi1*-expressing cells as enteroendocrine progenitors, and further demonstrate the capacity of progenitors from this lineage to

dedifferentiate and replenish the $Lgr5^+$ ISC pool in case of injury (Jadhav et al., 2017; Yan et al., 2017). Taken together, all these studies call into question the existence of a dedicated reserve stem cell population and rather highlight the plasticity of the intestinal epithelium along with the importance of maintaining the active ISC pool. Questions regarding the molecular drivers and the degree of this plasticity remain open. As a hypothesis, it could be argued that upon injury leading to loss of CBC, committed progenitors regain direct contact with the stem cell niche, which may participate to their dedifferentiation and revert them to stemness.

More than a way to visualize ISC and analyze their contributions to intestinal pathology, these markers allow for a better understanding of the interaction of ISC with their surroundings. Many questions regarding the functional relation between ISC and their direct microenvironment, or niche, can now be approached. What molecular signals are involved in crypt homeostasis and how do they affect ISC? What cell types constitute the niche and provide these signals? How are ISC capable of sensing and responding to microenvironmental changes? What delimits the “positional address” of ISC? Are ISC actively anchored to this position or, on the contrary, does the niche define “stemness”?

3. SIGNALING PATHWAYS REGULATING CRYPT HOMEOSTASIS

Proliferation, differentiation, and migration along the crypt-villus axis are tightly regulated by an array of interconnected signaling pathways. Both epithelial signals and epithelial–mesenchymal crosstalk act in concert to create a complex molecular environment supporting ISC activity. Below, we discuss the principal and best-characterized pathways essential to ISC homeostasis and the pathological consequences of their deregulation.

3.1 Wnt/ β -Catenin Signaling

The major regulatory pathway of the ISC compartment is the Wnt/ β -catenin signaling pathway. In the absence of Wnt signals, free cytosolic β -catenin is recognized by a protein complex containing the tumor suppressors Apc (adenomatous polyposis coli), Axin1, CK1 α (casein kinase 1 α), and GSK-3 β (glycogen synthase 3 β), called the β -catenin destruction complex. Indeed, CK1 α and GSK-3 β will phosphorylate serine and threonine residues of the β -catenin N-terminus, leading to its ubiquitination by E3 ubiquitin ligase β -TrCP and subsequent proteasomal degradation. Wnt ligands bind a

Frizzled (Fzd) receptor and coreceptor lipoprotein-related protein (Lrp5/6 in the intestine). This interaction triggers a cascade of events involving phosphorylation of disheveled and membrane sequestration of Axin1 which, through mechanisms that remain unclear, inhibits the destruction complex, and prevents β -catenin degradation. β -Catenin is therefore free to accumulate and translocate to the nucleus, where it will bind transcription factor Tcf/Lef (T-cell factor/lymphoid enhancer factor) to induce Wnt/ β -catenin target genes (Logan & Nusse, 2004). Numerous studies have illustrated that CBC homeostasis is dependent on the activity of the Wnt/ β -catenin signaling cascade. Wnt/ β -catenin signal strength is dependent on several agonists and antagonists which may participate to the very context-dependent transcriptional response of the pathway. In CBC, Lgr5 and its homologs, Lgr4 and Lgr6, constitute the receptors for R-spondins, potent Wnt signal enhancers and CBC growth factors that work by neutralizing Rnf43 and Znf3, two transmembrane E3 ligases that remove Wnt receptors from the cell surface (de Lau et al., 2011).

β -Catenin also plays a role at cell adhesion complexes, linking E-cadherin to α -catenin and the actin cytoskeleton. Consequently, throughout the intestinal epithelium, β -catenin is mainly localized to the plasma membrane between epithelial cells, but also displays strong nuclear staining at the crypt bottom. Indeed, Wnt/ β -catenin signaling is particularly active in CBC and Paneth cells and exhibits a decreasing gradient of activity from the crypt bottom up. The major indicator of the role of Wnt/ β -catenin signaling in the intestinal epithelium is the extensive effects of its deregulation. Patients with familial adenomatous polyposis (FAP) carry a germline mutation in one *APC* allele. They develop hundreds to thousands polyps in the large intestine and are at high risk for developing CRC (Miyoshi et al., 1992). Genetically engineered mouse models have been useful for modeling FAP; the adenomas that arise in *Apc*-mutant mice are similar to those in FAP patients (Taketo & Edelman, 2009). *Apc*-mutant mice develop large numbers of adenomas in their small intestine and fewer in the large intestine which rarely progress to invasive adenocarcinoma, likely linked to the short lifespan of these mice (Colnot et al., 2004). Use of a conditional *Apc* allele showed that loss of Apc is a critical event to initiate intestinal tumor development. Conditional deletion of both alleles of *Apc* specifically in the intestinal epithelium leads to β -catenin relocalization to the nucleus, intense cell proliferation in the crypt compartment, along with impaired migration and differentiation throughout the epithelium (Andreu et al., 2005; Sansom et al., 2004). Moreover, a vast majority of human sporadic CRC carry

early driver mutations of *APC* or, less frequently, mutations on other genes encoding components of the canonical Wnt pathways like Axin2, R-spondins, Rnf43, or β -catenin itself (Fodde et al., 2001; Koo et al., 2012; Morin et al., 1997; Powell et al., 1992; Seshagiri et al., 2012). These tumors have a high level of nuclear β -catenin, and the intestine-specific expression of β -catenin mutants lacking its N-terminal phosphorylation sites in mice also leads to the development of such lesions (Harada et al., 1999; Romagnolo et al., 1999). The protumorigenic effects of *Apc* mutation therefore result in the constitutive activation of canonical Wnt/ β -catenin signaling, which drives tumorigenesis. The fact that *Apc* deficiency acts as a driver and not just an initiating event in colorectal tumorigenesis has recently been underlined by Dow et al. In their study, restoring *Apc* in established tumors, even those carrying additional *Kras* and *Tp53* mutations, leads to their regression and the reestablishment of normal epithelial architecture (Dow et al., 2015).

Multiple studies have demonstrated that the Wnt/ β -catenin pathway is essential for the homeostatic maintenance and proliferation of CBC. In the intestine, disruption of the pathway by deletion of either *Tcf4* or the β -catenin gene *Ctmb1* results in a fatal disruption of intestinal architecture with ablation of the proliferative crypts (Fevr, Robine, Louvard, & Huelken, 2007; Ireland et al., 2004; Korinek et al., 1998; van Es, Haegebarth, et al., 2012; van Es, Sato, et al., 2012). More upstream disruption of the pathway by ectopic expression of Wnt antagonist Dickkopf (*Dkk1*) also results in the loss of stem and progenitor cells (Kuhnert et al., 2004; Muncan et al., 2006; Pinto, Gregorieff, Begthel, & Clevers, 2003). Conversely, overstimulation of the pathway by injecting recombinant human R-spondin1 into mice induces rapid crypt proliferation involving β -catenin stabilization (Kim et al., 2005). Accordingly, conditional deletion of both *Znf3* and *Rnf4* expands proliferation in the crypt and is protumorigenic (Hao et al., 2012; Koo et al., 2012). Although deletion of *Lgr5* has no effect on intestinal cell proliferation, inactivation of *Lgr4* severely decreases crypt cell proliferation. Genetic deletion of *Lgr4* and *Lgr5* together completely abolishes proliferation and confirms that R-spondins act as major drivers of crypt self-renewal (de Lau et al., 2011). *Lgr5*⁺ CBC were shown to be highly responsive to such modulations in Wnt signaling, expanding upon stimulation by R-spondin1 or *Apc* loss and dying upon *Dkk1*-mediated inhibition of the pathway (Barker et al., 2009; Yan et al., 2012). A recent report demonstrated that *Ascl2*, a Wnt target gene expressed in CBC, acts as a master regulator of the *Lgr5* ISC transcriptional program: *Ascl2*

cooperates with β -catenin/Tcf4 complexes to switch on transcription of signature genes that define CBC identity (Schuijers et al., 2015).

In addition to proliferation, Wnt/ β -catenin signaling also affects differentiation of ISC daughters. *Apc*-deficient murine tumors overexpress Paneth cell-specific genes, and this surprisingly also applies to human colorectal tumors despite the absence of these cells in the healthy colonic mucosa (Andreu et al., 2008; Crawford et al., 1999; van Es, Jay, et al., 2005; van Es, van Gijn, et al., 2005). Furthermore, in this context, Paneth cells are no longer restricted to the crypt bottom and while they do express the AMP cryptidin, they do not present mature secretion granules, suggesting that their differentiation is incomplete. The fact that Paneth cells express a unique Wnt receptor, *Fzd5*, as opposed to other epithelial cells, which express *Fzd6*, supports a distinct Wnt response in these cells (van Es, Jay, et al., 2005; van Es, van Gijn, et al., 2005). Intestine-specific deletion of *Fzd5* also results in mislocalized Paneth cells lacking nuclear β -catenin as well as *Cryptidin* and *Eplhb3* expression. In fact, Paneth cell-restricted expression of genes encoding Cryptidins, EphB3, and matrix metalloprotease (*Mmp7*) is transcriptionally regulated by β -catenin and Tcf4 (Andreu et al., 2008; Crawford et al., 1999; van Es, Jay, et al., 2005; van Es, van Gijn, et al., 2005). Precise control of Wnt signaling, in addition to its importance in proliferation and ISC cell maintenance, is therefore also essential for Paneth cell differentiation and maturation (Andreu et al., 2008; van Es, Jay, et al., 2005; van Es, van Gijn, et al., 2005).

The scattered localization of Paneth cells upon deregulation of Wnt signaling hints at its implication in migration along the crypt-villus axis as well. *Eplhb3*- or *Ephrin-B1*-knockout intestines also display delocalized Paneth cells (Batlle et al., 2002; Cortina et al., 2007). Ephrin-B ligands and their EphB receptors are cell-sorting molecules notably involved in axon guidance in the nervous system. In accord with their transcriptional activation by β -catenin, expression of EphB2 and EphB3 is the highest at the crypt bottom. Specifically, Paneth cells express EphB3, *Lgr5*⁺ cells express both EphB3 and EphB2, and TA cells express decreasing levels of EphB2 as they migrate up the crypt. Expression of the Ephrin-B ligands is repressed by β -catenin, so it follows the opposite pattern with the highest expression in differentiated cells and the lowest expression at the crypt bottom. Therefore, as cells migrate up the crypt and Wnt signaling decreases, they gradually decrease *Eplhb2* and increase *Ephrin-B* expression. Since the interaction between EphB receptors and Ephrin-B ligands has a repellent effect, this mechanism keeps EphB-expressing Paneth and ISC restricted to the crypt

bottom and prevents the downward migration of differentiating progenitors (Batlle et al., 2002). Knockout of both *EphB2* and *EphB3* in mice therefore results in dispersion of proliferative cells throughout the epithelium, intermingled with the differentiated cells. The effects of EphB/Ephrin-B interaction are thought to act, at least in part, through the regulation of tight junctions via activation of ADAM (a disintegrin and metalloproteinase domain) family mediated cleavage of E-cadherin at interfaces between neighboring EphB- and Ephrin-B-expressing cells (Solanas, Cortina, Sevillano, & Batlle, 2011). Despite the overactivation of Wnt signaling in CRC, *EphB* expression is often silenced at the adenoma-carcinoma transition, promoting cancer progression through a mechanism dependent on E-cadherin-mediated adhesion (Batlle et al., 2005; Cortina et al., 2007).

β -Catenin can also directly affect cell adhesion and thereby migration through its direct interaction with E-cadherin. In fact, β -catenin was historically first recognized for its role in adherens junction complexes. Modulation of E-cadherin levels affects migration speed and organization along the crypt-villus axis—with overexpression of the E-cadherin gene *Cdh1* inducing slower, structured migration and loss of E-cadherin resulting in disordered, rapid migration (Bondow, Faber, Wojta, Walker, & Battle, 2012; Hermiston, Wong, & Gordon, 1996; Schneider et al., 2010). Similarly, overexpression of *Apc* results in disordered migration (Wong, Hermiston, Syder, Gordon, & Kipnis, 1996). Proliferation in the crypt is also inversely linked to E-cadherin levels (Hermiston et al., 1996) and β -catenin is known to interact with E-cadherin and Tcfs through an overlapping-binding region, suggesting that the two functions could be in stoichiometric competition (Choi, Huber, & Weis, 2006; Graham, Weaver, Mao, Kimelman, & Xu, 2000; Huber & Weis, 2001). Supporting this notion, high expression of E-cadherin correlates with lower tumorigenic potential of mutant β -catenin (Huels et al., 2015). It has even been proposed in cell culture models that E-cadherin complexes could modulate more upstream Wnt receptor signaling (Maher, Flozak, Stocker, Chenn, & Gottardi, 2009). Contrarily, intercellular junctions appear unaffected by the deletion of β -catenin in the intestinal epithelium (Fevr et al., 2007). Evidently, the link between the opposing functions of β -catenin in Wnt signaling-mediated proliferation and E-cadherin-dependent cell adhesion still needs to be untangled.

The Wnt activity gradient from the crypt bottom is in part established by the localized production of Wnt ligands. Crypt epithelial cells produce Wnt3, Wnt9b, and Wnt6 (Farin, Van Es, & Clevers, 2012; Gregorieff et al., 2005). Of these, Wnt3 is specifically expressed in Paneth cells. The

intestinal mesenchyme also produces Wnt ligands, namely Wnt2b, Wnt4, and Wnt5a, which have been linked to noncanonical Wnt signaling. Crypt epithelial cells in the small intestine and colon also express various Fzd receptors as well as different Tcfs. Furthermore, the mesenchyme near the differentiated epithelium also produces Wnt antagonists like Dkk in a localized manner (Gregorieff et al., 2005). All this combined makes for a rather intricate and complex regulation of Wnt signaling resulting in a Wnt activity-permissive niche in the stem cell and Paneth cell compartment and repression of canonical Wnt signaling in the differentiated epithelium.

3.2 Hedgehog, BMP, and PI3K/PTEN Signaling

Hedgehog-family proteins and BMPs link epithelial homeostasis to mesenchymal signals. The Hedgehog receptor, Patched (Ptc), normally inhibits the constitutively active smoothened (Smo) receptor, thereby permitting cleavage of Gli transcription factors (Gli1, 2, and 3) into their repressive forms. Upon Hedgehog binding, Ptc is repressed, and Smo signaling allows Gli-mediated transcription of Hedgehog target genes. Hedgehog signaling is exclusively paracrine in the adult intestine; Indian Hedgehog and Sonic Hedgehog (Ihh and Shh, the main Hedgehog ligands in the intestine) secreted by enterocytes binds Ptc receptors on neighboring mesenchymal cells, inducing BMP production from these cells (Madison et al., 2005; van Dop et al., 2009). Binding of BMP to its type 2 receptor, Bmpr2, results in the phosphorylation and activation of its type 1 receptor, Bmpr1, which subsequently phosphorylates Smad proteins 1, 5, or 8. These will then heterodimerize with Smad4 before translocating to the nucleus to induce transcription of target genes. In the intestine, BMP2 and BMP4 are produced by intravillus and intercryptic mesenchymal cells, and Bmpr1 is expressed in differentiated cells and ISC but not in the proliferative TA cells (Haramis et al., 2004; He et al., 2004). BMP antagonists like Noggin, Gremlin1, Gremlin2, or Chordin-like 1 are expressed in subcryptic myofibroblasts and smooth muscle (He et al., 2004; Kosinski et al., 2007). It thus follows that BMP activity affects only in villus epithelial cells. The constitutive activation of Hedgehog signaling results in increased BMP signaling and depletion of proliferative and stem cells (van Dop et al., 2009). Conversely, either inhibition of Hedgehog or deregulation of the BMP pathway results in expansion of the ISC compartment, excessive crypt formation, and the formation of hamartomatous polyps (Davis et al., 2015; Haramis et al., 2004; He et al., 2004; Madison et al., 2005; Qi et al., 2017). These lesions are

typical of those found in patients with juvenile polyposis syndrome, often associated with *BMPRIA* or *SMAD4* mutations. Therefore, Hedgehog and BMP signaling restrain proliferation and promote differentiation, in opposition with Wnt/ β -catenin signaling.

In fact, modulation of either Hedgehog or BMP signaling affects nuclear β -catenin levels and the expression of its target genes (Madison et al., 2005; van den Brink et al., 2004; van Dop et al., 2009). Explaining this phenomenon, BMP signaling was shown to directly interfere with Wnt-mediated β -catenin activation through a mechanism involving PI3K and PTEN activation and subsequent inhibition of Akt (He et al., 2004; Waite, Waite, & Eng, 2003). Similar to disruption of BMP signaling, deletion of PTEN leads to de novo crypt formation, induced Wnt/ β -catenin signaling, and polyp formation (He et al., 2007). This parallels the development of hamartomatous lesions found in Cowden disease patients, known to inherit PTEN mutations (Liaw et al., 1997). Interestingly, the involvement of the stroma in the development of tumors associated with deregulated Hedgehog, BMP, and PTEN signaling is underlined in multiple studies (Auclair, Benoit, Rivard, Mishina, & Perreault, 2007; Beppu et al., 2008; Büller et al., 2015; Gering et al., 2016; He et al., 2007; Howe et al., 1998; Sneddon et al., 2006; van den Brink et al., 2004). These signaling pathways therefore simultaneously contribute to differentiation along the crypt-villus axis, determination of β -catenin active or inactive state despite a gradient of Wnt, and interactions with the crypt-adjacent mesenchyme.

3.3 Notch Signaling

The Notch pathway is also key to ISC maintenance and daughter cell-fate determination. Contrary to the previously described signaling pathways, Notch signaling works by lateral inhibition between two adjacent cells. Notch ligands Dll1, Dll4, or Jagged1 at the cell surface will bind the Notch1 or 2 receptors on neighboring cells (Sander & Powell, 2004; Schröder & Gossler, 2002). Upon ligand binding, the receptor is cleaved by ADAM10, shedding its extracellular portion, and by a γ -secretase complex, releasing its intracellular domain (NICD). The NICD translocates into the nucleus and, alongside cofactors like RBPJk (recombination signal-binding protein for immunoglobulin κ region), promotes the transcription of target genes involved in proliferation and differentiation, including Hes (hairy and enhancer of split)-family transcription factors. The best-characterized NICD target, *Hes1*, represses transcription of Notch ligands and of *Atoh1* (atonal

BHLH transcription factor 1, *Math1* in mice), both key to secretory lineage commitment, as well as that of genes encoding cyclin-dependent kinase inhibitors p27^{Kip1} and p57^{Kip2} (Milano et al., 2004; Obata et al., 2012; Stanger, Datar, Murtaugh, & Melton, 2005; Tsai et al., 2014; Yang, Bermingham, Finegold, & Zoghbi, 2001).

Signal inhibition all along the pathway by genetic deletion of *Dll1* and *Dll4* (Pellegri et al., 2011), *Notch1* and *Notch2* (Carulli, Keeley, Demitrac, Chung, & Samuelson, 2016; Riccio et al., 2008), *ADAM10* (Tsai et al., 2014), *RBPJk* (van Es, Jay, et al., 2005; van Es, van Gijn, et al., 2005), or multiple *Hes* genes (Ueo et al., 2012) or by treatment with γ -secretase inhibitors (Milano et al., 2004; van Es, Jay, et al., 2005; van Es, van Gijn, et al., 2005; VanDussen et al., 2012) converts all proliferative cells in the crypt into postmitotic secretory cells. This includes Lgr5⁺ ISC, which are consequently lost (Carulli et al., 2016; VanDussen et al., 2012), leading to lack of epithelial regeneration, nutrient malabsorption, weight loss, and death. Accordingly, lineage tracing experiments reveal active Notch signaling in ISC (Fre et al., 2011; Pellegri et al., 2011), and *Olfm4* was established as a direct target of the NICD and RBPJk (VanDussen et al., 2012). On the other hand, expression of a constitutively active NICD results in increased proliferation and secretory cell depletion (Fre et al., 2005; Stanger et al., 2005). *Math1* deletion also results in depletion of all secretory lineages (Durand et al., 2012; Kim, Escudero, & Shivdasani, 2012; Yang et al., 2001). Notch signaling therefore maintains the proliferating ISC pool and suppresses secretory lineage engagement. Beyond this, however, Notch signaling does not appear necessary for absorptive lineage differentiation as simultaneous *Math1* deletion and Notch disruption does not prevent enterocyte differentiation (Kazanjian, Noah, Brown, Burkart, & Shroyer, 2010; Kim & Shivdasani, 2011; van Es, de Geest, van de Born, Clevers, & Hassan, 2010). Furthermore, while the effects of Notch activation on proliferation are only possible in Wnt-activated cells, its effects on differentiation are Wnt independent (Fre et al., 2009). The current model postulates that Wnt-induced stem cells are maintained in a Notch-high state by neighboring Notch ligand-expressing cells, and that upon exit from the ISC niche, the bimodal nature of the pathway will result in some daughters' expression of *Dll1* and *Math1* and repression of *Notch*, leading to their secretory lineage commitment. These cells will then repress such a commitment in their neighboring TA cells, ensuring an appropriate absorptive-to-secretory lineage ratio. Paneth cells express Notch ligands (Sato, van Es, et al., 2011) and thereby sustain active Notch signals in their neighboring

ISC, although other sources of Notch ligands also likely exist in the crypt as stem cells are maintained despite the absence of Paneth cells in the colon and in the small intestine of *Math1*-knockout mice (Durand et al., 2012; Kim et al., 2012).

Unsurprisingly, Notch signaling has been detected in both human and mouse intestinal adenomas, and aberrant Notch activation promotes adenoma formation in *Apc*-mutant mice (Fre et al., 2009; van Es, Jay, et al., 2005; van Es, van Gijn, et al., 2005). Conversely, γ -secretase inhibitor treatment partially converted adenoma cells of *Apc*-mutant mice into secretory cells, although this treatment is limited by its concurrent effects in the normal epithelium (van Es, Jay, et al., 2005; van Es, van Gijn, et al., 2005) and on other tissues. Furthermore, blocking Notch signaling does not prevent the initiation of *Apc*-mutant tumors, suggesting that β -catenin activation overrides the expected forced differentiation of proliferative cells (Peignon et al., 2011). Notably, Notch signaling is weaker in more advanced human carcinomas compared to adenomas, and its activation never leads to adenocarcinoma formation in *Apc*-mutant mice, indicating that Notch activation may be favorable (although not required) for tumor development but not for malignant progression (Fre et al., 2009).

4. ORGANOID CULTURE AND THE EXPERIMENTAL NICHE

Although in vitro culture of intestinal crypts has often been labeled as “niche independent” growth of ISC, it actually highlights the importance of a minimal molecular niche for proper ISC function. Among the key supplements to organoid culture medium are Wnt agonist and Lgr5 ligand R-spondin1, the BMP antagonist Noggin, and epidermal growth factor (EGF) (Sato et al., 2009). Little is currently known about the effects of EGF on ISC. In the adult intestine, EGF receptor (EGFR) is mainly expressed in the epithelium, especially in crypt CBC and TA cells (Yang et al., 2017), whereas Paneth cells highly express EGF ligands EFG and TGF α (transforming growth factor- α) (Sato, van Es, et al., 2011). EGFR-family receptor ErbB2 is also highly expressed on CBC (Sasaki et al., 2016), although its activity appears to be negatively regulated by Lrig1 (Wong et al., 2012). Production of another EGF-family ligand, Amphiregulin, from intestinal lymphocytes has recently been implicated in epithelial regeneration (Monticelli et al., 2015). Furthermore, EGFR signaling activates the RAS pathway, of which several components are constitutively activated in later-stage CRC.

When isolated ISC rather than whole crypts are cultured, exogenous Wnt ligands (typically Wnt3a) must be added to the culture medium. Although this allows for ISC maintenance and proliferation, the resulting organoids no longer contain all the differentiated cells of the homeostatic epithelium but rather seem to consist largely of undifferentiated progenitors that form a spheroid structure (Sato, van Es, et al., 2011). This is likely due to the ubiquitous delivery of Wnt ligand, as opposed to the more homeostatic gradient created by coculture with Paneth cells. The implication of different signaling pathways in intestinal homeostasis can be tackled in vitro as organoids recapitulate the in vivo epithelial response—a hyperplastic crypt might develop into an undifferentiated spheroid whereas stem cell failure will lead to organoid death. This system also has the added advantage of easier manipulation of treatments and the possibility to follow epithelial response over time while excluding mesenchymal response (Yin et al., 2014).

The supplemented growth factors described above apply for murine small intestinal crypts (without exogenous Wnt) or isolated stem cells (with added Wnt3a). Isolated colon crypts produce insufficient amounts of Wnt ligands to maintain colonic ISC in culture, therefore Wnt3a must be added for colonic organoid growth (Sato, Stange, et al., 2011). Like small intestinal organoids with exogenous Wnt, however, these do not properly differentiate. In the case of human small intestinal and colon crypts, addition of further factors is required for efficient long-term culture—gastrin, nicotinamide, an ALK receptor inhibitor, and an inhibitor of p38 MAPK (mitogen-activated protein kinase)—suggestive of additional important pathways in human ISC function. It is interesting to note that ISC presenting mutations in genes commonly mutated in CRC, or organoids derived from human colorectal tumors have decreasing needs for growth factors in the culture medium, suggesting a decreasing niche factor dependence in tumor stem cells (Drost et al., 2015; Fujii et al., 2016).

A final component that must not be neglected for ISC maintenance and organoid growth in vitro is the substrate upon which ISC are plated. In vivo, the epithelium is surrounded by an extracellular matrix (ECM) enriched in laminin at the crypt base. For organoid growth, ISC are embedded in laminin-rich matrigel. Isolated epithelial cells die by anoikis, a form of apoptosis following loss of matrix interactions, and the addition of ROCK signaling inhibitor Y-27632 to the culture medium also improves organoid formation, further highlighting the importance of matrix interactions (Sato et al., 2009). In vivo, the basal side of intestinal epithelial cells is in direct contact with a network of extracellular proteins that form the

basement membrane (BM), including type IV collagen, laminin, and proteoglycans. BM components are produced both from epithelial and mesenchymal cells including subepithelial myofibroblasts. In the lamina propria, similar components including type I collagen and fibronectin form a three-dimensional ECM. Integrins recognize laminin, collagen, and fibronectin, inducing cytoplasmic signaling cascades that affect cytoskeleton remodeling, cellular growth, and cell cycle progression. Differential distributions of laminins and integrins have been observed along the crypt-villus axis, affecting not just adhesion, but also proliferation, migration, and sensitivity to anoikis of epithelial cells. BM and ECM components as well as integrin signaling have been implicated in CRC. Like EGF, integrins also induce the Ras signaling cascade, therefore the activating *KRAS* and *BRAF* mutations in later-stage CRC could promote anchorage-independent growth of tumor cells. Mmps are soluble or membrane-bound proteases produced both by the epithelium and mesenchyme that can remodel the BM and ECM, and several of these have also been implicated in CRC progression and inflammatory bowel disease. Lastly, the ECM can also sequester different growth factors, like transforming growth factor β (TGF β), which become accessible by matrix remodeling or during epithelial damage (Hynes, 2009; Lussier, Basora, Bouatrous, & Beaulieu, 2000; Murgia et al., 1998; Worthley, Giraud, & Wang, 2010).

5. THE EPITHELIAL NICHE: PANETH CELLS AND DEEP CRYPT SECRETORY CELLS

One epithelial cell type stands out with regard to its involvement in the ISC niche. Even as far back as the 1970s, Cheng and Leblond proposed a tight functional link between CBC and their position between Paneth cells. In link with their demarcating feature, large secretory granules filled with α -defensins (HD5 and HD6 in humans) and other AMP, Paneth cells were initially recognized for their role in innate immunity. Paneth cells normally release their granule contents into the crypt lumen in basal conditions and increasingly so in response to stimuli such as bacterial surface products and other TLR (Toll-like receptor) agonists (Ayabe et al., 2000; Vaishnava, Behrendt, Ismail, Eckmann, & Hooper, 2008) or proinflammatory cytokines like interferon gamma (Farm et al., 2014). Although α -defensins are constitutively expressed by Paneth cells under the control of the Wnt pathway and Tcf4 (Andreu et al., 2008; van Es, Jay, et al., 2005; van Es, van Gijn, et al., 2005), even in the absence of luminal microbes before birth or in

germ-free conditions, other AMP are upregulated in the presence of bacteria (Ayabe et al., 2000; Harris, 1996; Pütsep et al., 2000). These AMP include lysozyme, secretory phospholipase A2, and Reg3 (regenerating islet-derived protein 3, Reg3A in humans, Reg3 γ and Reg3 β in mice). These secreted AMP not only protect the crypt epithelium from enteric pathogens (Vaishnava et al., 2008), but also directly affect the composition of the microbiota (Salzman et al., 2010). In this manner, Paneth cells regulate homeostatic interactions between the epithelium and the intestinal microbiota, which could have huge repercussions on the inflammatory, and therefore regenerative, state of the intestine.

Paneth cells also produce many of the essential trophic factors regulating the ISC niche, including Wnt3, Wnt6, Wnt9, Dll4, Dll1, EGF, and TGF α (Gregorieff et al., 2005; Sato, van Es, et al., 2011). This hints at a major role of these cells in the maintenance of ISC and regulation of their proliferation. On this basis, it has been proposed that the Paneth cell zone defines the ISC niche. As ISC divide and their numbers increase, they will neutrally compete to either remain between Paneth cells and therefore maintain an ISC identity or drift out of the niche and adopt a TA fate (Risima et al., 2014; Snippet et al., 2010). An opposing hierarchical model argues that ISC divide mostly asymmetrically, giving rise to both a new stem daughter and a TA daughter that exits the niche. Both models are compatible with early lineage tracing experiments and the observed drift toward monoclonality of ISC within a single crypt (Gordon et al., 1992). Consistent with Paneth cells delimiting the ISC niche, partial ablation of Paneth cells results in a decrease in the number of Lgr5⁺ CBC, with the remaining CBC crowded around the remaining Paneth cells (Sato, van Es, et al., 2011). Surprisingly, the total elimination of Paneth cells does not result in the loss of ISC (Durand et al., 2012; Kim et al., 2012; Shroyer et al., 2007). The space normally occupied by Paneth cells is filled by normal amounts of CBC with active Wnt/ β -catenin signaling. In fact, proliferation is increased in Paneth cell-deficient crypts (Durand et al., 2012; Kim et al., 2012; Shroyer et al., 2007) and the regenerative capacity of ISC following irradiation is not affected (Durand et al., 2012). Paneth cells are therefore not required for ISC survival and proliferation. They are also not essential for stem cell localization, as CBC are retained at the crypt bottom despite the lack of Paneth cells. Although this may be shocking in light of the importance attributed to Paneth cells, several lines of evidence comfort the dispensability of Paneth cells for ISC function. Not only is the colon devoid of Paneth cells, but

the small intestines of certain mammalian species, namely dogs and pigs, also lack Paneth cells (Potten, Booth, & Pritchard, 1997).

In vitro, however, isolated Paneth cell-deficient crypts cannot form organoids (Durand et al., 2012). The same is true of Paneth cell-derived Wnt3, the expression of which is dispensable for stem cell maintenance in vivo but essential for the mesenchyme-independent growth of intestinal organoids in vitro (Farin et al., 2012). The absence of Paneth cells in vivo must therefore be compensated by other as-of-yet unknown niche cells likely providing compensatory Wnt in response to the lack of Paneth cell signals. Wnt3- or Paneth cell-deficient organoids can be maintained in culture by addition of exogenous Wnt3a or by coculture with intestinal mesenchymal cells (Durand et al., 2012; Farin et al., 2012; Kabiri et al., 2014; Sato, van Es, et al., 2011). Wnt2b is the only Wnt ligand normally expressed by the mesenchyme capable of rescuing Wnt3- or Paneth cell-deficient organoid growth; it has therefore been suggested as the potential compensatory Wnt signal in vivo (Farin et al., 2012). Of course, such a rescue has not been formally demonstrated in vivo, and the existence of another compensatory mechanism should not be excluded. Of note, exogenous Wnt6 or Wnt9b also rescue Wnt3-deficient organoid growth, suggesting that the amount produced by the crypt epithelial cells is not sufficient to compensate Wnt3 loss. Recently, Farin et al. further examined the establishment of the Wnt3 gradient from Paneth cells. Surprisingly, they found Wnt3 to act not as a diffusible gradient but as a Fzd-bound signal on the basolateral membrane of cells at the crypt bottom that is diluted up the crypt by cell division (Farin et al., 2016). They also report that Wnt3 does not localize in Paneth cell secretion granules, implying that it is secreted through another mechanism. This is in agreement with a previous report that put forward a Rab8a vesicle-dependent secretion of Wnt ligands (Das et al., 2015).

Paneth cells have been shown not just to provide a defined niche for ISC, but also to orchestrate their response to nutrient availability. It makes sense for the intestinal epithelium, whose principal function is nutrient absorption, to be highly responsive to diet and nutrient availability. In response to calorie restriction, villi shorten along with the TA compartment, while CBC numbers increase. This increase in ISC numbers and proliferation is accompanied by an increase in Paneth cells. Inhibition of mTORC1 (mammalian target of rapamycin complex 1) is a classically described cellular response to nutrient deprivation. In 2012, Yilmaz and coworkers showed that the augmented ISC renewal following calorie restriction is dependent on reduced

mTORC1 activity in Paneth cells, resulting in increased extracellular cyclic ADP ribose production (Yilmaz et al., 2012). Igarashi and Guarente complemented this study by showing that this paracrine signal activates Sirtuin1 and mTORC1 signaling in ISC, promoting their self-renewal. Paneth cell signaling therefore overrides direct nutrient sensing in ISC to coordinate a proper response (Igarashi & Guarente, 2016). A new study by Rodriguez-Coman et al. showed that while Lgr5⁺ CBC have high mitochondrial activity, Paneth cells show high glycolysis levels. Blocking the glycolytic activity of Paneth cells hindered their ability to support organoid formation in culture, while blocking oxidative phosphorylation in CBC also reduced organoid formation. On the other hand, providing lactate to Lgr5⁺ CBC enhanced organoid formation. The authors therefore propose that Paneth cells, through their high glycolytic activity, provide lactate to CBC, enabling their high mitochondrial metabolism and proliferative capacity (Rodríguez-Colman et al., 2017). These studies put forward an entirely new niche function of Paneth cells: supporting ISC metabolism, and orchestrating their adaptation to their metabolic environment. Paradoxically, a high-fat diet also leads to an increase in ISC numbers along with decreased Paneth cell numbers (Beyaz et al., 2016). This is supposedly by a mechanism involving peroxisome proliferator-activated receptor δ (PPAR δ) signaling in CBC conferring Paneth-independent growth in vitro.

Although Paneth cells are absent in the colon, Lgr5⁺ CBC are still found intercalated between secretory cells in the colon mucosa. These cells, first coined “deep crypt secretory” (DCS) cells in 1983 by G. G. Altmann, are distinct from goblet cells but still contain mucous vacuoles in their cytoplasm. Morphologic analysis of DCS cells reveals many shared features with small intestine Paneth cells: they have a highly developed endoplasmic reticulum and Golgi complex for glycoprotein production, they mature as they migrate down from the midcrypt to the crypt base, and their numbers vary from the proximal to the distal colon (Altmann, 1983). With this background in mind, Rothenberg et al. identified a new goblet cell type that expresses several factors involved in ISC maintenance, such as Dll1, Dll4, and EGF. These cells can be recognized by marker cKit, also expressed by small intestinal Paneth cells, and intercalate between Lgr5⁺ CBC in the colon (Rothenberg et al., 2012). These cells were also shown to express Reg4 (regenerating islet-derived 4) and correspond to Altmann’s DCS cells. Just recently, Sasaki et al. characterized the gene expression profile of Reg4⁺ DCS cells and found that they express several other Notch and EGF ligands along with goblet cell markers, although their signature matches that of

Paneth cells more closely than that of goblet cells. Specific ablation of DTR-expressing DCS cells upon DT treatment led to a loss of Lgr5⁺ stem cells (Sasaki et al., 2016). Consistently, coculture of Lgr5⁺ stem cells with cKit⁺ and Reg4⁺ DCS cells improves organoid growth while the disruption of DCS cells hinders it. Finally, DCS cell numbers are regulated by Notch signaling, as is the case for Paneth cells (Rothenberg et al., 2012; Sasaki et al., 2016). One key difference stands out between the two cell types: DCS cells, unlike Paneth cells, do not produce any Wnt ligands (Sasaki et al., 2016), although colon Lgr5⁺ cells do depend on them for growth in vitro (Sato, Stange, et al., 2011). As in the Paneth cell-deficient small intestine, another extraepithelial source of Wnt likely exists in the colon to maintain ISC homeostasis.

6. THE MESENCHYMAL NICHE

The lamina propria comprises various mesenchymal cell types, including fibroblasts and myofibroblasts, smooth muscle cells, vascular pericytes and endothelial cells, enteric neurons, and hematopoietic cells. Of course, decrypting the complex interactions between mesenchymal cells and the intestinal epithelium relies on ongoing characterization of the different subsets of stromal cells and their functions. Stromal cells have been shown to play a crucial role in homeostasis of the intestinal epithelium as well as in its response to different physiological stresses. Several key signaling pathways regulating epithelial proliferation and differentiation involve epithelial-mesenchymal communication, although the specific mesenchymal cell types involved are not yet fully established. These signals include the aforementioned Hedgehog and BMP signals, canonical and noncanonical Wnt ligands and Wnt antagonists, EGF ligands and R-spondins, as well as ECM components. Moreover, as small intestinal organoids lack the formation of extruding villi, the lamina propria is likely also necessary for complete crypt-villus axis patterning and morphology. Importantly, stromal cells have additionally been shown to contribute to intestinal tumor development and progression (Powell, Pinchuk, Saada, Chen, & Mifflin, 2011).

As previously noted, stromal cells provide Wnt signals both in the colon, where epithelial niche cells do not produce Wnt ligands, and in the small intestine, in complement to Paneth cells. Similar to the epithelial deletion of Wnt3 by Farin et al. or Paneth cell ablation by Durand et al., Kabiri and coworkers used epithelium-specific genetic ablation of *Poren*, encoding the ER O-acetyltransferase Porcupine required for Wnt secretion and

activity, to demonstrate that epithelial Wnts were dispensable in vivo but necessary for organoid growth in vitro (Kabiri et al., 2014). On the other hand, pharmacologic inhibition of Porcupine (affecting both the epithelium and surrounding mesenchyme) or whole-body deletion of Wntless (another protein required for Wnt secretion) results in Lgr5⁺ ISC loss (Kabiri et al., 2014; Valenta et al., 2016). Kabiri et al. further demonstrate that Porcupine-deficient organoid growth can be rescued by coculture with intestinal stromal cells (Kabiri et al., 2014), proving the compensatory ability of the intestinal stroma in this context. Several stromal cell types located adjacent to the epithelium, including subepithelial myofibroblasts, secrete Wnt2b and Wnt5a (Valenta et al., 2016). The fact that simultaneous deletion of Porcupine in both epithelial cells and myofibroblasts still does not disrupt crypt homeostasis suggests that other Wnt-producing cells of the lamina propria balance out the lack of Wnt ligand from these cells (San Roman, Jayewickreme, Murtaugh, & Shivdasani, 2014). A new report focused on CD34⁺ gp38⁺ mesenchymal cells, which are negative for endothelial (CD31), hematopoietic (CD45), and myofibroblast (α -smooth muscle actin) markers and surround crypts in both the small intestine and colon. Coculture of organoids with these cells results in the formation of highly proliferative spheroids, similar to addition of exogenous Wnt. Moreover, this overrules the need for R-spondin supplementation. Indeed, CD34⁺ gp38⁺ crypt stromal cells express high levels of *Wnt2b*, *Grentlin1*, and *Rspo1* (encoding R-spondin1) as compared to other lamina propria mesenchymal cell types (Szepounginski et al., 2016). These cells could therefore be an essential mesenchymal component of the ISC niche. In line with this, depletion of mesenchymal cells expressing Foxl1 (forkhead box L1, previously known as Fkh6), a transcription factor notably expressed in CD34⁺ gp38⁺ cells, results in disrupted epithelial morphology including loss of stem and proliferative cells (Aoki et al., 2016).

Immune cells also reside in the lamina propria and can interact with the epithelium and its stem cells. Defined subsets of hematopoietic cells such as $\gamma\delta$ T lymphocytes (Komano et al., 1995) or macrophages (Bansal, Trinath, Chakravorty, Patil, & Balaji, 2011) have been reported to affect epithelial homeostasis or recovery following infection or tissue damage, although the signals involved in these interactions remain unclear. Coculture of intestinal organoids with isolated lamina propria lymphocytes stimulates Lgr5⁺ CBC expansion and growth of the organoids in a way that is dependent on interleukin (IL)-22 production. The same effect is recapitulated both with isolated group 3 innate lymphoid cells (ILCs) and with recombinant IL-22

in a dose-dependent manner. This process is independent of Paneth cells or altered Wnt, Notch, or EGF activity, instead relying on Stat3 signaling in ISC (Lindemans et al., 2015). Upon injury in vivo, crypt-adjacent ILCs, which lack antigen receptors but respond to IL-23, produce IL-22 while epithelial cells upregulate their expression of the IL-22 receptor, overall promoting epithelial regeneration (Hanash et al., 2012; Lindemans et al., 2015). In counterpart, exacerbated IL-22 signals can promote tumorigenesis in the intestine (Huber et al., 2012; Kirchberger et al., 2013), emphasizing its effects on ISC.

7. THE MICROBIAL NICHE

Opposite to the lamina propria, the intestinal epithelium is exposed to the contents of the intestinal lumen, including passing digested foods but also the resident microbiota. In germ-free mice and rats, epithelial kinetics are slowed (Abrams, Bauer, & Sprinz, 1963; Alam, Midtvedt, & Uribe, 1994; Reikvam et al., 2011), suggesting a direct or indirect effect of the microbiota on ISC dynamics. Aside from an indirect effect on immune cell-epithelium cross-talks, Jones et al. identified one mechanism through which commensal bacteria could affect ISC. Intestinal epithelial cells express NADPH oxidases like Nox1 that produce reactive oxygen species (ROS) as an antimicrobial defense mechanism. Well-regulated levels of ROS can stimulate proliferation of stem and progenitor cells (Jones et al., 2013; Myant et al., 2013). Specific bacterial species (including *Lactobacillus rhamnosis* but not *Escherichia coli*) induce epithelial ROS and subsequent proliferation in a Nox1-dependent manner (Jones et al., 2013). Later, Jones et al. further showed a cytoprotective effect of *Lactobacillus* on crypt cells in the context of irradiation via the activation of an antioxidant response mediated by Nrf2 (nuclear factor erythroid 2) (Jones et al., 2015). Another mechanism by which the microbiota affects ISC is through microbial metabolites, several of which were discovered to suppress colon stem and progenitor cell proliferation in a recent screen by Stappenbeck's group (Kaiko et al., 2016). Of these, butyrate, a product of bacterial dietary fiber fermentation, had the most drastic effects, suppressing proliferation at low concentrations and inducing apoptosis at concentrations closer to those found in the colon lumen. The authors postulate that the crypt architecture and colonocytes' ability to oxidize and metabolize butyrate create a luminal gradient of butyrate down the crypt regulating stem and progenitor cell proliferation. Interestingly, butyrate-mediated inhibition of proliferation depends on epigenetic changes

involving histone deacetylase 3 (HDAC3), previously reported to link bacterial signals to epithelial response in the intestine (Alenghat et al., 2013).

Because of their localization furthest from luminal contents in the mucus- and AMP-protected crypt bottom, it was long thought that homeostatic ISC benefited from a sterile environment. Challenging this notion, work by Sansonetti's group revealed the existence of a restricted set of bacteria in colonic crypts of healthy mice, which they termed the "crypt-specific core microbiota" (Pédron et al., 2012). Interestingly, this predominantly aerobic population resembles the microbiota found in the midgut of invertebrates, hinting at a potential coevolutionary selection of commensals favoring proper gut dynamics. Host cells can recognize microbial signals via pattern recognition receptors (PRR), including transmembrane TLR and cytoplasmic Nod-like receptors (NLR). The stimulation of PRR by their corresponding antigens induce a signaling cascade via their common downstream adaptor molecule MyD88 (myeloid differentiation primary response gene 88), often resulting in induction of MAPK and NF- κ B (nuclear factor κ -light-chain-enhancer of activated B cells) signaling and production of proinflammatory cytokines. Both the commensal microbiota and MyD88 activity are required for epithelial recovery from DSS-induced colitis, revealing a surprising regenerative effect of microbiota-induced PRR signaling in the gut (Rakoff-Nahoum, Pglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004). MyD88 signaling also contributes to intestinal tumorigenesis (Rakoff-Nahoum & Medzhitov, 2007). These observations were made all the more relevant by reports of direct expression of PRR TLR4 and Nod2 (nucleotide-binding oligomerization domain-containing protein 2) by Lgr5⁺ ISC directly. The stimulation of these PRR in vivo or in organoid cultures has drastically different effects on ISC; whereas treatment with TLR4 antigen lipopolysaccharide induces apoptosis of Lgr5⁺ ISC (Neal et al., 2012), treatment with the Nod2 agonist muramyl dipeptide protects them from regenerative or ROS-induced stress (Nigro, Rossi, Commere, Jay, & Sansonetti, 2014). Stem cells are therefore in direct communication with luminal microbes and can thereby adjust their response to the luminal microenvironment. These studies also bring a new perspective on the reported link between the presence of particular commensals and pathogens and the development of CRC (Castellari et al., 2011; Dejea et al., 2014; Kostic et al., 2012, 2013; Machida-Montani et al., 2007).

Through its unique organization and multifaceted microenvironment, the intestinal system truly expands the boundaries of what is classically called the stem cell niche. Taking into account not just epithelial signals but also

those from the mesenchyme and lumen, ISC are able to continuously regenerate an epithelium adapted to the microenvironment they are faced with. Despite the continuous and rapid regeneration of this epithelium, cross-talk and redundancy between ISC niche components allow for a tightly controlled, robust system, and prevent unrestrained proliferation and tumorigenesis. Understanding the intricate regulatory networks behind this is made considerably easier with the development of modern techniques of genetic manipulation and ex vivo culture that will no doubt lead to great advances in the field in the years to come.

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Titre : Rôles de l'autophagie dans l'homéostasie des cellules souches intestinales

Mots clés : cellules souches, autophagie, intestin, cancer colorectal

Résumé : Le renouvellement de l'épithélium intestinal repose sur la prolifération incessante de cellules souches intestinales (CSI) capables de régénérer l'intégralité de l'épithélium en 3 à 5 jours. Des altérations de ces dernières sont à l'origine de la transformation tumorale. L'étude des mécanismes impliqués dans la protection des CSI face à différents stress est donc essentielle pour mieux comprendre l'homéostasie et les pathologies intestinales. Dans un modèle de souris prédisposées à développer des tumeurs suite à la perte du gène *Apc*, notre équipe a pu précédemment démontrer une activation de l'autophagie nécessaire à la croissance tumorale. Nos travaux visent à étudier le rôle de ce processus catabolique dans l'homéostasie des CSI. Pour ce faire, nous utilisons des modèles murins génétiquement modifiés et des cultures d'organoïdes afin d'étudier les effets de l'inhibition de l'autophagie dans l'homéostasie intestinale et en particulier dans les CSI.

Nos travaux indiquent que l'inhibition de l'autophagie par l'invalidation du gène *Atg7* conduit à une activation de p53 et à l'apoptose spécifique des CSI. L'invalidation simultanée du gène *Tp53* empêche la mort des CSI déficientes en autophagie. De plus, au long terme, ces souris développent des tumeurs, contrairement aux souris invalidées uniquement pour les gènes *Atg7* ou *Tp53*. Nous avons donc émis l'hypothèse que l'inhibition de l'autophagie sensibilisait les CSI à l'apoptose suite à une accumulation de dommages cytotoxiques. Par une analyse d'expressions géniques des CSI issues de cryptes contrôles et invalidées pour le gène *Atg7*, nous avons mis en évidence une altération des réponses associées au stress oxydant et à la réparation de l'ADN. Confirmant ces signatures, nous avons observé des dommages de l'ADN dans les cryptes déficientes en autophagie et un défaut de réparation de ces dommages suite à une irradiation. Nous observons également une accumulation d'espèces réactives de l'oxygène dans les CSI déficientes en autophagie associée à une atténuation de la réponse antioxydante médiée par NRF2. Des traitements antibiotiques à large-spectre ou antioxydants améliorent la survie des CSI déficientes en autophagie et soutiennent l'influence des espèces réactives de l'oxygène et de la flore intestinale sur la mort des CSI. Nos travaux indiquent donc un rôle important de l'autophagie dans la protection et le maintien des CSI, de par son contrôle des espèces réactives de l'oxygène, du microenvironnement bactérien et des voies de réparation de l'ADN.

Title: Role of autophagy in intestinal stem cell homeostasis

Key Words: stem cells, autophagy, intestine, colorectal cancer

Abstract: The renewal of the intestinal epithelium relies on the continuous proliferation of stem cells capable of regenerating the entire epithelium every 3 to 5 days. These intestinal stem cells (ISC) are thought to be the cell of origin for colorectal cancer. Thus, characterizing the mechanisms involved the protection of ISC against different stresses is key to understanding both intestinal homeostasis and tumor development. In tumoral tissue from mice predisposed to intestinal tumor development following the loss of the tumor suppressor gene *Apc*, our laboratory previously showed an upregulation of autophagy required for tumor growth. Our work aims to understand the role of this catabolic mechanism in the homeostasis of ISC. To this end, we use genetically modified mouse models and intestinal organoid culture to study the effects of autophagy inhibition in intestinal homeostasis and in particular in ISC.

We found that the inhibition of autophagy upon deletion of the gene *Atg7* results in p53 activation and apoptosis of ISC specifically. The simultaneous deletion of *Tp53* prevents the death of autophagy-deficient ISC. Moreover, over time, mice deficient for both *Atg7* and *Tp53* develop tumors, contrary to those deficient for either *Atg7* or *Tp53* alone. We therefore hypothesized that the inhibition of autophagy sensitizes ISC to p53-mediated apoptosis as a result of accumulated pro-tumorigenic damages. Transcriptomic analysis on sorted control or *Atg7*-deficient ISC revealed alterations in oxidative stress and DNA damage responses. Confirming these signatures, we observed DNA damages in autophagy-deficient crypts along with a defect in the repair of induced damages following irradiation. We additionally observed an accumulation of reactive oxygen species in autophagy-deficient ISC linked to a downregulation of the NRF2-mediated antioxidant response. Wide-spectrum antibiotic or antioxidant treatments improve the survival of autophagy-deficient ISC and support the contribution of both reactive oxygen species and the intestinal microbiota to the death of ISC. Our work therefore reveals an important function of autophagy in the integrity and maintenance of ISC by controlling reactive oxygen species, the microbial microenvironment and DNA repair pathways.