

Elucidating the mechanisms of colorectal cancer

THE ROLE OF URI IN COLORECTAL CANCER

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Abstract

Colorectal cancer is the third leading cause of cancer-related death, and its incidence is expected to increase. It is mandatory to understand the molecular mechanisms associated with this pathology. Adenomatous Polyposis Coli (APC) forms a complex with other proteins to interact with β -catenin and unable the Wnt signaling pathway. Familial Adenomatous Polyposis (FAP) is associated with mutations in the *APC* locus and polyp formation in the colon. Further loss of expression of the tumor suppressor p53 potentiates the progression of adenomas to adenocarcinomas. Therefore, *Apc* mice models have been used to study the pathogenesis of colorectal cancer, although they form more tumors in the small intestine. Unconventional Prefoldin RPB5 Interactor-like protein (URI) is a molecular chaperone and an oncogene. Its levels are increased in colorectal cancer patients, especially when the protein is mutated, associating with a worse prognosis.

In this work, the effects of URI loss of function have been studied in *Apc* wild-type and mutant mice. The tumors formed in the intestine were analyzed, regarding p53 expression, activation of Unfolded Protein Response (UPR), apoptosis, senescence, and immune infiltration.

All the mice with *Apc* mutation developed tumors in the small intestine, however in the ones lacking URI the tumors were less frequent, smaller, and less aggressive, revealing that URI loss impairs tumor formation in *Apc* mutant mice. These mice were also able to express p53, unlike the *Uri* wild-type animals. However, they did not reveal an increase in apoptosis or in UPR. URI loss of function potentiated cell-cycle arrest, demonstrated by an increase in protein and mRNA levels. CD3 and NK1.1. positive cells within the tumors were quantified, and URI loss did not affect tumor infiltration by immune cells. Taken together, these results suggest that URI ablation activates cell senescence, although further confirmation must be performed.

This work was able to reveal some mechanisms associated with URI loss of function and how this can weaken the ability of *Apc* mutant mice to form tumors in the intestine by (1) increasing the expression of p53; (2) increasing the expression of antiapoptotic proteins; (3) promoting cell cycle arrest; while (4) not affecting UPR or immune cell infiltration.

We also analyzed the stability of mutated versions of mouse and human URI, that can be related to an increase oncogenic effect. We detected that deleting Aspartic Acid aminoacids in mouse has the same effects that in human, and URI gets more stable. This discovery is leading the generation of new mice models, with more oncogenic versions of URI.

Keywords: colorectal cancer, *Apc*-mutant mice, URI protein

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Abbreviations and Symbols

List of abbreviations

APC	Adenomatous Polyposis Coli
ASK1	Apoptosis Signal-regulating Kinase 1
ATF4	Activating Transcription Factor 4
ATF6	Activating Transcription Factor 6
BAK	BCL-2 Antagonist/Killer 1
BAX	BCL-2 Associated X-protein
BCL-2	B-cell lymphoma 2
BCL-XL	BCL-extra Large
BI-1	BAX inhibitor 1
BID	BH3 Interacting-Domain
BIM	BCL-2-Interacting Mediator of cell death
BiP	Binding immunoglobulin protein
bZip	Basic Leucine Zipper
CC3	Cleaved Caspase 3
CD3	Cluster of Differentiation 3
CD8	Cluster of Differentiation 8
CHOP	C/EBP Homologous Protein
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
CMSs	Consensus Molecular Subtypes
CNAs	Copy-Number Alterations
CRC	Colorectal Cancer
CRCSC	Colorectal Cancer Subtyping Consortium
CREB/ATF	cAMP-Response Element-Binding protein/Activating Transcription Factor
DAB	3,30-diaminobenzidine
<i>E. coli</i>	<i>Escherichia coli</i>
eIF2	Eukaryotic Translation Initiation Factor 2
ER	Endoplasmic Reticulum
ERAD	ER-Associated Degradation
ERO1 α	ER Oxidoreduction-1 α
FAP	Familial Adenomatous Polyposis
GADD	Growth Arrest and DNA Damage-inducible protein

GALT	Gut-Associated Lymphoid Tissue
GPCRs	G-Protein-Coupled Receptor
GRP78	Glucose-Regulated Protein 78
GRP94	Glucose-Regulated Protein 94
GSK3B	Glycogen Synthase Kinase 3 Beta
H&E	Hematoxylin and Eosin
HNF4A	Hepatocyte Nuclear Factor 4 Alpha
HSP	Heat Shock Protein
HSPA5	Heat Shock 70 kDa Protein 5
hURI	Human URI
IFN- γ	Interferon gamma
IkB	Inhibitor of kappa B
IL-2	Interleukin-2
ILFs	Isolated Lymphoid Follicles
IRE1	Inositol Requiring Kinase 1
ISCs	Intestinal Stem Cells
JNK	c-Jun N-terminal Kinase
<i>KRAS</i>	Kirsten Rat Sarcoma Viral oncogene homolog
LDL	Low-Density Lipoprotein
Lgr5 ⁺	Leucine-rich repeat-containing G protein-coupled Receptor 5-positive
LPR	Receptor-Related Protein
LRCs	DNA-label-Retaining Cells
MAP	MUTYH-Associated Polyposis
MDM2	Murine/human Double Minute 2
MET	Mesenchymal-to-Epithelial Transition
MMLV	Moloney Morine Leukaemia Virus
MMR	Mismatch Repair
mURI	Mouse URI
NAD ⁺	Nicotinamide Adenine Dinucleotide
NF- κ B	Nuclear transcription Factor- κ B
NK	Natural Killer
NRF2	Nuclear factor E2 Related Factor 2
P58IPK	DNAJ homolog subfamily C member 3
PAQosome	Particle for Arrangement of Quaternary structure
PBS	Phosphate-Buffered Saline
PBS-T	Phosphate-Buffered Saline with Tween
PCR	Polymerase Chain Reaction
PERK	Pancreatic ER eIF2 α Kinase

PI3K	Phosphatidylinositol 3-kinase
PJS	PEUTZ-JEGHERS Syndrome
PUMA	p53 Upregulated Modulator of Apoptosis
RIDD	Regulated IRE1-Dependant Decay of mRNA
RIPA	Radio Immuno Precipitation Assay
ROS	Reactive Oxygen Species
RPB5	RNA Polymerase Binding subunit 5
rpm	Rotations per minute
RT	Room Temperature
SASP	Senescence-Associated Secretory Phenotype
SCNAs	Somatic Copy Number Alterations
SDS-PAGE	Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis
SILTs	Solitary Isolated Lymphoid Tissues
STAP1	Signal-Transducing Adaptor Protein 1
sXBP1	Spliced XBP1
TA	Transit Amplifying
TAE	Tris-Acetate-EDTA
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline with Tween
TCGA	The Cancer Genome Atlas
TGF- β	Transforming Growth Factor beta
Th1	T Helper lymphocytes 1
TNFR2	Tumour Necrosis Factor Receptor-associated factor 2
TRAF2	IRE1-TNF Receptor-Associated Factor 2
UPC	URI PFDN-like Complex
UPR	Unfolded Protein Response
URI	Unconventional Prefoldin RPB5 Interactor-like protein
uXBP1	Unspliced XBP1
VEGF	Vascular Endothelial Growth Factor
XBP1	X-box-binding protein 1
γ H2AX	Phosphorylated Histone H2AX

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Chapter 1

1. Introduction

1.1. The intestinal tract

The primary function of the intestinal tract is food digestion, absorption of nutrients and water, and production of vitamins. The mammalian one is covered by a single layer of epithelial cells¹ renewed every 4-5 days, which besides enabling digestion, and water and nutrient absorption, also forms a barrier against luminal pathogens (van der Flier & Clevers, 2009).

The intestinal tract is anatomically divided into the small intestine and the large intestine (or colon). The intestines share a basic structure - an outer layer of smooth muscle with enteric nervous system, an intermediate layer of connective tissue, with nerves and lymphatic vessels, and an inner absorptive epithelium (Barker et al., 2010). This epithelial layer is the one that differentiates the small intestine and the colon. While the small intestine has a large surface area compared to its luminal volume, to potentiate its absorption functions, the large intestine has a larger volume and a smaller surface, potentiating its capacity as a reservoir (Helander & Fändriks, 2014).

The small intestine, around 7 meters long in humans, can be divided into the duodenum, the jejunum, and the ileum (Helander & Fändriks, 2014). Microscopically, it is composed by the villi, finger-like protrusions of the gut wall, which project into the gut lumen and maximize the absorptive surface area (Clevers, 2013), and the crypts of Lieberkühn, that surround the villi and compose the proliferative area, comprised by intestinal stem cells² (ISCs) (Clevers, 2013), as represented in Figure 1. Within the intestinal epithelium reside different types of cells - the absorptive lineage is composed of enterocytes, while the secretory lineage is formed by Goblet cells, enteroendocrine cells and Paneth cells. The enterocytes are highly polarized columnar cells, that play an important role in the secretion of hydrolases and in the absorption of molecules from the lumen (Moor et al., 2018). Goblet cells, simple columnar epithelial cells, and enteroendocrine cells are specialized in secreting, respectively, mucins and peptide hormones, and are present in both the villi and the crypts (Lee et

¹ Epithelial cells form a continuous sheet of tightly linked cells that lines both the surfaces and inside cavities of the body - the epithelium.

² Stem cells are undifferentiated cells, with the ability to self-renew by symmetric division (generating two daughter stem cells), non-self-renewing symmetric division (creating two progenitor cells, but not stem cells) and asymmetric division (generating one stem cell and one progenitor cell). Stem cells reside in their niche, within a tissue, which promotes their activities and maintenance of their characteristics.

al., 2002; Pelaseyed et al., 2014). Paneth cells occupy the bottom positions in the crypt and secrete antimicrobial peptides, such as lysozyme and defensins, which regulate the composition of the intestinal flora and maintain a sterile environment (Sato et al., 2011). Finally, the microfold cells reside in the specialized epithelium and intervene in the immunity, while Tuff cells serve to sense luminal contents and occur in the crypt-villi axis (Clevers, 2013).

The epithelia of the villi extend to the lamina propria, where they form the crypts, where ISCs and their progeny, and transit-amplifying (TA) cells reside (van der Flier & Clevers, 2009). Under homeostatic conditions, the rapid turnover of the intestinal epithelium is driven by leucine-rich repeat-containing G protein-coupled receptor 5-positive ($Lgr5^+$) ISCs, located at the base of the crypts (Chaves-Pérez et al., 2019). ISCs give rise to $Lgr5^{low}$ progenitors (or TA cells), which progressively migrate and differentiate toward the luminal surface. The fully differentiated and functional epithelial cells undergo apoptosis after reaching the surface, after around five days (Chaves-Pérez et al., 2019; van der Flier & Clevers, 2009). Paneth cells migrate downwards to the bottom of the crypt, forming the stem cell niche (Simons & Clevers, 2011). Above the Paneth cells reside the DNA-label-retaining cells (LRCs), also known as “position 4” or “+4” cells, that are $Lgr5^+$ and provide a reserve pool of stem cells (Clevers, 2013).

Based on the ability of stem cells to divide and differentiate, a hierarchical model consisting of stem cells, transit-amplifying cells and terminally differentiated cells has been proposed, allowing adult organs, as the intestine and the skin, to recover from injury. Indeed, the intestinal epithelium is a very dynamic tissue and deregulation of cell pathways can lead to various pathologies, such as cancer.

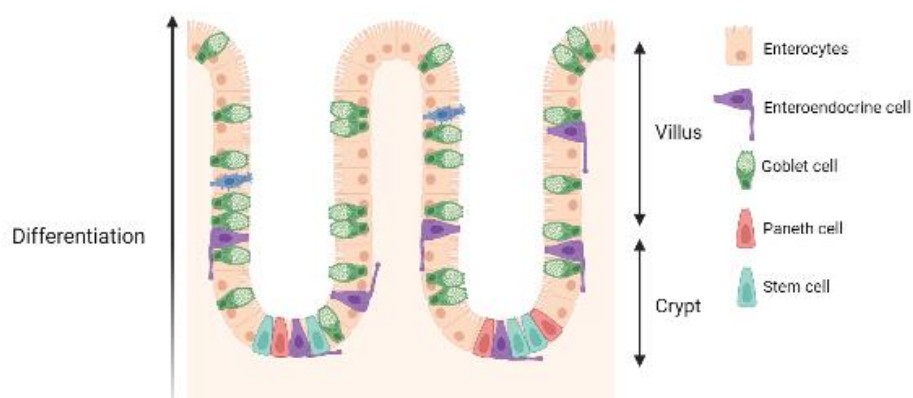


Figure 1 - Schematic representation of the crypt-villus unit that composes the small intestine. The degree of differentiation of intestinal cells increases from the crypts, bottom part, to the villi. Cells migrate towards the tip of the villi, where they undergo apoptosis. The location of the intestinal cells is represented in the scheme - Enterocytes secrete hydrolases and participate in the absorption of molecules from the lumen, Goblet cells secrete mucins, Enteroendocrine cells secrete peptide hormones, Paneth cells secrete antimicrobial peptides, regulating the intestinal flora, and Stem cells maintain the turnover of the intestinal epithelium. Created with BioRender.com.

The large intestine, varying between 110 and 190 cm in humans, is the last part of the gastrointestinal track and is where water and salts are absorbed, waste material is stored before defecation, and where undigested food gets fermented by the bacterial microflora (Helander & Fändriks, 2014). This organ is composed by the cecum, the ascending colon, transverse colon, descending colon, sigmoid colon, and rectum, as shown in Figure 2. The colonic epithelium consists of a plane surface with multiple epithelial invaginations that form crypts, but lack villi (Simons & Clevers, 2011). As in the ileum, the colon reveals a big amount of mucus-secreting Goblet cells, reflecting a need to lubricate the passage of feces, as they get more compact (Barker et al., 2010).

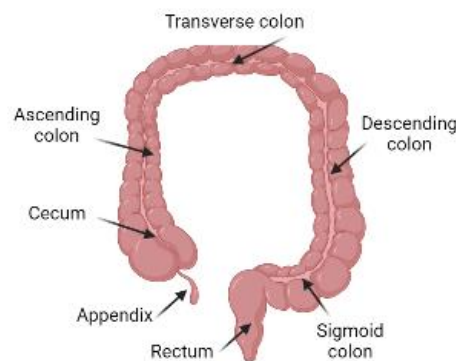


Figure 2 - The large intestine anatomy. The picture represents different anatomic areas of the large intestine. Created with Biorender.com.

1.2. Colorectal Cancer

Cancer is a multifactorial neoplastic disease, resulting from a combination of genetic and environmental factors that affect cell proliferation, differentiation, and survival.

Colorectal cancer (CRC) is a gastrointestinal malignancy, that begins with an aberrant, yet benign, cell growth in the colon or rectum epitheliums, which develops a polyp. In 10 to 15 years, this neoplastic precursor lesion can progress, with cells getting the ability to invade the different layers of the epithelium and form colorectal cancer (Dekker et al., 2019). Metastases arise when tumor cells penetrate the blood or lymphoid vessels, spreading to distant parts of the organism (Paduch, 2016).

The relative survival of colorectal cancer patient decreases with age and the stage of diagnosis is the most important prognostic factor. For example, in the USA, in the beginning of the decade of 2000, the 5-year relative survival of patients diagnosed with colorectal cancer with localized stage was 90.1%, while for patients with regional spread it reached 69.2%, and decreased to 11.7% for

patients with distant tumors (Brenner et al., 2014). However, the prognosis has been improving during the last decades, especially in the high-income countries.

1.2.1. Incidence and mortality of colorectal cancer

Colorectal cancer was the fourth most diagnosed cancer type worldwide, in 2020, with a higher incidence in developed countries, as described by the International Agency for Research on Cancer (Ferlay J, 2020), data shown in Figure 3 and Figure 4. Incidence is higher in men than women (Dekker et al., 2019), and strongly increases with age, with an average diagnosis age of 70 years in the developed countries (Brenner et al., 2014). The highest incidence is registered in countries in Oceania, North America and Europe, while the lowest incidence rates are reported in south and central Asia and Africa (Brenner et al., 2014; Ferlay J, 2020).

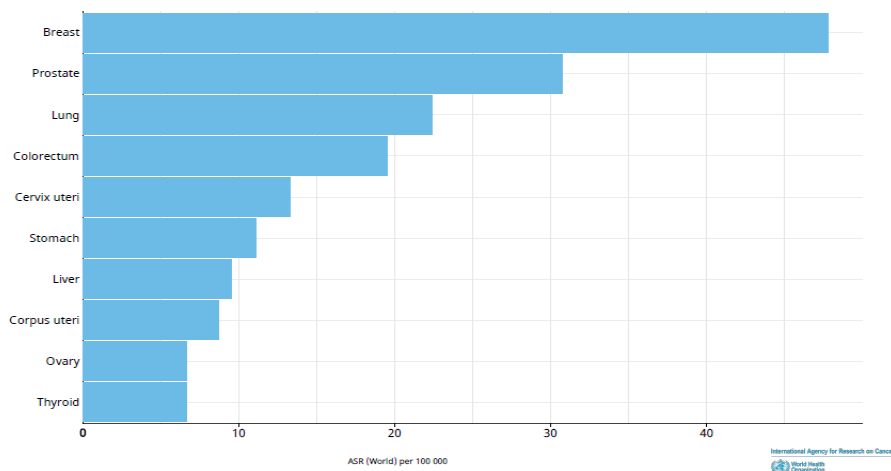


Figure 3 - Estimated age-standardized incidence rates for all cancer types, in 2020, worldwide, for both sexes and all ages, excluding non-melanoma skin cancer (Ferlay J, 2020). Age-standardised rates (ASR) are summary measures of the rate that a population would have if it had a standard age structure. Standardisation is necessary when comparing several populations that differ with respect to age because age has a powerful influence on the risk of being diagnosed with or dying from cancer.

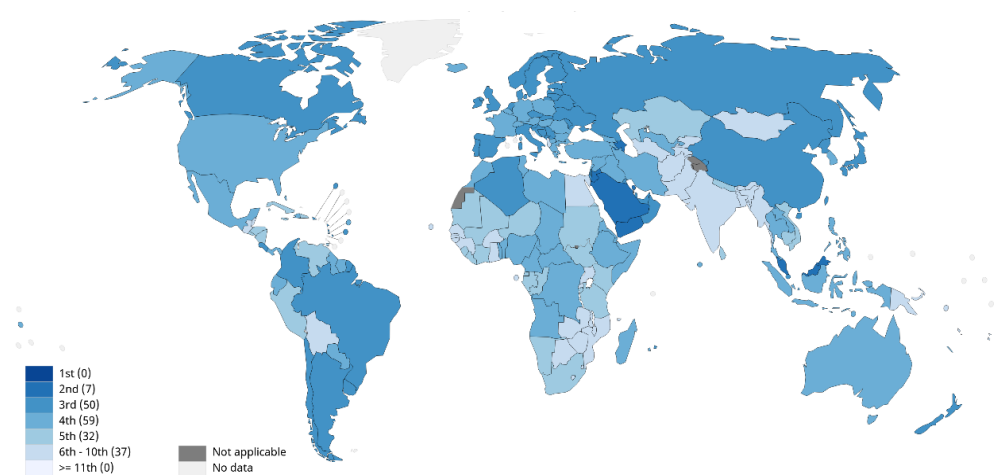


Figure 4 - Estimated age-standardized incidence rates in the world for colorectal cancer, in 2020, for both sexes, all ages, excluding non-melanoma skin cancer (Ferlay J, 2020). Age-standardised rates (ASR) are summary measures of the rate that a population would have if it had a standard age structure. Standardisation is necessary when comparing several populations that differ with respect to age because age has a powerful influence on the risk of being diagnosed with or dying from cancer. Dotted and dashed lines on maps represent approximate borderlines for which there might not yet be full agreement.

Regarding mortality, colorectal cancer is the third-leading cause of cancer-related deaths, as described by the International Agency for Research on Cancer (Ferlay J, 2020), data shown in Figure 5. However, evaluating the incidence of these cancers in the last decades, one can realize that in east Asia and eastern Europe there has been a decrease in the number of deaths, probably due to improved early detection methods and better treatments (Brenner et al., 2014).

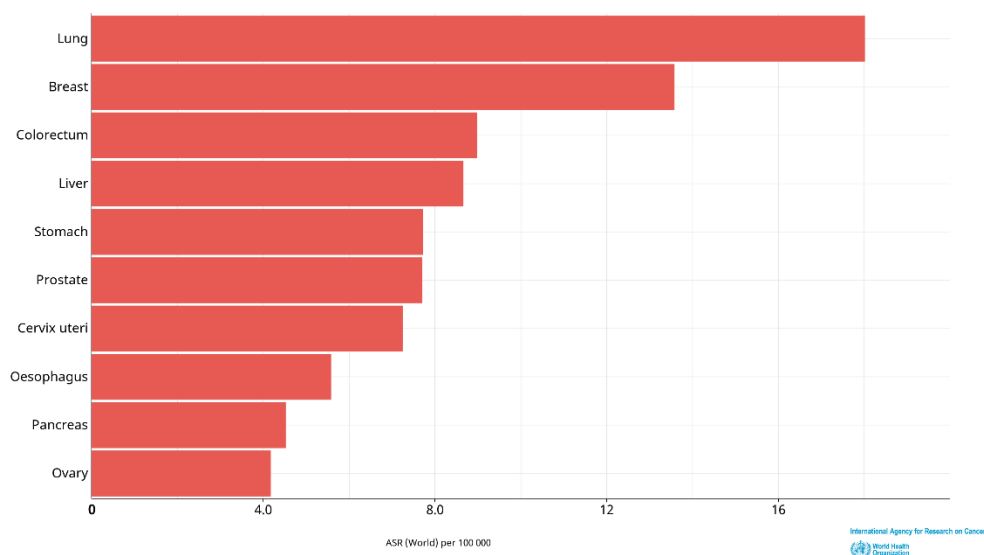


Figure 5 - Estimated age-standardized mortality rates for all cancer types, in 2020, worldwide, for both sexes and all ages, excluding non-melanoma skin cancer (Ferlay J, 2020). Age-standardised rates (ASR) are summary measures of the rate that a population would have if it had a standard age structure. Standardisation is necessary when comparing several populations that differ with respect to age because age has a powerful influence on the risk of being diagnosed with or dying from cancer.

Elucidating the mechanisms of colorectal cancer

Although screening strategies, such as fecal occult blood test (FOBT), flexible sigmoidoscopy, and colonoscopy, have been proven effective trying to reduce the incidence and mortality of the disease (Taylor et al., 2010), the number of patients with either colon or rectum patients is expected to increase in the next decades, as described by the International Agency for Research on Cancer (Ferlay J, 2020), represented by Figure 6. Thus, there is a need to try to understand the mechanisms of colorectal cancer, in order to improve early detection and treatments.

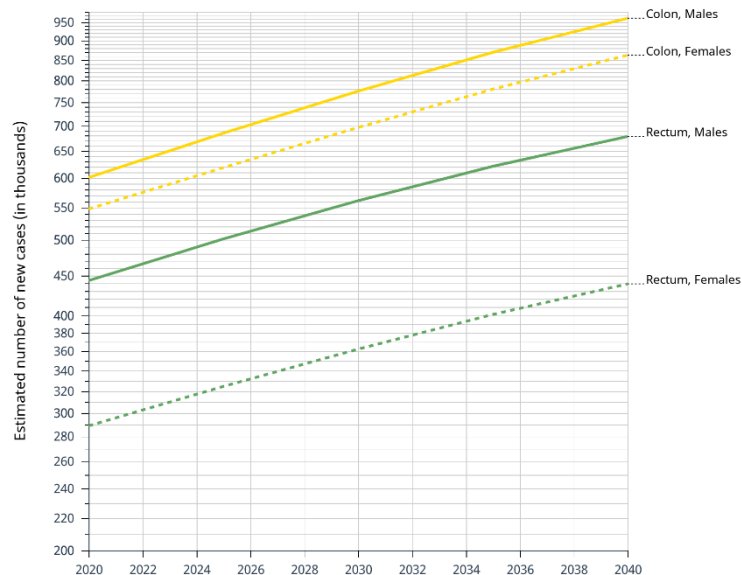


Figure 6 - Estimated number of new cases of colon and rectum cancers, in males and females, from 2020 to 2040, worldwide (Ferlay J, 2020).

1.2.2. Risk factors for colorectal cancer

Colorectal cancer can have both hereditary and environmental risk factors. Unlike other types of cancer, e.g., lung cancer, there is not a definition of a concrete risk factor accounting for most cases of colorectal cancer, although ageing and male sex have a role in this. The following risk factors have also been identified and established by different epidemiological studies - ulcerative colitis (Jess et al., 2012), smoking (Liang et al., 2009), family history of colorectal cancer (Taylor et al., 2010), increased alcohol consumption (Fedirko et al., 2011), high consumption of red and processed meat (Chan et al., 2011), obesity (Ma et al., 2013), and diabetes (Jiang et al., 2011). Further evidence suggests that infection with *Helicobacter pylori* (Sonnenberg & Genta, 2013), *Fusobacterium spp* (Kostic et al., 2012), and other infectious agents might be associated with an increased risk of developing colorectal cancer. Moreover, the family history plays a role in less than 35% of all patients, with the risk depending on both the number and the degree of affected relatives and age at diagnosis (Dekker et al., 2019; Taylor et al., 2010).

The most common hereditary colorectal cancer predisposing syndrome is the Lynch syndrome, also known as hereditary non-polyposis colorectal cancer, and is related to 2-4% of colorectal cancer cases (Ma et al., 2018). Lynch syndrome patients develop colorectal cancer at an average of 45 years-old, while the general population develops the disease at the age of 67. The syndrome is acquired in an autosomal dominant mutation in one allele of a DNA mismatch repair (MMR) gene, usually *MLH1* and *MSH2* (up to 90%), or less frequently *MSH6* (approximately 10%) and *PMS2* (rarer). Moreover, a deletion in the epithelial cell adhesion molecule *EPCAM*, located upstream of *MSH2*, can occur, leading to *MSH2* silencing, causing Lynch syndrome. Loss of MMR proteins is used to identify tumor with microsatellite instability and is thought to be an early step in the progression from adenomas to carcinomas (Ma et al., 2018).

The second most common inherited colorectal cancer syndrome is Familial Adenomatous Polyposis (FAP), accounting for approximately 1% of all colorectal cancer cases (Ma et al., 2018). FAP is characterized by the development of adenomatous polyps, from late childhood to early adolescence, within the colon. The crypts become dysplastic and these lesions are precursors of adenomas and subsequent cancers. FAP is an autosomal dominant hereditary cancer syndrome caused by a mutation in the adenomatous polyposis coli gene (*APC*), known as a gatekeeper³ gene for colorectal cancer. In addition to the germline *APC* mutation, colorectal tumors from FAP patients develop additional somatic *APC* mutations or loss of heterozygosity at this locus. Therefore, *APC* loss is described as one of the first steps in tumorigenesis - the *APC* mutations are followed by additional genetic mutations in, amongst others, *KRAS* and *TP53* genes (Ma et al., 2018).

MUTYH-Associated Polyposis (MAP) is a recessively inherited syndrome, caused by biallelic mutations in *MUTYH* (or *MYH*). The patients develop 10 to 100 adenomas in the colorectum, correlating with an increased risk of colorectal cancer, at an average of 48 years old (Ma et al., 2018). *MUTYH* functions as part of the base excision repair pathway, which repairs DNA damage caused by reactive oxygen species, functioning as a molecular switch for apoptosis under oxidative stress.

Besides, PEUTZ-JEGHERS Syndrome (PJS) patients have a 90% life-time risk of developing various cancers, including a 70% risk of gastrointestinal cancer and a 50% risk of breast cancer (Ma et al., 2018).

³ Tumor suppressor genes can be separated into two major categories - gatekeepers and caretakers. Gatekeepers directly inhibit tumor growth or promote tumor death. Thus, inactivation of these genes contributes directly to cancer formation and progression.

1.2.3. Molecular pathogenesis of colorectal cancer

The molecular pathogenesis of colorectal cancer, presented in Figure 7, is heterogenous and relate to patients' prognosis and treatment response (Brenner et al., 2014). The disease can develop during the course of ten years and *APC* gene mutations are an important and early event in the formation of colorectal cancer, occurring in more than 70% of these adenomas (Brenner et al., 2014).

In sporadic colorectal cancer patients, mutations in the normal epithelium can form an early adenoma, benign tumor of epithelial tissue with glandular origin, which evolves into a late adenoma, and, finally, an adenocarcinoma, its malignant form. Damage in the epithelium can also promote inflammation-driven mutations.

The consensus states that there are two major pathways to develop colorectal cancer. The traditional adenoma-carcinoma pathway, also known as chromosomal instability sequence, that leads to 70-90% of all colorectal cancer, and the serrated neoplasia pathway that leads to close to 10-20% of new cases (Dekker et al., 2019). In the first scenario, the *APC* mutations result in the formation of an adenoma and are followed by activating mutations in the *KRAS* oncogene and loss of the tumor suppressor p53, which promote cell expansion and invasion, respectively (Brenner et al., 2014; Leslie et al., 2002). On the other hand, the serrated neoplasia pathway associates with *RAS* and *RAF* mutations, and epigenetic instability, which leads to microsatellite stable and unstable cancers (Dekker et al., 2019).

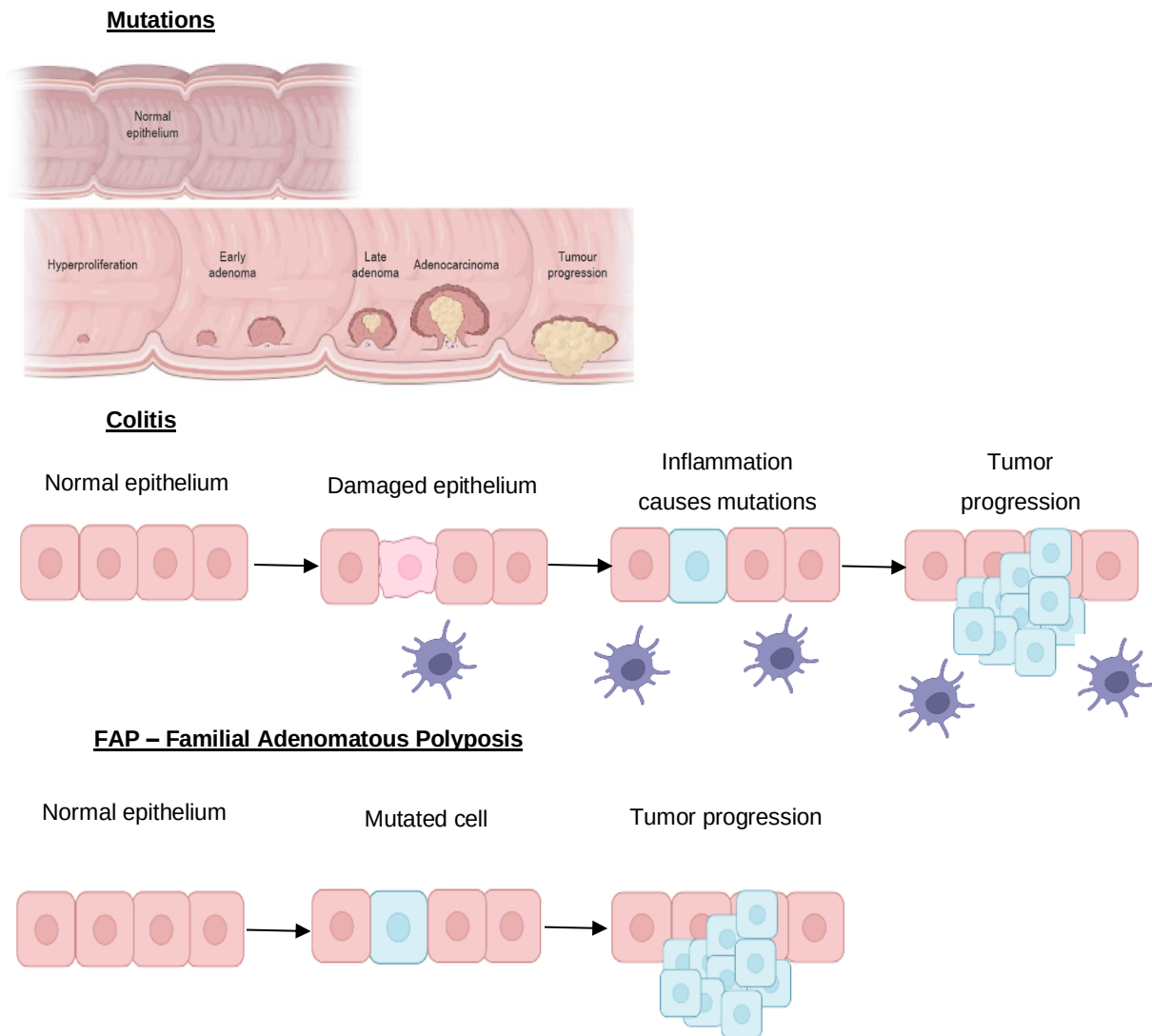


Figure 7 - The molecular pathogenesis of colorectal cancer. When mutations are involved, cells start to hyperproliferate, creating an adenoma, which involves into an adenocarcinoma. In colitis patients, the damage in the epithelium potentiates an inflammation state, that causes mutations and consequent tumor progression. In patients with Familial Adenomatous Polyposis (FAP), associated with the hereditary cases of colorectal cancer, cells present mutations in the *APC* gene and promote the development of colorectal cancer. Created with BioRender.com.

1.2.4. Tumor suppressors and oncogenes altered in colorectal cancer

1.2.4.1. *Adenomatous Polyposis Coli* tumor suppressor

The most common mutations occurring in colorectal cancer cause inactivation of the Adenomatous Polyposis Coli (*APC*) gene, which encodes for the APC tumor-suppressor protein. APC is involved in a variety of cellular functions, including adhesion, apoptosis, and cell cycle control.

While wild-type APC forms a complex with GSK-3 β , Axin and other cofactors to degrade β -catenin, limiting the activity of the Wnt signaling pathway, APC loss leads to accumulation of β -

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catenin and activation of the Wnt signaling pathway (Ma et al., 2018), as demonstrated in Figure 8. Somatic inactivation of APC leading to Wnt overactivation, through stabilization of β -catenin and its nuclear translocation, is found in about 80% of non-inherited colorectal cancer cases (Goss & Groden, 2000).

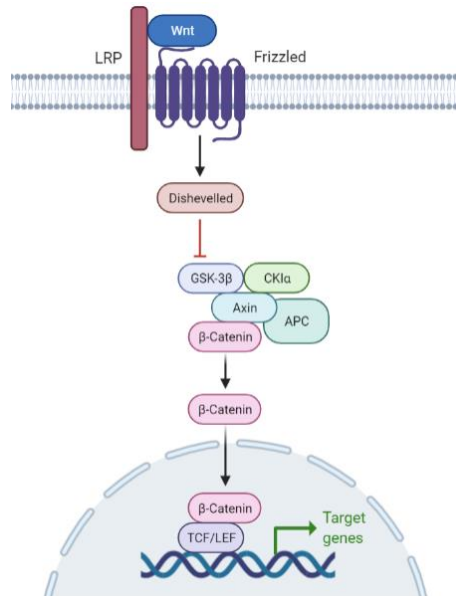


Figure 8 - The β -catenin pathway. APC forms a complex with other proteins, limiting the activity of Wnt signaling pathway. When APC is mutated, β -catenin accumulates and Wnt pathway is activated. Created with Biorender.com.

Wnts constitute a family of growth factors, which mediate biological processes, as embryogenesis, organogenesis, and tumorigenesis. They form a ligand-receptor complex with a G-protein-coupled receptor (GPCRs) and a low-density lipoprotein (LDL) receptor-related protein (LPR). This initiates intracellular signaling cascades, including the β -catenin pathway, signaling through LRP-5 or LRP-6 (Bodine & Komm, 2007). In the intestine, Wnt signaling imposes a crypt progenitor phenotype, while also influencing Paneth cell positioning in the crypt. The highest Wnt activity is observed in the niche, with a decrease in its activity while moving up to the surface, indicating that the Wnt signaling pathway is needed to maintain normal crypt homeostasis (Ma et al., 2018).

Intestinal epithelium cells after APC loss are no longer capable of expressing the villi cell markers alkaline phosphatase or Villin, and present perturbed positioning. Besides, this inactivation abolishes migration along the crypt-villi axis and leads to the production of undifferentiated daughter cells with increased proliferation, properties of premalignant cells. The increased apoptosis reported in APC ablated-cells can be a consequence of c-Myc up-regulation, in a mechanism shown by Pelengaris

et al. (2002). Also, there are matrix interactions, stimulating anoikis⁴ signal, demonstrated by the changes in the expression of antiadhesive molecule tenascin and laminins. Furthermore, mitosis is aberrant in this context. Regarding β -catenin, it is relocalized to the nuclei upon loss of APC (Sansom et al., 2004).

Thus, APC mutant cells are more prone to develop tumors.

1.2.4.2. *TP53* tumor suppressor

The second most important tumor suppressor gene in colorectal cancer is *TP53* gene, located on chromosome 17p, which encodes for a protein of 53 kDa, p53. Once activated, p53 upregulates its negative regulator murine/human double minute 2 (MDM2), which regulates the ubiquitination of p53 and leads to its degradation, maintaining low levels of p53 in normal cells (Ma et al., 2018). p53 is involved in the protection of the DNA integrity, cell cycle arrest, apoptosis, or senescence, in the presence of cellular stress (Hernández Borrero & El-Deiry, 2021). Usually, *TP53* gets inactivated by a combination of a missense mutation that inhibits its transcriptional activity and deletion of 17p chromosomal region, losing its second allele. This gives cancer cells a survival advantage, promoting the transformation of adenomas into invasive carcinomas (Markowitz & Bertagnolli, 2009).

1.2.4.3. *TGF- β* Tumor-suppressor pathway

TGF- β is inactivated in about one third of the colorectal cancer cases. Inactivation of the tumor suppressor is related to inactivating missense mutations, affecting TGFBR2 kinase domain, and inactivation of genes involved in the downstream signaling, as *SMAD2* and *SMAD4* (Markowitz & Bertagnolli, 2009). These mutations correlate with the transition from adenoma to high-grade dysplasia or carcinoma.

⁴ Anoikis is a form of programmed cell death, which occurs in anchorage-dependent cells when they detach from the surrounding extracellular matrix.

1.2.4.4. Kirsten Rat Sarcoma Viral oncogene homolog

Several oncogenes play roles in the development of colorectal cancer. Rat Sarcoma Viral oncogene homolog (*KRAS*) encodes for the RAS protein, which activates the MAPK signaling pathway. Modifications in *KRAS* occur in 37% of colorectal cancers (Markowitz & Bertagnolli, 2009), leading to worse prognosis than in wild-type tumors (Wang & Fakih, 2021).

RAS proteins are part of guanosine triphosphatase families, alternating between the active GTP-bound state and the inactive GDP-bound state. The mutations in *KRAS* that promote tumorigenesis disable GTP hydrolysis and lock RAS proteins into an activated state (Huang et al., 2018; Wang & Fakih, 2021). *KRAS* mutations occur early during the progression of colorectal adenoma to carcinoma and culminate in the activation of Ras/Raf/MEK/ERK and Ras/PI3K/PTEN/Akt/mTOR signaling cascades. The events that lead to this activation are mediated by kinases, phosphatases and proteins, and generally start from the cell surface and lead to control gene expression (Chappell et al., 2011). Dysregulations in these pathways cause uncontrolled cellular growth and proliferation.

1.2.4.5. *BRAF* oncogene

BRAF oncogene mutations signal BRAF serine-threonine kinase activity, that drives the MAPK signaling cascade (Markowitz & Bertagnolli, 2009). These mutations are very common in hyperplastic polyps, serrated adenomas, and proximal colon cancers.

1.2.4.6. *Phosphatidylinositol 3-kinase* oncogene

Phosphatidylinositol 3-kinase (PI3K) gene encodes the catalytic subunit of PI3K. Inactivating mutations in this gene promote the transition from adenoma to carcinoma and the most frequent occur in the catalytic subunit of PIK3CA (Markowitz & Bertagnolli, 2009).

1.2.5. Subtypes of Colorectal Cancer

The molecular pathways of colorectal cancer formation involve genomic and epigenomic instabilities, and four have been well described - chromosome instability, the most common, microsatellite instability (MSI), CpG island methylator phenotype (CIMP) and DNA global hypomethylation (YIU & YIU, 2016).

The Cancer Genome Atlas (TCGA) (The Cancer Genome Atlas) presents data from over 10 000 cancer patients, and together with other sources, was used by the Colorectal Cancer Subtyping Consortium (CRCSC) to described common characteristics among six independent classification systems (YIU & YIU, 2016). Thus, four consensus molecular subtypes (CMSs) were described - CMS1, CMS2, CMS3 and CMS4. However, around 13% of the samples had heterogenous patterns of CMS, which did not constitute a fifth subtype (Guinney et al., 2015). The characteristics of these subtypes are summarized in Table 1.

CMS1, or MSI or immune, subtype was found in 14% of samples. This was characterized by hypermutation, low prevalence of somatic copy number alterations (SCNAs), overexpression of proteins involved in DNA damage repair, occurrence of *BRAF* mutations, hypermethylation, increased expression of genes related to diffuse immune infiltration consisting mainly of Th1⁵ and cytotoxic T-cells, and activation of immune evasion pathway. Patients with CMS1 present worse prognosis after relapse and exhibited up-regulation in the levels of immune response proteins (Guinney et al., 2015; YIU & YIU, 2016).

CMS2, or canonical, was found in 37% of samples, CMS3, or metabolic, in 13% and CMS4, or mesenchymal, in 23% of samples, and exhibited higher chromosomal instability (CIN) (Guinney et al., 2015; YIU & YIU, 2016).

CMS2 is the subtype displaying more chromosome instability, with more copy number gains in oncogenes and losses in tumor suppressor genes, when compared with other subtypes. CMS2 samples revealed amplifications of the gene encoding the transcription factor HNF4A and were related to marked up-regulation of WNT and downstream targets associated with colorectal cancer. This subtype was associated with better survival upon recurrence (Guinney et al., 2015; YIU & YIU, 2016).

On the other hand, CMS3 samples have a distinctive global genomic and epigenomic profile, comparing to other CIN tumors - there were less SCNAs, 30% of the samples were hypermutated,

⁵ T helper lymphocytes 1 (Th1) are a subtype of effector T cells, characterized by the production of IL-2 and IFN- γ .

there was a higher prevalence of CpG island methylator phenotype (CIMP)-low clusters in TCGA samples, and an occurrence of *KRAS* mutations (Guinney et al., 2015; YIU & YIU, 2016).

CMS4 was associated with high SCNA, confirming chromosome instability, TGF β activation, stromal infiltration, angiogenesis, matrix remodeling and complement-mediated inflammatory system, confirmed by the up-regulation of epithelial-to-mesenchymal transition (MET). Patients with CMS4 were usually diagnosed at cancers stages⁶ III and IV and had worse survival, according to clinicopathological features, MSI status and *KRAS* and *BRAF* mutations. Proteins involved in stromal invasion, mesenchymal activation and complement pathways were up-regulated in CMS4 colorectal cancer subtype (Guinney et al., 2015; YIU & YIU, 2016).

Table 1 - Summary of the characteristics of the Colorectal Cancer. The characteristics of each Consensus Molecular Subtype (CMSs) are described, including its incidence and characteristics. CIMP, CpG island methylator phenotype; MSI, microsatellite instability; SCNA, somatic copy number alterations.

CMS1	CMS2	CMS3	CMS4
MSI Immune	Canonical	Metabolic	Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermethylation <i>BRAF</i> mutations	SCNA high	Mixed MSI status, SCNA low, CIMP low <i>KRAS</i> mutations	SCNA high
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF- β Activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

This work focuses on the Canonical subtype of colorectal cancer, associated with *APC* mutations and absence of immune cells infiltration.

⁶ Cancer staging is the process of determining the extent to which a cancer has developed. It assigns a number from I to IV, describing the evolution of an isolated cancer to a cancer that has spread.

1.2.6. Immune system in colorectal cancer

Tumors can be infiltrated by immune cells, supporting an immunosurveillance or tumor-promoting environment. This leads to a differentiation into low, intermediate, and high immunoscore categories, based on the Cluster of Differentiation 3 (CD3) positive and Cluster of Differentiation 8 (CD8) positive T cell densities in the tumor. In clinic, this means, for example, that patients with stage I-III colorectal cancer, with a high level of T cells, reveal better overall survival (Ijsselsteijn et al., 2019). In general, tumors can be infiltrated by a polyclonal population of memory CD4⁺ and CD8⁺ T cells, that respond to a variety of antigens - cancer antigens, neoantigens, self- or viral-derived antigens. Other immune cells can be found in colorectal tumors, especially regulatory T cells and M2 macrophages, with an anti-inflammatory phenotype, which leads to a poor prognosis (Ijsselsteijn et al., 2019).

In the last decades, cancer immunotherapy revealed how effective it can be to use immune cells to treat cancer. Therefore, understanding how the immune system works in the colorectal cancer microenvironment can lead to more effective treatments.

1.3. Mouse models for Colorectal Cancer

In humans, most *APC* mutations that cause colorectal tumors are inactivating mutations, that result in loss of function of the APC protein and, consequently, nuclear accumulation of β -catenin (Robanus-Maandag et al., 2010). In order to understand the role of this protein in tumorigenesis, many animal models derived from models of FAP have been proposed, harboring mutant alleles of the *Apc* gene. Mice with these constitutional *Apc* mutations, in heterozygous state, mimic the tumor phenotype of FAP patients, however tumors mostly develop in the small intestine (Robanus-Maandag et al., 2010; Young et al., 2013). While heterozygous *Apc*-mutant mouse models succumbed to these tumors at a young age, homozygous *Apc*-mutant mice are embryonic lethal (Robanus-Maandag et al., 2010).

The Cre/lox recombination system allows for the induction of a somatic mutation in a given gene, at a given time, in a specific cell type. The Cre recombinase enzyme catalyzes the recombination between two loxP-sites, which flank a specific DNA sequence, inactivating the targeted gene.

In this work, *Uri*^{+/Δ}, models were generated by the Cre/lox recombination system. The exon 4 of *Uri* was flanked with two loxP sites. The Cre recombinase was fused to a mutated ligand-binding domain of the human estrogen receptor, resulting in a tamoxifen-dependent activation of Cre recombinase, named Cre-ERT2 (Chaves-Pérez et al., 2019). Villin is a cell type-specific promoter

expressed in absorptive tissues, as the small intestine and the colon, restricting the inactivation of the target gene to these specific cells (West et al., 1988). Thus, the inducible activation of Cre-ERT2 by tamoxifen leads to a specific depletion of the target gene in intestinal cells. Crossing Villin-CreERT2 and *Uri*^{lox} mice generated URI heterozygous mice in the small intestine and colon. *Uri* loss must be maintained in heterozygosis, as *Uri*^(Δ/Δ)^{Int} mice have been described to die within 7 to 9 days after tamoxifen treatment, besides presenting reduced body weight and intestinal length (Chaves-Pérez et al., 2019).

The *Apc* mutants were also obtained by the Cre-lox recombination system, as described for the *Uri* lox mice model. The loxP-flanked exon 15 of the *Apc* gene was deleted, as it represents the last exon of *Apc*, encoding all domains involved in regulation of Wnt/β-catenin signaling (Robanus-Maandag et al., 2010). This model has been used and described in previous work developed in the lab (Brandt et al., 2018).

1.4. Protein Folding and Unfolded Protein Response

In eukaryotic cells, the endoplasmic reticulum (ER) is responsible for the synthesis, folding⁷, modification, and quality control of numerous secretory and membrane proteins.

Protein must fold into their final three-dimensional structure, to meet their biological functions (Herranz-Montoya et al., 2021). The process of protein folding can be altered under ER stress conditions, such as oxidative stress, hypoxia, inflammation, depletion of calcium, or altered glycosylation, as well as increases in levels of unfolded/misfolded proteins or unassembled protein subunits (Huang et al., 2021). Disturbed protein folding leads to unfolded/misfolded proteins, which are transmitted to the ER-associated degradation (ERAD), instead of to the Golgi apparatus (Cao & Kaufman, 2012).

Molecular chaperones interact in the folding of proteins or in the assembly of protein complexes, contributing to proteostasis (Herranz-Montoya et al., 2021). Chaperones are supported by co-chaperones, regulating, and selecting the binding of chaperones to their targets. Prefoldins are co-chaperones, found from archaea to humans, that regulate the assembly of cytoskeletal proteins and are implicated in several pathologies, such as cancer. They can capture non-native actin and tubulin

⁷ Protein folding is the physical process by which a protein chain is translated to its three-dimensional structure, the biologically functional conformation.

monomers, and conduct them to the cytosolic chaperonin, which undergoes major ATP-dependent conformational changes, facilitating folding (Chaves-Pérez et al., 2018).

The cell resort to ERAD to ease the accumulation of unfolded proteins, enhance protein folding capacity in the ER, and increase the expression of chaperones to stabilize the process, including glucose-regulated protein 78 (GRP78, or BiP, or HSPA5), glucose-regulated protein 94 (GRP94) and calnexin (Huang et al., 2021).

In mammals, the unfolded protein response (UPR) signal pathway is mediated by three ER transmembrane protein sensors - inositol requiring kinase 1 (IRE1), pancreatic ER eIF2 α kinase (PERK), and activating transcription factor 6 (ATF6) (Cao & Kaufman, 2012; Huang et al., 2021). Under homeostasis, the chaperone GRP78 binds to the three ER transducer sensors' luminal domain. Upon ER stress, the UPR is activated, and GRP78 dissociates from IRE1, PERK and ATF6. A schematic representation of UPR pathways is presented in Figure 9.

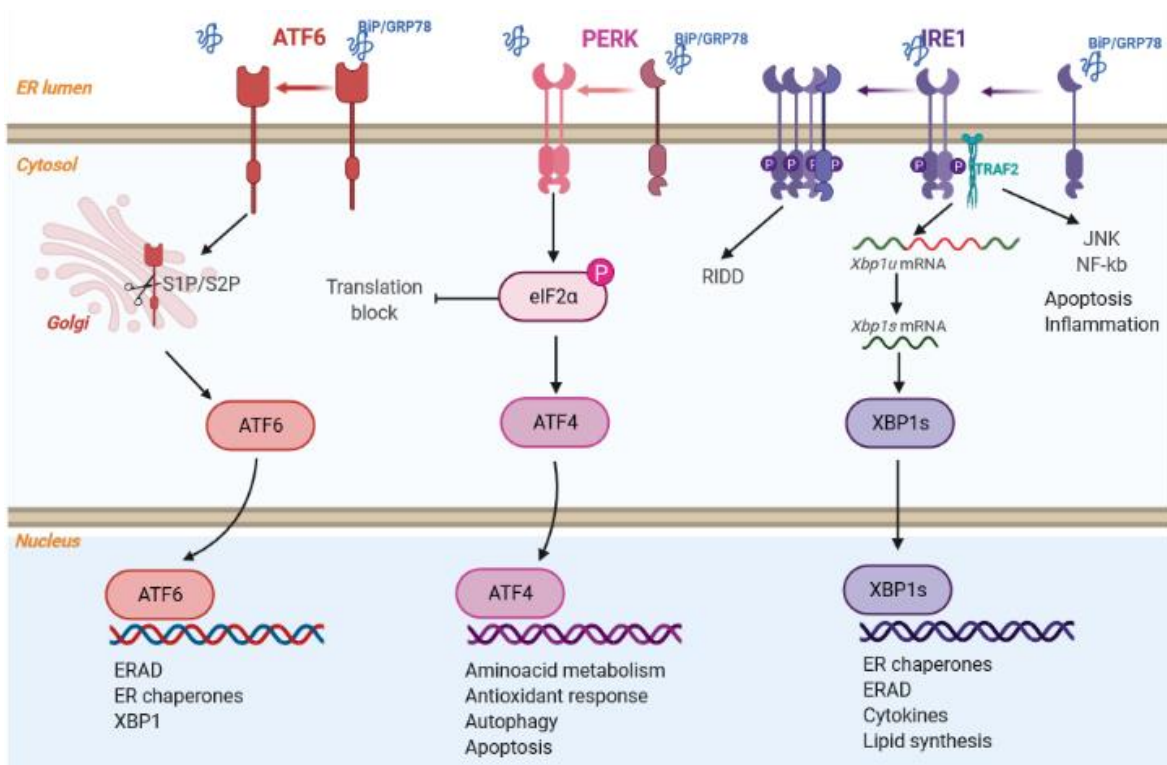


Figure 9 - The Unfolded Protein Response pathways. These three main pathways initiate from the dissociation of GRP78 from the transmembrane protein sensors - activating transcription factor 6 (ATF6) pancreatic ER eIF2 α kinase (PERK), and inositol requiring kinase 1 (IRE1). This leads to different effectors, leading to apoptosis, inflammation, translation block and production of endoplasmic reticulum (ER) chaperones. Created with BioRender.com.

1.4.1. IRE1 signaling pathway

IRE1 is a type I transmembrane protein⁸, with a serine/threonine kinase domain and an endoribonuclease (RNase) domain in its cytosolic portion (Huang et al., 2021). It is the most conserved branch of UPR. IRE1 α is expressed ubiquitously in mammals, while IRE1 β restricts its expression to the intestine and other mucosal surfaces.

IRE1 signaling can induce opposite effects on cell fate, through the unconventional splicing of encoding X-box-binding protein 1 (*XBP1*) mRNA, regulated IRE1-dependant decay of mRNA (RIDD), formation of UPORosome⁹ and post-translational modifications (Maurel et al., 2015).

To get activated, IRE1 needs to dissociate from GRP78. Usually, the luminal domain of the IRE1 α subtype forms homodimers in the ER membrane, but it can also be assembled with IRE1 β , boosting the kinase and RNase activities. These initiate the removal of a 26-base intron from the mRNA *XBP1*, generating a cAMP-response element-binding protein/activating transcription factor (CREB/ATF) basic leucine zipper (bZip)-containing transcription factor (Huang et al., 2021). Spliced *XBP1* (*sXBP1* or *XBP1s*) launches a transcriptional program, potentiating the production of chaperones, transcription factors, proteins involved in ER biogenesis, and phospholipid synthesis. Furthermore, IRE1 degrades a subset of ER-localized mRNAs in a stress-dependent decay of mRNAs, revealing that protein synthesis is diminished to relieve ER stress (Huang et al., 2021). The activation of IRE1 α also recruits adaptor protein tumor necrosis factor receptor-associated factor 2 (TNFR2) and apoptosis signal-regulating kinase 1 (ASK1), which finally promotes apoptosis. Thus, *XBP1* mRNA splicing is cytoprotective (Maurel et al., 2015).

On the other hand, IRE1 allows a basal RIDD activity, which can either maintain ER homeostasis or induce cell death. While the decay of specific RIDD substrates required for gene expression may contribute to the inhibition of protein synthesis, RIDD activity also increases proportionally to ER stress intensity, inducing the degradation of mRNA substrates required for cell survival, leading to cell death (Maurel et al., 2015).

The composition of the UPORosome depends on ER stress intensity and modulates IRE1 signaling. For example, the interaction of B-cell lymphoma 2 (BCL-2) associated X-protein (BAX) and BCL-2

⁸ Type I transmembrane proteins are anchored to the lipid membrane with a stop-transfer anchor sequence. The N-terminal domains target to the ER lumen during synthesis, or the extracellular space, in the case of cell membrane proteins.

⁹ IRE1 α signals by assembling a dynamic protein platform, the UPORosome, where different modulator and adaptor proteins assemble to regulate the kinetics and amplitude of UPR effector responses.

antagonist/killer 1 (BAK) with the cytosolic domain of IRE1 promotes *XBP1* splicing, while p53 upregulated modulator of apoptosis (PUMA) and BCL-2-interacting mediator of cell death (BIM) interact with IRE1 to maintain *XBP1* splicing (Maurel et al., 2015). On the other hand, BAX inhibitor 1 (BI-1) competes with BAX/BAK for the binding site for IRE1, which inhibits *XBP1* splicing and survival (Maurel et al., 2015).

Under irreversible ER stress, in response to BIM and PUMA, BAK induces cytochrome c release, in a mechanism depend on calcium release, and an IRE1-TNF receptor-associated factor 2 (TRAF2) interaction, triggering cell death. TRAF2 is recruited to the ER membrane, forming a protein complex with the cytosolic domain of IRE1 and phosphorylated apoptosis signal-regulating kinase 1 (ASK1). This IRE1/TRAF2/ASK1 complex activates c-Jun N-terminal kinase (JNK), that phosphorylates BCL-2 and BCL-extra-large (BCL-XL), enhancing apoptosis, and BIM and BH3 interacting-domain death agonist (BID), promoting their pro-apoptotic activities (Maurel et al., 2015).

The cells may be protected of ER stress-induced apoptosis by heat shock protein (HSP) 72, that binds to IRE1, enhancing *XBP1s* production. IRE1 activity is also controlled by post-translational modifications, such as phosphorylation, ADP-ribosylation and ubiquitination. The first allows for the activation of its RNase activity, while IRE1 oligomerization states regulate *XBP1* splicing and RIDD. Moreover, IRE1 is ADP-ribosylated and activated by poly polymerase 16, an ER transmembrane protein upregulated, upon ER stress, protecting cells against cell death. Besides, ubiquitination of IRE1 by the protein CHOP is required for IRE1/TRAF2/JNK signaling (Maurel et al., 2015).

1.4.2. PERK signaling pathway

PERK is a type I transmembrane protein with a cytosolic serine/threonine kinase domain, which functions as a sensor for ER stress. As in the other pathways, GRP78 dissociates from this protein, upon ER stress. PERK undergoes oligomerization and trans auto-phosphorylation¹⁰ (Huang et al., 2021).

Activated PERK phosphorylates Ser51 of the subunit of eukaryotic translation initiation factor 2 (eIF2 α ¹¹), which attenuates translation initiation. However, not all translation is stopped, as is the case of activating transcription factor 4 (ATF4), involved in aminoacid metabolism, autophagy and antioxidant response (Harding et al., 2000). The PERK/eIF2 α /ATF4 axis mediates both pro-survival and pro-death outcomes, by unknown mechanisms. Pro-death signaling occurs through CHOP/GADD153¹², one of ATF4 target genes, as CHOP downregulates anti-apoptotic BCL-2 and upregulates pro-apoptotic BIM, BCL-XL and BAX, and promotes the expression of caspase-3¹³ (Maurel et al., 2015).

CHOP also promotes oxidative protein folding in the ER, through the induction of ER oxidoreduction-1 alpha (ERO1 α), generating reactive oxygen species (ROS), which can also lead to apoptosis (Huang et al., 2021). An antioxidant response is activated by the UPR to cope with ROS's effects, by the activation of nuclear factor E2 related factor 2 (NRF2). Phosphorylation of NRF2 by PERK triggers its dissociation and nuclear import from the cytoplasm. The pathway also activates nuclear transcription factor-kB (NF-kB), by repressing the inhibitor of kappa B (I κ B), regulating anti-apoptotic genes, especially the TRAF1 and TRAF2.

¹⁰ Autophosphorylation is a type of post-translational modification of proteins, usually defined by a kinase phosphorylating itself. In eukaryotes, a phosphate group is added to serine, threonine, or tyrosine residues within kinases, normally to regulate its catalytic activity. Cis autophosphorylation occurs when a kinases' own active site catalyzes the phosphorylation reaction, while trans autophosphorylation occurs when the provider of the active site is another kinase of the same type, as is the case when kinases dimerize. In general, the phosphate groups introduced originate from ATP.

¹¹ eIF4 α is an initiation factor required for most forms of eukaryotic translation initiation, mediating the binding of tRNA to the ribosome in a GTP-dependent manner. eIF2 is a heterotrimer consisting of an alpha, a beta, and a gamma subunit. Its phosphorylation prevents translation initiation.

¹² DNA damage-inducible transcript 3, also known as C/EBP homologous protein (CHOP), is a pro-apoptotic transcription factor, encoded by the DDIT3 gene.

¹³ Caspase-3 is a caspase protein that interacts with caspase-8 and caspase-9. It exists as an inactive proenzyme and must undergo proteolytic processing. The sequential activation of caspases is essential for cell apoptosis.

PERK signaling pathway is inhibited by the interaction of co-chaperone P58IPK¹⁴ with the kinase domain of PERK and the impairing of eIF2 α phosphorylation (Huang et al., 2021).

1.4.3. ATF6 signaling pathway

ATF6 is a type II transmembrane protein with a CREB/ATF bZip transcription factor domain at the amino terminus. Upon ER stress, GRP78 dissociates from ATF6, which migrates to the Golgi apparatus, where it is sequentially cleaved by site 1 and site 2 proteases. Its cytosolic fragment ATF6 p50 migrates to the nucleus and activates gene expression.

The ER transmembrane proteins, ATF6 α ATF6 β , are cleaved in response to ER stress (Thuerauf et al., 2004). ATF6 α target genes include chaperone *GRP78*, ERAD components, UPR genes *XBP1*, *P58IPK*, and *CHOP*, and it is also able to heterodimerize with *XBP1s*, regulating UPR elements in target genes. On the other hand, ATF6 β is cleaved and transferred to the nucleus upon ER stress and serves as an endogenous inhibitor of ATF6 α (Huang et al., 2021; Thuerauf et al., 2004).

1.4.4. Unfolded Protein Response activation and cancer

Studies have revealed the interaction between the three UPR pathways. For example, ATF6 can be activated by PERK/eIF2 α /ATF4 pathway (Teske et al., 2011) and ATF6 α heterodimerizes with XBP1 for the induction of ERAD (Yamamoto et al., 2007).

Moreover, the UPR has been studied in the context of cancer. During ER stress, cells either survive by adaption mechanisms or undergo apoptosis. UPR attenuates mRNA translation and increases the expression of chaperones, to either decrease the levels or repair unfolded/misfolded proteins. Cancer cells, under ER stress, tend to modify their ER proteins and chaperones, increasing cell viability. Cancer progression and development of metastasis are impaired when using UPR inhibitors (Lee et al., 2014), while activated IRE1 can promote the expression of XBP1, induce ERAD and facilitate cell survival. However, the role of RIDD remains unclear, as depending on the context, it can be anti- or pro-oncogenic.

¹⁴ DNAJ homolog subfamily C member 3 (P58IPK) is a member of the heat shock protein 40 family. In resting cells, P58IPK localizes in the cytosol, and translocates to the ER during ER stress, due to its N-terminal ER-targeting signal.

PERK-regulated pathways are important for cancer cell survival and cells with impaired PERK, ATF4 or NRF2 signaling are more sensitive to ER stress (Maurel et al., 2015). Indeed, ATF4 is upregulated in various cancer patients, helping cells survive hypoxia and detach from the extracellular matrix. Even when the cells are not under ER stress, PERK pathway can be activated and promote MYC-induced tumorigenesis and angiogenesis, by vascular endothelial growth factor (VEGF) signaling (Maurel et al., 2015).

Regarding ATF6 signaling pathway, it has been described that ATF6f is essential for the adaptation of dormant, and not proliferating, squamous carcinoma cells to chemotherapy, nutritional stress and *in vivo* tumor environment (Maurel et al., 2015). ATF6f up-regulates unspliced *XBP1* (*XBP1u*), the major substrate for IRE1 RNase. *XBP1u* is known to be rapidly degraded by the proteasome and does not play a specifically role in the cellular response to ER stress, however it plays an important role in autophagy and survival in human colon cancer. *XBP1u* also inhibits the expression of *XBP1s* target genes and enhances ATF6f and *XBP1s* degradation by the proteasome upon prolonged ER stress, acting a negative feedback regulator of the UPR. In the context of cancer, PDIA5-mediated ATF6 activation may contribute to the resistance of leukemia cells to imatinib (Maurel et al., 2015).

1.4.4.1. Unfolded Protein Response pathways in colorectal cancer

The expression of GRP78 increases in many types of solid tumors, including colorectal cancer and its functions may differ. While it can restrain early tumor development, by inducing suppressive mechanisms (Arap et al., 2004), it can also promote cancer progression through its pro-survival mechanisms, when cancer cells in advanced stages are exposed to excessive ER stress (Dong et al., 2008).

Colorectal cancer tissues display higher levels of GRP78, compared to normal colon tissues, regulating its sensitivity to apoptosis induced by chemotherapy (Mhaidat et al., 2016). Therefore, many drugs have been developed to inhibit GRP78 levels in colorectal cancer patients.

Angiogenesis represents a crucial step in tumor development, and it is usually induced concomitantly with UPR. IRE1 has been identified as a therapeutic target to control both angiogenic switch and cancer development, as IRE1-dependent signaling pathways play a crucial role in the cell's response to ischemia (Chalmers et al., 2019). Besides, the inhibition of XBP1, an output of IRE1, decreases the levels of VEGF receptor-2, which regulates angiogenesis. Therefore, XBP-1 enhances colorectal cancer cell invasion (Mhaidat et al., 2015).

ATF4 and NRF2, transcription factors of PERK signaling pathways, contribute to cellular adaptation and oncogenesis, mediating the angiogenic switch (Wang et al., 2012). PERK can also

phosphorylate and activate FOXO transcription factors family, known tumor suppressors. Thus, the loss of FOXO promotes the progression of colorectal cancer (Huang et al., 2021).

1.4.5. Unconventional RPB5 interactor

The unconventional prefoldins (PFDN) are represented in Figure 10. There are two related classes of prefoldin subunits, α and β , both of which feature N- and C-terminal α -helical coiled-coil structures connected by either one or two β -hairpins, in β -class prefoldins or α -class prefoldins, respectively. Additionally, it has both nuclear and cytoplasmic localization signals, allowing for the subcellular localization.

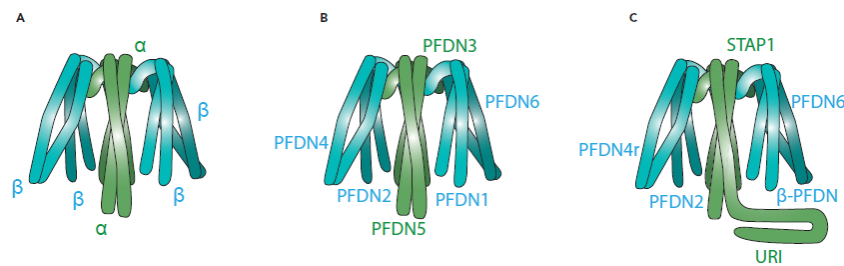


Figure 10 - Structure of chaperone unconventional prefoldin (PFDN). (A-C) Schematic representation of the structure of PFDN in Archaea (A), of the classic PFDN complex in eukaryotes (B) and of the eukaryotic UPC (C). Two α - (in green) and four β -class (in blue) PFDNs assemble through hydrophobic interactions to form a jellyfish-like structure, as evidenced by x-ray crystallography analyses performed in Archaea. Similar structural model has been proposed for the classic eukaryotic PFDN complex. Yet, the structure of the UPC remains to be determined.

The chaperone PFDN RNA polymerase binding subunit 5 (RPB5) interactor (URI), with a molecular weight of 90 kDa, controls and protects genome integrity in response to environmental insults (Chaves-Pérez et al., 2019). URI is a α -class prefoldin and forms a heterohexameric prefoldin-like complex ($\alpha 2\beta 4$) with a α -class prefoldin SKP2-associated α -prefoldin 1 or UXT (STAP1), three known β -class prefoldins (PFDN2, PFDN4r and PFDN6), and another unidentified β -class prefoldin, probably a second copy of one of the PFDN proteins (Chaves-Pérez et al., 2018). Its schematic representation is presented in Figure 11.

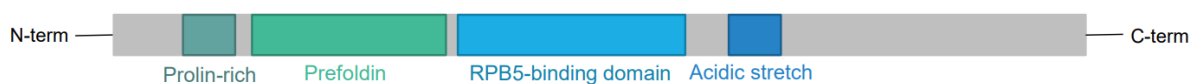


Figure 11 - Schematic representation of URI protein.

The human URI PFDN-like complex (UPC) interacts with different partners, including DNA polymerases-related genes (POL3A, RPAP3, RPB5, RPB5MP) and proteins related to chromatin-remodeling complexes. Several studies show a contribution of the UPC in the assembly of RNA polymerases I and III, directly linked to the ability of URI to bind RPB5, a common subunit of the

three RNA polymerases (Herranz-Montoya et al., 2021). Besides, URI has been linked to DNA damage and chromatin remodeling.

The URI prefoldin-like complex associates with the R2TP (Rvb1-Rvb2-Tah1-Pih1) module, which, besides other functions, recruits diverse proteins to the HSP90, and is implicated in multiple biological processes, including apoptosis (Chaves-Pérez et al., 2018). This complex is also referred as the PAQosome, from Particle for Arrangement of Quaternary structure. URI expression is regulated by environmental factors, and while its overexpression is related to uncontrolled protein substrate regulation, its ablation may induce over-functioning of proteins. URI also coordinates cell growth and survival, with nutrient availability (Chaves-Pérez et al., 2018).

It has organ-specific oncogenic activities, regulating the functions of diverse proteins, enzymes, and tumor suppressors. In the intestine, URI is predominantly expressed in the upper part of the crypts, colocalizing with its binding partner heat shock protein 90 (Chaves-Pérez et al., 2019). URI depletion specifically in the intestine of adult mice destroys the organ architecture and leads to mice lethality (Chaves-Pérez et al., 2018). Besides, URI depletion activates the β -catenin-c-MYC axis in LR cells, by inducing TA-LR cell proliferation and stemness.

Our group performed an analysis of URI mutations, based on the COSMIC database, analyzing cancer patients and cell lines. This unpublished data revealed that the Acidic Stretch region of URI is the region most affected by mutations, specifically deletions, in cancer patients. The wild-type version of human URI (hURI) presents 13 Aspartic Acid aminoacids (13D), while the most common mutated versions present a deletion of one or two Aspartic Acids (12D or 11D). These deletions are more common in the upper aerodigestive tract, large intestine, and stomach. It is indeed important to understand why these versions of URI are more common and tumorigenic. In mice, the sequence of URI protein (mURI) is different from hURI, ending with 1 Glutamic Acid (E) and 4 Aspartic Acids (D), while it starts with a variable number of Aspartic Acids (D), Glutamic Acids (E) and Aspartic Acids (D). The wild-type form sequence (18D) is 3D_4E_7D.

Besides, our group has also understood that high *URI* mRNA levels are positively associated with *TP53* mutations or loss and Wnt signaling pathway activation, in canonical colorectal cancer patients. On the other hand, overexpression of URI protects against the effects of high dose irradiation, preserving the quiescent state of LR cells, and it is involved in double-strand break repair by non-homologous end-joining (Herranz-Montoya et al., 2021). Thus, URI labels a population of cells considered as a facultative stem cell pool, required for intestinal regeneration following radiation-induced injury.

A study by our lab also elucidated on the functional roles of PFDN genes in cancer, based on genomic aberrations (copy-number alterations (CNAs) - amplification, deletion and somatic

mutations) of 10 000 tumors, from 33 cancer types sequenced in The cancer Genome Atlas - Table 2. Notably, a low frequency of point mutations in the PFDN genes was observed, demonstrating that they are not highly mutated genes in cancer. The highest CNAs amplification frequencies were found in URI, PFDN2 and PDRG1. URI is one of the highest CNA amplification frequencies in various cancer types, which correlates with poor disease-free survival (Herranz-Montoya et al., 2021).

Studies revealed that some colorectal cell lines are URI-dependent, as cells died when exposed to URI shRNA. This can be explained by a protective effect of URI against apoptosis - depletion of URI activates p53-mediated apoptosis - demonstrating its possible role as an oncogene (Herranz-Montoya et al., 2021).

Table 2 - The cancer genome atlas (TCGA) samples analyzed. NT - Normal Tissue; DEA - Differential Expression Analysis; NP - Not Possible; LN - Low Number; N - Number of samples available; CNV - Copy Number Variations; OK - Possible.

Cohort	Description	mRNA tumor	mRNA NT	DEA	N CNVs
ACC	Adrenocortical carcinoma	79	0	NP	89
BLCA	Bladder Urothelial Carcinoma	408	19	OK	406
BRCA	Breast invasive carcinoma	1093	112	OK	996
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma	304	3	LN	278
CHOL	Cholangiocarcinoma	36	9	OK	36
COADREAD	Colorectal adenocarcinoma	379	51	OK	526
DLBC	Lymphoid Neoplasm Diffuse Large B-cell Lymphoma	48	0	NP	37
ESCA	Esophageal carcinoma	184	11	OK	182
GBM	Glioblastoma multiforme	153	5	LN	378
HNSC	Head and Neck squamous cell carcinoma	520	44	OK	496
KICH	Kidney Chromophobe	66	25	OK	65
KIRC	Kidney renal clear cell carcinoma	533	72	OK	354
KIRP	Kidney renal papillary cell carcinoma	290	32	OK	274
LAML	Acute Myeloid Leukaemia	0	0	NP	109
LGG	Brain Lower Grade Glioma	516	0	NP	507
LIHC	Liver hepatocellular carcinoma	371	50	OK	353
LUAD	Lung adenocarcinoma	515	59	OK	507
LUSC	Lung squamous cell carcinoma	501	51	OK	649
MESO	Mesothelioma	87	0	NP	82
OV	Ovarian serous cystadenocarcinoma	302	0	NP	398

PAAD	Pancreatic adenocarcinoma	178	4	LN	175
PCPG	Pheochromocytoma and Paraganglioma	179	3	LN	692
PRAD	Prostate adenocarcinoma	497	52	OK	488
SARC	Sarcoma	259	2	NP	232
SKCM	Skin Cutaneous Melanoma	103	1	NP	363
STAD	Stomach adenocarcinoma	415	35	OK	434
STES	Stomach and Esophageal carcinoma	599	46	OK	265
TGCT	Testicular Germ Cell Tumors	150	0	NP	144
THCA	Thyroid carcinoma	501	59	OK	482
THYM	Thymoma	120	2	NP	123
UCEC	Uterine Corpus Endometrial Carcinoma	176	24	OK	509
UCS	Uterine Carcinosarcoma	57	0	NP	56
UVM	Uveal Melanoma	80	0	NP	80

1.5. Main objectives and hypothesis

Apc mutations in the intestine and colon unable the phosphorylation of β -catenin. Thus, β -catenin translocates to the nucleus and activates Wnt signaling, potentiating proliferation and tumorigenesis. Moreover, URI is related to the UPR, apoptosis and β -catenin activity. Thus, based on the group's published and unpublished data, we hypothesize that URI loss can counteract the effects of *APC* mutations in the intestine and colon.

We aim to elucidate the role of URI in colorectal cancer development, based on mouse models for colorectal cancer, with *Apc* mutations. We intend to reveal molecular mechanisms linking URI and colorectal cancer, such as its effects on apoptosis induction, unfolded protein response and tumor size.

On the other hand, we want to investigate the stability of different URI variants, in mice and human, as a preliminary action before creating mice models with these URI versions.

Chapter 2

2. Materials and Methods

2.1. Cell cultures

HCT-116 cell line is originated from of a human male with colonic carcinoma. Handling of culture of adherent cells was performed until near confluence, in standard culture medium (Gibco Dulbecco's Modified Eagle Medium, DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin and 0.1 mg/mL streptomycin. Cells were sub-culture when reaching 80-90% confluence following standard cell culture protocols.

i. Plasmids preparation by bacteria transformation followed by mini or midiprep

The acidic acid stretch sequence of each type of URI protein is presented in Table 3. The human influenza hemagglutinin (HA) epitope-tagged pcDNA3 plasmids able to transfect cells with the presented forms of URI are also expressed in Table 3.

The competent bacteria¹⁵ DH5 α competent *E. coli* strain or Stbl3™ *E. coli* strain were transformed by heat shock with one of the human or mouse plasmids for URI protein and controls from Table 3. 1ul of the different plasmids was added to 30 ul of competent bacteria. The bacteria were incubated in ice for 30 minutes, underwent a heat shock at 42°C for 1 minute (Thermomixer Compact, Eppendorf), and were further incubated in ice for 15 minutes. The competent bacteria were incubated with 500μL of the nutritionally rich medium, Lysogeny Broth (LB), at 37°C, at 300 rpm (Thermomixer Compact, Eppendorf). Ampicillin was added to 100mL of LB (1:100) with the bacteria transformed with the human plasmids, or to 20mL of LB (1:100) with the bacteria transformed with the mouse plasmids. The bacteria cultures were incubated overnight at 37°C, at 200 rpm.

The following day, plasmids were purified by pelleting bacteria cultures, lysing the cells and extracting the plasmids using the mini or midi-prep, for mouse or human plasmids, respectively, with

¹⁵ Competent bacteria are bacteria that can undertake foreign DNA from the surroundings, by a process called transformation.

the NZYTech Plasmid kits, following the kits' specifications. Stock solutions of 100 ng/uL were prepared.

Table 3 - Acidic Strech Sequence for each variant of human URI protein (hURI) and mouse URI protein (mURI) and corresponding plasmid used. Both the wild-type proteins (WT) and mutated versions are represented. Aspartic Acid is represented by D and Glutamic Acid by E, NI demonstrates that the aminoacid in that position is not defined. The plasmids vector is pcDNA3, tagged with a human influenza hemagglutinin (HA) epitope.

Alias	Acidic Stretch Sequence	Name of the plasmid
hURI WT 13D	-DDDDDDDDDDDDDD-NI-DDDD-	pcDNA3.1(+)-HA-hURI_13D
hURI 14D	-DDDDDDDDDDDDDDDD-NI-DDDD-	pcDNA3.1(+)-HA-hURI_14D
hURI 12D	-DDDDDDDDDDDDDD--NI-DDDD-	pcDNA3.1(+)-HA-hURI_12D
hURI 11D	-DDDDDDDDDDDD---NI-DDDD-	pcDNA3.1(+)-HA-hURI_11D
hURI 10D	-DDDDDDDDDDDD----NI-DDDD-	pcDNA3.1(+)-HA-hURI_10D
mURI WT 18D	-DDD-EEEE-DDDDDDDD-E-DDDD-	pcDNA3.1(+)-HA-mURI-3D_4E_7D_1E_3D
mURI 0D	-----EEEE-DDDDDDDD-E-DDDD-	pcDNA3.1(+)-HA-mURI-0D_4E_7D_1E_3D
mURI 3E	-DDD-EEE--DDDDDDDD-E-DDDD-	pcDNA3.1(+)-HA-mURI-3D_3E_7D_1E_3D
mURI 0E	-DDD-----DDDDDDDD-E-DDDD-	pcDNA3.1(+)-HA-mURI-3D_0E_7D_1E_3D
mURI 6D	-DDD-EEEE-DDDDDD--E-DDDD-	pcDNA3.1(+)-HA-mURI-3D_4E_6D_1E_3D
mURI 5D	-DDD-EEEE-DDDDDD---E-DDDD-	pcDNA3.1(+)-HA-mURI-3D_4E_5D_1E_3D
mURI 4D	-DDD-EEEE-DDDDDD----E-DDDD-	pcDNA3.1(+)-HA-mURI-3D_4E_4D_1E_3D

ii. Cell transfection

HCT-116 cells were seeded at a density of 2×10^5 cells/mL per well, in a twelve wells plate, cultured in supplemented DMEM. Transfection was performed using Lipofectamine 3000 and following the manufacturer's guidelines. 1000 ng of plasmid were mixed with 2 μ L of lipofectamine in 150 μ L of Opti-MEM (low serum cell culture media). Cells were washed and incubated with the transfection solution for 4 hours, at 37°C, 5% CO₂. After that time, 1 mL of DMEM was added. Cells were harvested for analysis of protein levels by Western Blot 24 hours later.

iii. Proteasome inhibition

Proteasomes are protein complexes that degrade unneeded or damaged proteins by proteolysis, which is a chemical reaction that breaks peptide bonds. Proteasomes are part of a major mechanism by which cells regulate the concentration of particular proteins and degrade misfolded proteins.

The transfected cells were cultured in the absence or presence of a proteasome inhibitor, MG132 (Z-Leu-Leu-Leu-al) or Carfilzomib at 10 μ M, for 24 hours, at 37°C, 5% CO₂. MG132 is a synthetic peptide aldehyde that inhibits the 26S proteasome, while Carfilzomib is an anti-cancer drug that inhibits the proteasome by covalently and irreversibly binding to the chymotrypsin-like activity of the 20S proteasome. The inhibition of the proteasome was used to detect if the proteins tested are degraded by this mechanism, in normal conditions.

2.2. Generation and Handling of mice

C57BL/6 mice were handled in pathogen-free conditions and all experiments approved by the Centro Nacional de Investigaciones Oncológicas (CNIO)-Instituto de Salud Carlos III Ethics Committee and performed in accordance with the guidelines for ethical conduct in the care and use of animals, as stated in the international guiding principles for biomedical research involving animals, developed by the Council for International Organization of Medical Sciences. A schematic representation of the mice models used and treatments performed is present in Figure 12.

At 8 weeks of age, Cre-ERT2-mediated recombinase was activated, by feeding the adult mice a tamoxifen diet, at a concentration of 400mg/kg, for 2 weeks. The expression of Cre-ERT2 recombinase deleted the targeted exon 4 and generated a *Uri*^Δ allele and the *Apc*^Δ allele. Mice were sacrificed at 25 weeks old, after 15 weeks of the end of the tamoxifen treatment.

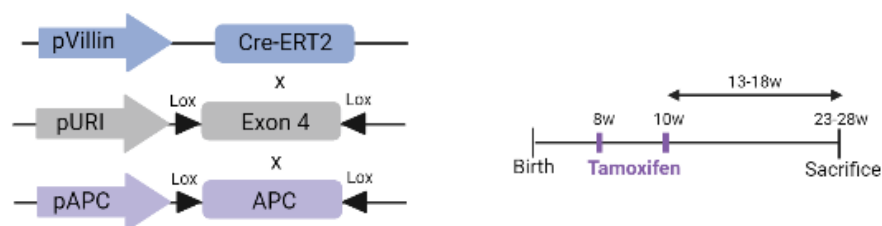


Figure 12 - Schematic representation of the mouse models. On the left, the cross between URI-CreERT2 mice with *Apc*^Δ mice. On the right, the experimental design for the specific depletion of the target genes in intestinal cells. Created with BioRender.com.

2.3. Genotyping

To verify the mice genotypes, DNA was extracted from fingers and tails. Tissue was digested overnight, at 56°C, in 500 µL of lysis buffer (100 mM NaCl, 20 mM tris-HCl pH8, 10 mM EDTA and 0.5% SDS) and proteinase K (25 mg/mL). The following day, proteins, lipids, polysaccharides, and contaminants were precipitated with 250 µL of NaCl 5M. Samples were centrifuged, at room temperature, at 13 000 rpm, for 10 min (Eppendorf, Centrifuge 5425R), and the supernatant, with DNA, was collected. 300 µL of isopropanol were added to the solution, to precipitate the DNA. Samples were centrifuged, again, for 30 min, at 13 000 rpm and the supernatant was discarded. The pellet was washed with 250 µL of 75% ethanol and centrifuged at 13 000 rpm, for 10 min. The final DNA pellet was dried and re-suspended in 500 µL of distilled water and used for Polymerase Chain Reaction afterwards.

Polymerase Chain Reaction (PCR) was performed with the NZYTaQ II 2x Green Master Mix and specific primers for each allele used (Table 4). 8 µL of master mix containing DNA polymerase and nucleotides was plated together with 1 µL of the purified DNA solution and 1 µL of a primer mix at a concentration of 10 µM. PCR conditions were set in accordance with each primer.

After PCR, the samples run on a 2.5% agarose gel in Tris-acetate-EDTA (TAE) buffer, for 35 min, at 150V. BioRad system and Quantity One software were used to detect specific amplicons, accordingly to each genotype, after incubation of the gels in TAE and 3 µL of Gel Red and exposure to UV light.

Table 4 - Primers used to detect each allele by PCR. For each primer, at least one forward and one reverse primer were used for each gene of interest.

Gene	Primer	Primer Sequence (5'-3')
<i>Cre</i>	Ins1_Cre Fw	CCATCTGCCACCAGCCAG
	Ins1_Cre Rv	TCGCCATCTTCCAGCAGG
	Ins1_Cre CTR Fw	ACTGGGATCTTCGAACCTTTGGAC
	Ins1_Cre CTR Rv	GATGTTGGGGCATGCTCATTACCC
<i>Apc lox</i>	<i>Apc</i> Fw	AACTTCTGAGTATGATGGAGG
	<i>Apc</i> Fw2	AGGGTTATTGAATATGATCGG
	<i>Apc</i> Rv	AGCAGCAAACCTTACTTTACAG
<i>Uri lox</i>	<i>Uri</i> Fw	CCCTCTTGCCTTCATGCC
	<i>Uri</i> Rv	AAACCAAGTGTAATAATGTCCC

2.4. Collection and preparation of tissue samples

Mice were sacrificed with CO₂ and weighted. Blood samples were analyzed, spleen and liver weighted. The intestines were opened longitudinally for macroscopic detection of tumors. Tissue samples were frozen and stored at -80°C for protein and RNA further analysis. Part of the intestine was rolled, and incubated overnight in 10% formalin, at room temperature, for fixation. Tissue was then embedded in paraffin. Paraffin blocks were sectioned (2.5 mm thick) and affixed on positively charged coated slides for further testing.

These procedures were carried out by qualified personal with the corresponding animal handling certificates.

2.5. Determination of tumor size and tumor grade

Hematoxylin and eosin (H&E) staining was performed on the paraffin-embedded sections, for histopathological analysis. First, tissue sections were deparaffinized in xylol, rehydrated through graded washes of ethanol (100%, 96% and 70%) and washed with distilled water, in the Leica Autostainer XL. In the Leica Autostainer XL, slides were rinsed twice with xylene, for 2 min each, hydrated with 100% and 96% ethanol, for 2 min, followed by a 2 min washing step. Slides were rinsed in hematoxylin (5 min), eosin (2min), washed in water (30 seconds), dehydrated in 96% (30 seconds) and 100% ethanol (first 2 min, and after 30 seconds), and placed in xylene for 30 seconds. Afterwards, the slides were then mounted with xylol.

Microscopic fields images at 10x were analyzed, in Leica DFC420 microscope, with Leica Application Suite Software and tumor size was measured in ImageJ (1.49J).

A pathologist studied the slides stained for H&E. The tumors in the colon and intestine were graded them between 0 and 4 - from normal tissue to carcinoma. This analysis revealed the maximum tumor grade reached on each mouse.

2.6. Immunohistochemistry

Sections were deparaffinized, as described in the previous section. Antigen retrieval procedure was performed with a pressure cooker 2100 Retriever Chamber (Biocare Medical), with citrate buffer (1 M sodium citrate, pH 6.5), and endogenous peroxidases blocked by incubating the slides, for 10 min, in hydrogen peroxide (H₂O₂, 3%), followed by washing phase with 1x phosphate buffered saline (PBS; pH 7.4). Tissue was blocked and permeabilized with a 1-hour incubation in 5% bovine serum albumin (BSA), 0.2% Triton in PBS (pH 7.4), at room temperature (RT). The primary antibodies were diluted in 5% BSA, 0.2% Triton in PBS (pH 7.4) in the dilutions described in Table 5, and slides incubated with these overnight, at 4°C.

The following day, slides were washed with 1x phosphate buffered saline with Tween 20 (PBST; pH 7.4) and incubated with the corresponding secondary antibody - anti-mouse, anti-rat or anti-rabbit - at a dilution of 1:200 in 5% BSA, 0.2% Triton in PBS (pH 7.4) for 1h, at room temperature. The section was then washed twice with PBST 1x (pH 7.4) for 10 min each.

To detect the biotinylated secondary antibody, Avidin DH and Bioinylated peroxidase H were mixed and incubated for 30 min. The slides were incubated with Vectastain ABC reagent for 1 hour. After a new washing step with PBST 1x (pH 7.4), the cells were incubated with 0.1% 3,30-diaminobenzidine (DAB) solution (Deco DAB). To stop the reaction, the slides were rinsed in tap water.

Slides were counterstained in Leica Autostainer XL, by dehydration in 70%, 96% and 100% ethanol, 3 times changes of 100% ethanol and xylene for 2 min each, and mounted with xylol, as described in the previous section. Microscopic fields images at 10x or 20x were analyzed in Leica DFC420 microscope, with Leica Application Suite Software, and positive cells quantified with Cell Counter plug-in in ImageJ (1.49J).

Table 5 - Antibodies used for Immunohistochemistry assays and the concentration used. The antibodies were used for the following cell components: cleaved caspase 3 (CC3) is the active form of caspase 3; cluster of differentiation 3 (CD3) is a protein complex found in T cells; NK1.1 is expressed by natural killer (NK) cells and NK T cells in C57BL/6 mice.

Target	Origen	Concentration
Cleaved Caspase-3 (CC3)	Rabbit	1:300
CD3	Rabbit	1:100
NK1.1	Mouse	1:100

2.7. Immunofluorescence with amplification

Sections were deparaffinized and antigen revealed, as described in the previous sections. Slides were first incubated in H₂O₂ 3%, and blocked with a 1-hour incubation in 5% bovine serum albumin (BSA), 0.2% Triton in PBS (pH 7.4), at RT.

Slides were incubated with Avidin blocking, for 45 minutes, at RT, washed in PBST 1x, and incubated with Biotin blocking, for 45 minutes, at RT. After a washing phase with PBST 1x, the slides were incubated overnight, at 4°C, with the primary antibody for mCSF (1:200), that recognizes CSF1 in bone marrow and mononuclear cells - monocytes, lymphocytes, and macrophages.

The following day, the slides were washed and incubated with the rat secondary antibody (1:200), for 1 hour, at RT. After washes with PBST 1x, slides were incubated with Streptavidin conjugated with horseradish peroxidase (HRP) enzyme (1:500), for 30 minutes, at RT. Slides were washed with PBST 1x, and incubated with H₂O₂ 1% in TSA-Borax Buffer, for 30 minutes, at RT. After a washing phase with PBST 1x, the slides were incubated with Streptavidin-Alexa555 (1:1000) and DAPI (1:1000). Slides were then washed again in PBST 1x and mounted with Mowiol.

2.8. Cell lysis

i. Cells lysis from transfected HCT-116 cells

The transfected HCT-116 cells were washed with PBS after 24 hours of transfection. Cells were lysated for protein extraction, by scratching the surface with 100µL of Radio Immuno Precipitation Assay (RIPA) lysis buffer supplemented with Ethylenediaminetetraacetic Acid (EDTA) final concentration 1:50, Phenylmethylsulfonyl Fluoride (PMSF) final concentration 1:200, Sodium Fluoride (NaF) final concentration 1:200, sodium orthovanadate (OV, 200nM) final concentration 1:200, Aprotinin final concentration 1:1000, Dithiothreitol (DTT, 0.1M) final concentration 1:100. The samples were centrifuged (Centrifuge 5425R, Eppendorf), at 4°C, for 10 minutes, at maximum speed and supernatant was collected.

ii. Cell lysis from mice samples

Small parts of frozen intestine from mice were lysated with 750 μ L RIPA lysis buffer supplemented as described above and homogenized using Precellys® Evolution tissue homogenizer. The samples were centrifuged at 4°C, for 20 minutes, at maximum speed, to collect the supernatant containing the proteins

2.9. Protein detection by Western Blot

The protein quantity in the supernatant from cell or tissue lysis was quantified by the Bradford Protein Assay, in Synerg 4 Izasa Scientific. Samples were prepared with Laemmli buffer (5x), water and equal amount of protein (15 μ g for the transfected HCT-116 cells and 20 μ g for the mice samples) were loaded on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% milk in tris-buffered saline and Tween 20 (TBST) and incubated overnight with one of the primary antibodies described in Table 6, with Vinculin as a loading control. Membranes were developed in Curix 60, AGFA, after 1 hour-incubation with the corresponding secondary antibody (1:5000 in 5% milk in TBST) and ECL following the manufacturer's instructions.

Table 6 - Antibodies used for Western Blot. The data indicates for which type of sample the antibody was used, its target protein, its origin, and the concentration at which they were used.

Experiment	Target	Origen	Concentration
Mice Samples	CHOP	Mouse	1:1000
	GRP78	Rabbit	1:1000
	p21	Mouse	1:200
	p53	Mouse	1:1000
	Vinculin	Mouse	1:2000
Transfected HCT-116 cells	α -HA	Mouse	1:1000
	Human URI 71.9	Mouse	1:500
	Vinculin	Mouse	1:2000

2.10. mRNA detection by qPCR

Small parts of frozen intestine from mice were lysated with 1mL of Trizol reagent¹⁶, in Precellys® Evolution tissue homogenizer, and centrifuged at maximum speed, at 4°C, for 10 minutes (Centrifuge 5425R, Eppendorf). 200 µL of chloroform were added to the supernatant and after an incubation at room temperature, at 1400 rpm, for 10 min (Thermomixer Compact, Eppendorf), the samples were centrifuged at 12000G, at 4°C, for 15 min. This separates the sample into 3 phases - an aqueous phase containing the RNA, the interphase containing DNA, and an organic phase containing proteins.

The upper layer of the supernatant was collected and 500 µL of isopropanol added. The samples were centrifuged at 12000G, at 4°C, for 30 min. The pellet obtained was washed with 1mL of 75% ethanol and centrifuged at 7500 rpm, at room temperature, for 10 min. The supernatant was resuspended in 50 µL of nuclease-free water. The quantity and quality of RNA obtained was measured in NanoDrop One, Thermo Scientific.

cDNA was retrotranscribed from the mRNA samples by incubating 1 µL of random primers, 1 µL of dNTP mix and the obtained RNA solution at a concentration of 2 µg/µL, in nuclease free water. The samples were incubated at 65°C, for 5 min.

A master mix of 4 µL of first strand buffer and 2 µL of DTT 0.1M was added to each sample and the solutions incubated at 37°C, for 2 min. After the addition of 1 µL of Moloney Morine Leukemia Virus (MMLV) reverse transcriptase enzyme, isolated from *E. coli*, the samples were incubated for 50 min, at 37°C. The enzyme was inactivated by an incubation at 70°C, for 15 min.

The objective was to check the mRNA levels of the following genes: *Chop*, *Atf4*, *Atf6*, *Bcl-2*, *Cdkn2A* (for p16 protein), *Cdkn2D* (for p19 protein), *Cdkn1B* (for p27 protein), *Tp53*, *uXbp1*, *sXbp1* and the housekeeping gene *Gapdh*, resorting to the primers of Table 7. A primer mix - forward primer (final concentration 1 µM) and reverse primer (final concentration 1 µM) - was prepared for each pair of primers.

To measure the relative amount of each gene of interest, qPCR was performed by mixing 5 µL of syber green, 1 µL of the primers mix and 3 µL of the cDNA. qPCR plates were then closed with a Axygen Cyclorseal sealin film. Real-time quantization was performed using LightCycler based system, Eppendorf.

¹⁶ Trizol Reagent, a mixture of guanidine thiocyanate and phenol in a monophasic solution, is used for the simultaneous isolation of RNA, DNA and protein. The resulting RNA is intact with little or no contaminating DNA and protein.

Table 7 - Primers used for qPCR analysis and their sequence.

Gene	Primer	Primer Sequence (5'-3')
<i>Chop</i>	<i>Chop</i> mh Fw	GCTGTGGTAGTGAGCTGTTGCA
	<i>Chop</i> mh Rv	CACAGCCCAGGTATGGAATCA
<i>Atf4</i>	<i>Atf4</i> mh Fw	AAACCTCATGGGTTCTCCAG
	<i>Atf4</i> mh Rv	GGCATGGTTTCCAGGTCATC
<i>Atf6</i>	<i>Atf6</i> mh Fw	AATTCTCAGCTGATGGCTGT
	<i>Atf6</i> mh Fw	TGGAGGATCCTGGTGCCAT
<i>Bcl-2</i>	<i>Bcl-2</i> Fw	GTACCTGAACCGGCATCG
	<i>Bcl-2</i> Rv	GGGGCCATATAGTTCCACAA
<i>Cdkn2A</i>	<i>Cdkn2A</i> Fw	GTGTGCATGACGTGCGGG
	<i>Cdkn2A</i> Rv	GCAGTTCTGAATCTGCACCGTAG
<i>Cdkn2D</i>	<i>Cdkn2D</i> Fw	GCTCTGGCTTTTCGTGAACATG
	<i>Cdkn2D</i> Rv	TCGAATCTGCACCGTAGTTGAG
<i>Cdkn1B</i>	<i>Cdkn1B</i> Fw	AGGAGAGCCAGGATGTCAGC
	<i>Cdkn1B</i> Rv	CAGAGTTTGCCTGAGACCCAA
<i>Tp53</i>	<i>Tp53</i> Fw	ATGCCCATGCTACAGAGGAG
	<i>Tp53</i> Rv	AGACTGGCCCTTCTTGCTCT
<i>uXbp1</i>	<i>uXbp1</i> Fw	TCCGCAGCACTCAGACTACG
	<i>Xbp1</i> Rv	AGTTGTCCAGAATGCCCAACA
<i>sXbp1</i>	<i>sXbp1</i> Fw	CTGAGTCCGCAGCAGGTG
	<i>Xbp1</i> Rv	AGTTGTCCAGAATGCCCAACA
<i>Gapdh</i>	<i>Gapdh</i> Fw	CGTCCCGTAGACAAAATGGT
	<i>Gapdh</i> Rv	TCAATGAAGGGGTCGTTGAT

2.11. Statistical Analysis

For each experiment and assay, the number of replicas is presented. Statistical analyses were performed using GraphPad 8.3.0 (GraphPad Software, CA, United States). Statistical significance (p) (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$) between the means of the groups was tested by using the appropriate statistical test (Students t-test for 2 groups, one-way ANOVA with post-hoc Tukey HSD test for more than 2 groups). Results are represented as the mean $\pm$ Standard Error of the Mean (SEM).

Chapter 3

3. Results

3.1. URI loss prevents tumor formation in the *Apc* model

The genotyping protocol allows for the identification of the alleles of *Cre*, *Apc* and *Uri*. This procedure was performed when mice are born, and after their sacrificing them, to ensure that the genotyping was correct. An example of the PCR gels can be analyzed in Figure 13A, Figure 13B and Figure 13C.

The genotyping protocol used for *Cre* reveals which mice have the *Cre* allele, however it does not demonstrate which are heterozygous or homozygous. However, the mice analyzed are known to be heterozygous for *Cre*, as we followed a careful mating procedure, assuring that any possible off-target effects can occur in the same frequency in all the mice, either *Apc* wild-types or *Apc* mutants.

The Cre-mediated recombination leads to deletion of the loxP-flanked exon 15 of the *Apc* gene, resulting in the *Apc*^Δ allele. To confirm the genotype of the mice, by PCR, *Apc*⁺ and *Apc*^{lox} alleles were detected in a single reaction with a combination of two forward primers and one reverse primer, as described in (Robanus-Maandag et al., 2010).

These mice were also crossed with the *Uri* wild-type (*Uri*^{+/+}) or *Uri* mutant (*Uri*^{+/Δ}) mice. Thus, regarding the *Uri* genotype, mice can reveal a single smaller (and lower band) in the PCR gel, the *Uri*⁺ allele, or a bigger (and upper band), the *Uri*^{lox} allele, as well.

During the necropsy procedure, mice were analyzed based on different parameters. Regarding the body weight, demonstrated in Figure 13D, the *Apc* mutant mice reveal a decrease in body weight, however the *Uri* genotype does not reveal a clear influence in that.

The mice spleen weight can be associated with the mice well-being - sick mice experience an increase in the spleen weight (Simpson, 1975) and it is described that models with *Apc* mutations tend to present splenomegaly. Therefore, the mice spleen weight was normalized by their body weight (Figure 13E), revealing that the spleen weight is influenced by the mice genotype. In general, *Apc* mutant mice have bigger spleens, as expected, with URI loss significantly reverting that phenotype, while in *Apc* wild-type mice, the *Uri* genotype has no influence. *Apc*^{+/Δ} *Uri*^{+/+} mice show a significant increase in spleen weight, compared to *Apc*^{+/+} *Uri*^{+/+}, the same tendency is revealed in *Apc*^{+/Δ} *Uri*^{+/Δ} compared to *Apc*^{+/+} *Uri*^{+/Δ}. Therefore, we can conclude that the *Apc* mutation potentiates splenomegaly, but URI loss can revert that phenotype, which can correlate with mice being healthier.

Regarding liver weight normalized by the mice body weight, the *Apc* mutant mice have bigger liver, which can be associated with steatosis. However, comparing mice with *Uri*^{+/+} genotype, *Apc* mutant show an increase in liver weight (Figure 13F).

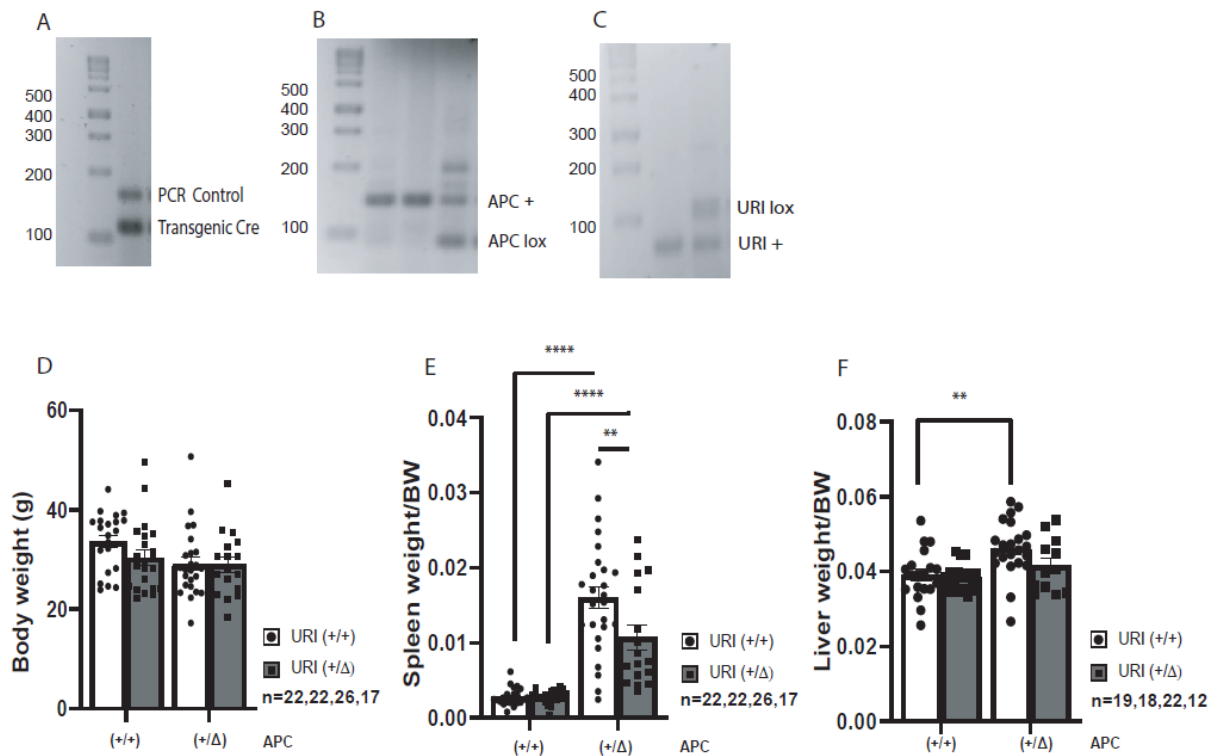


Figure 13 - Example of the genotype of the analyzed mice and data from the mice necropsy. (A) The *Cre* genotype, with an upper band that confirms that the PCR protocol worked, and a lower band that represents the transgenic *Cre*. (B) The *Apc* genotype, with an upper band representing the wild-type allele, and a lower band representing the *Apc*^{lox} allele. (C) The *Uri* genotype accounts for an upper band, the *Uri*^{lox}, and a lower band that represents the wild-type allele. (D) Mice body weight (g), for *Apc*^(+/+)_{int} and *Apc*^(+/Δ)_{int}, either *Uri*^(+/+)_{int} or *Uri*^(+/Δ)_{int}. (E) Mice spleen weight normalized by body weight, for *Apc*^(+/+)_{int} and *Apc*^(+/Δ)_{int}, either *Uri*^(+/+)_{int} or *Uri*^(+/Δ)_{int}. (F) Mice liver weight normalized by body weight, for *Apc*^(+/+)_{int} and *Apc*^(+/Δ)_{int}, either *Uri*^(+/+)_{int} or *Uri*^(+/Δ)_{int}. Data are mean±S.E.M. from n independent experiments, the n is indicated on each graph. Statistical analysis by one-way ANOVA (**=p<0.01, ****=p<0.0001).

The objective of this project is to analyze how mutations in *Uri* can alter and affect the tumor development in the intestines. In the time points used, as expected, the *Apc* wild-type mice do not form tumors. On the other hand, *Apc* mutant mice develop tumors in both the colon and small intestine, as demonstrated in Figure 14B and Figure 15B.

Focusing on the colon, the length of the organ is not affected by *Apc* genotype (Figure 14A). Furthermore, colon size is also similar in *Uri* wild-type and mutant mice, however they demonstrate a different number of tumors (Figure 14B). Unexpectedly, the *Uri* mutant mice have more tumors in the large intestine. A pathologist studied the tumors in the colon and graded them between 0 and 4 - from normal tissue to adenocarcinoma. This analysis revealed the maximum tumor grade reached in each mouse, that culminated in the results of Figure 14C. While with *Uri* loss, mice reveal more

tumors with grade 0, there also more mice with grade 4 tumors. Overall, these data reveal that, in the colon, URI loss seems to increase the number and aggressiveness of tumors.

A differential analysis based on sex was performed on the mice, as in humans it is clear that men develop more colorectal cancer than women. This can be explained by a protective role of estrogens, as pre-menopausal women are less likely to develop colorectal cancer compared to age-matched men (Ditonno et al., 2021). Besides, post-menopausal women experience a fall of estrogen levels and have a worse overall survival, compared to age-matched men after the diagnosis of metastatic colorectal cancer. Two isoforms of nuclear estrogen receptors have been described, and while the α isoform activates anti-apoptotic pathways, the β isoform activates caspase-3 and induces apoptosis. The latter also tends to be under-regulated in advanced adenomas and colorectal cancer, in opposition to the α (Ditonno et al., 2021). These effects of estrogen on carcinogenesis have also been studied in animal model. Treatment with β isoform estrogen receptors in male and female *Apc* mutant mice reduces the number of small intestinal polyp number and diameter. This isoform is also described as an inhibitory modifier of APC-dependent carcinogenesis in the proximal colon (Ditonno et al., 2021).

Based on this fact that men are more prone to develop colorectal cancer, we sought to determine if this same sex-drive tendency is present in our mice model. In both groups, male present more tumors in colon (Figure 14D). When analyzing the mice by sex, in female mice, the number of colon tumors is smaller when *Uri* is mutated (Figure 14E). However, in male, mutations in *Uri* increase the number of tumors (Figure 14E). The pathologist analysis of tumor grade was also assessed based on mice sex, and in female mice the tumors become less aggressive with URI loss, unlike what happens in males (Figure 14F).

Concluding, URI loss in the colon increases the number and grade of tumors, and this effect depends on the mice sex.

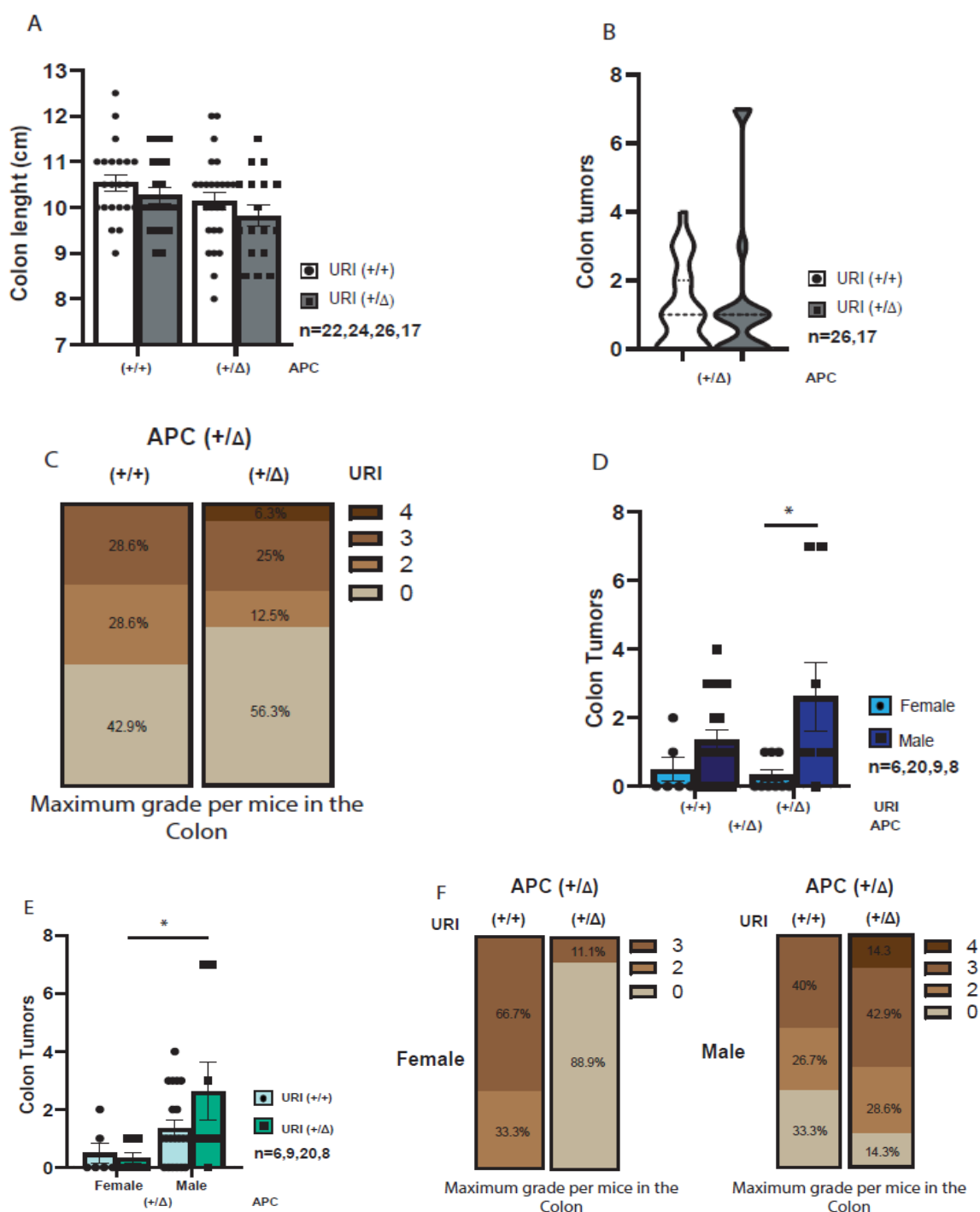


Figure 14 - Analysis of the colon length and tumors. (A) Colon length (cm) for $Apc^{(+/-)int}$ and $Apc^{(+/-)int}$, either $Uri^{(+/-)int}$ or $Uri^{(+/-)int}$. (B) Number of tumors in the colon, in $Apc^{(+/-)int}$, either $Uri^{(+/-)int}$ or $Uri^{(+/-)int}$. (C) Maximum grade of the tumors in the colon, in $Apc^{(+/-)int}$ mice, either $Uri^{(+/-)int}$ or $Uri^{(+/-)int}$. 0-normal; 2-Low grade adenoma; 3-High grade adenoma; 4-Carcinoma. (D) Number of tumors in the colon for $Apc^{(+/-)int}$, either $Uri^{(+/-)int}$ or $Uri^{(+/-)int}$, separated by the mice sex. (E) Number of tumors in the colon for $Apc^{(+/-)int}$ female and male, separated by the mice Uri genotype. (F) Maximum grade of the tumors in the colon, in $Apc^{(+/-)int}$ mice, either $Uri^{(+/-)int}$ or $Uri^{(+/-)int}$, separated by the mice sex. 0-normal; 2-Low grade adenoma; 3-High grade adenoma; 4-Carcinoma. Data are mean $\pm$ S.E.M. from n independent experiments, with n indicated by each graph. Statistical analysis by Students t-test or one-away ANOVA (*= $p < 0.05$).

When analyzing the small intestine, the data reveal that the organ's length is not altered by the *Apc* or *Uri* genotypes (Figure 15A). Focusing on the number of tumors in the intestine, URI loss potentiates a statistically significant decrease (Figure 15B).

The pathologist's analysis of the tumors was also performed in the intestine. In this case, *Uri* loss decreases the percentage of mice with more severe tumors - there are less mice with grades 3 and 4 (more aggressive tumors) and more with grades 0 to 2 (Figure 15C).

As explained previously, based on human data, it is expected that male mice have more tumors (Ditonno et al., 2021), but in the intestine this tendency is not very clear (Figure 15D). Regarding the effects of *Uri* mutation (Figure 15E), both sexes follow the same tendency - URI ablation decreases the number of intestine tumors. Figure 15F reveals the fold-change of the number of tumors compared to the wild-type female mice, further confirming the previous conclusions, that male and female have similar number of tumors and URI loss decreases this value. A better prognosis can be expected of female and male mice upon URI loss, as, in both sexes, the induced *Uri* mutation decreases the maximum tumor grade on the mice (Figure 15G).

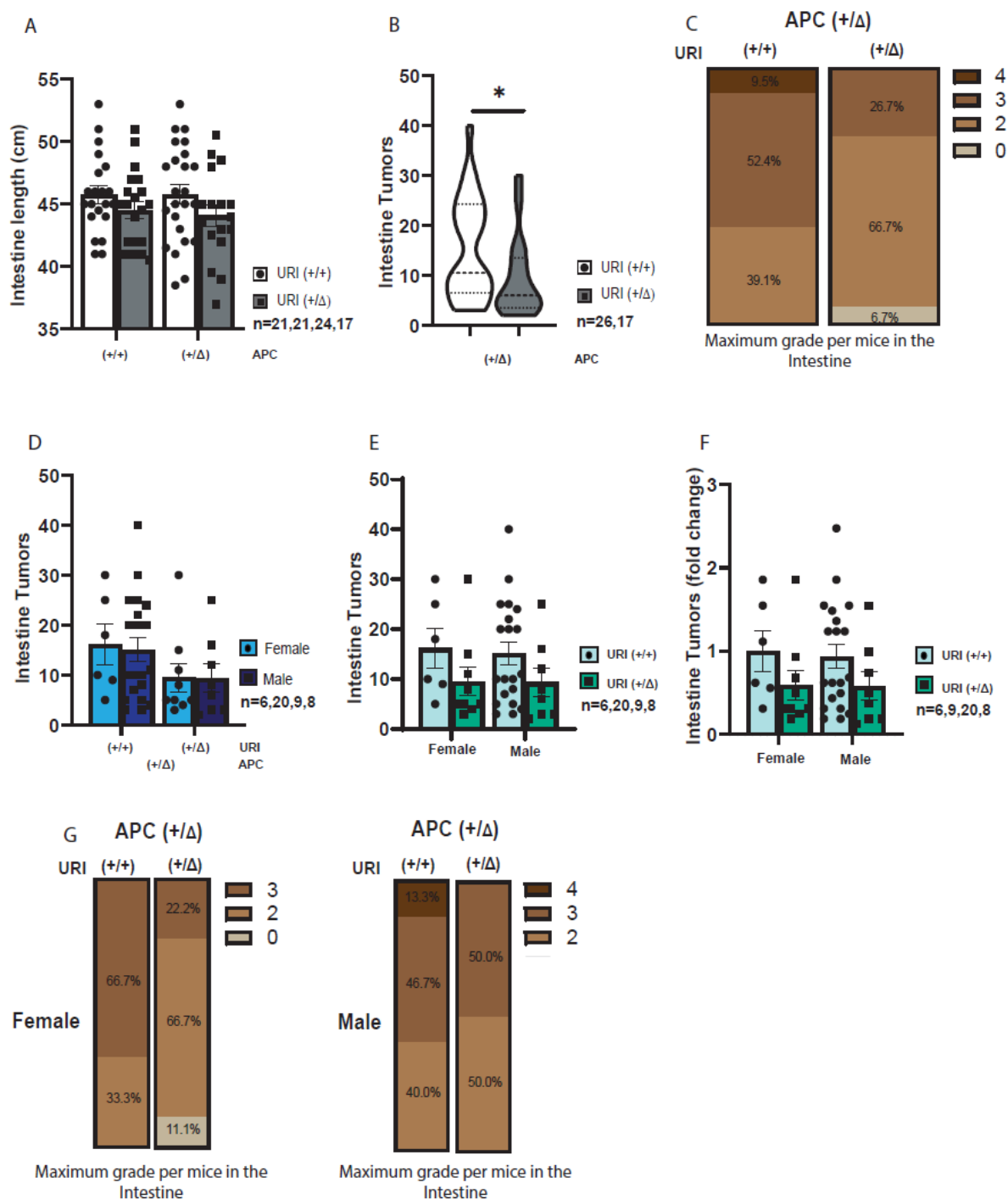


Figure 15 - Analysis of the small intestine length and tumors. (A) Intestine length (cm) for *Apc*^{(+/+)int} and *Apc*^{(+/Δ)int}, either *Uri*^{(+/+)int} or *Uri*^{(+/Δ)int}. (B) Number of tumors in the intestine, in *Apc*^{(+/Δ)int}, either *Uri*^{(+/+)int} or *Uri*^{(+/Δ)int}. (C) Maximum grade of the tumors in the intestine, in *Apc*^{(+/Δ)int} mice, either *Uri*^{(+/+)int} or *Uri*^{(+/Δ)int}. 0-normal; 2-Low grade adenoma; 3-High grade adenoma; 4-Carcinoma. (D) Number of tumors in the intestine for *Apc*^{(+/Δ)int}, either *Uri*^{(+/+)int} or *Uri*^{(+/Δ)int}, separated by the mice sex. (E) Number of tumors in the intestine for *Apc*^{(+/Δ)int} female and male, separated by the mice *Uri* genotype. (F) Fold-change of the number of tumors in the intestine for *Apc*^{(+/Δ)int} female and male, separated by the mice *Uri* genotype, compared to female *Apc*^{(+/Δ)int} *Uri*^{(+/+)int} mice. (G) Maximum grade of the tumors in the intestine, in *Apc*^{(+/Δ)int} mice, either *Uri*^{(+/+)int} or *Uri*^{(+/Δ)int}, separated by the mice sex. 0-normal; 2-Low grade adenoma; 3-High grade adenoma; 4-Carcinoma. Data are mean±S.E.M. from n independent experiments, with n indicated on each graph. Statistical analysis by Students t-test or one-way ANOVA (*= $p < 0.05$).

The different tendencies seen in the intestine and colon can be due to the different structure of the organs - they both have crypts, but the colon lack villi. Besides, the number of mice expressing tumors in the colon is smaller, making the n less significant when comparing to the small intestine, which leads to the necessity to further test the tumors in the colon, with a bigger cohort.

As expected, the mice *Apc* model for colorectal cancer develops more tumors in the small intestine. Therefore, the focus of our analysis is the small intestine and its tumors, based on the assumption that this can correlate to what happens in humans, although in humans the *APC* mutation affects the colon. Since both female and male mice show the same tendency in the number of tumors, we believe that the model has the same effect in both sexes, therefore no differential analysis must be performed based on mice sex.

The hematoxylin and eosin staining was used to measure the size of the tumors in the intestine. Figure 16A and Figure 16B present an intestinal tumor, that disrupts the intestinal epithelium architecture, with different dimensions. Figure 16C demonstrates the average area of the tumors in each mouse, while Figure 16D reveals the total tumor area on each mouse. Both these data, confirm the hypothesis upon URI loss, *Apc* mutant mice have decreased tumor size.

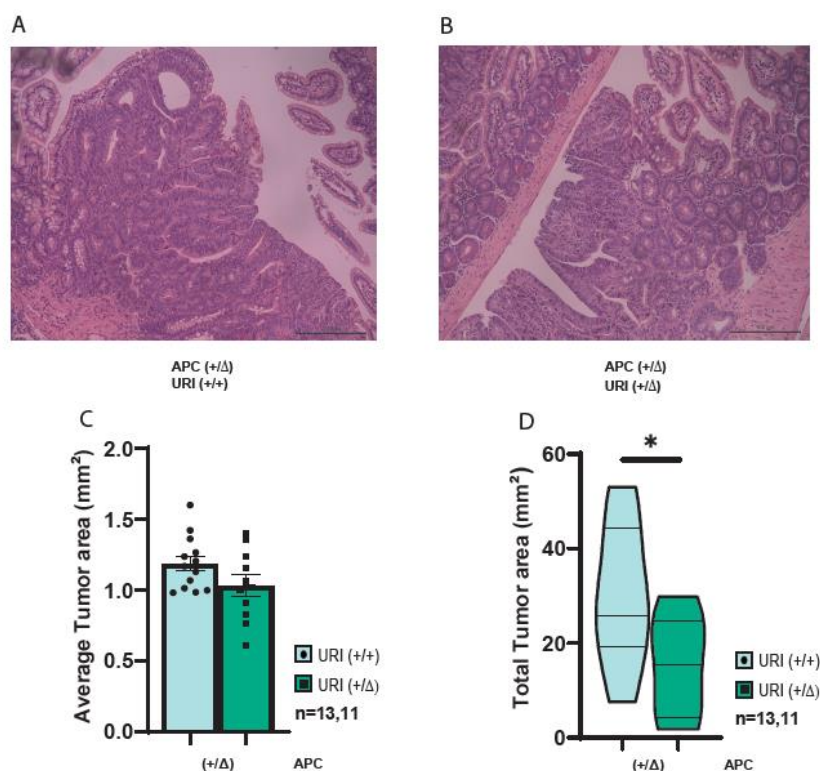


Figure 16 - The tumor area is smaller in mice upon URI loss. (A and B) H&E in intestinal sections of *Apc*^{(+/Δ)int} *Uri*^{(+/+)int} mice (A) and *Apc*^{(+/Δ)int} *Uri*^{(+/Δ)int} mice (B). (C) Average tumor area (mm²) on each *Apc*^{(+/Δ)int} mice, either *Uri*^{(+/+)int} or *Uri*^{(+/Δ)int}. (D) Total tumor area (mm²) (The areas of each individual tumor were summed) on each *Apc*^{(+/Δ)int} mice, either *Uri*^{(+/+)int} or *Uri*^{(+/Δ)int}. Data are mean±S.E.M. from n independent experiments, with n indicated on each graph. Statistical analysis by Students t-test (*=p<0.05).

As not only the size of the tumor decreases with URI loss, but there is also a smaller number of tumors, we conclude that it is possible that this mutation is preventing tumor formation. In fact, URI overexpression has been described to promote survival, while its deletion enhances cancer cell death (Tummala et al., 2014). The objective is to understand how this is happening in our model.

URI reduction enhances Nicotinamide Adenine Dinucleotide (NAD^+) levels, as URI inhibits aryl hydrocarbon and estrogen receptor-mediated transcription of enzymes implicated in NAD^+ metabolism (Tummala et al., 2014). NAD^+ acts as a cofactor playing an essential role in many enzymatic reactions of energy metabolism, such as glycolysis, oxidative phosphorylation, and fatty acid oxidation. A deregulation of the NAD^+ levels has been associated with metabolic diseases and aging-related diseases, including neurodegeneration, defective immune responses, and cancer (Navas & Carnero, 2021).

3.2. URI loss increases the levels of p53

To understand how URI loss can affect the tumor size and number in the *Apc* model, we first studied its effects on p53, as the decrease in the expression of p53 is one of the first milestones of APC mutations.

The activity of p53 is inactivated in nearly all tumors, either by mutations in the *TP53* locus or by oncogenic events that decrease the protein activity, such as overexpression of the repressor MDM2 (Sullivan et al., 2018). Therefore, the protein levels of the p53 were assessed by western blot and the levels of *Tp53* evaluated by qPCR (Figure 17).

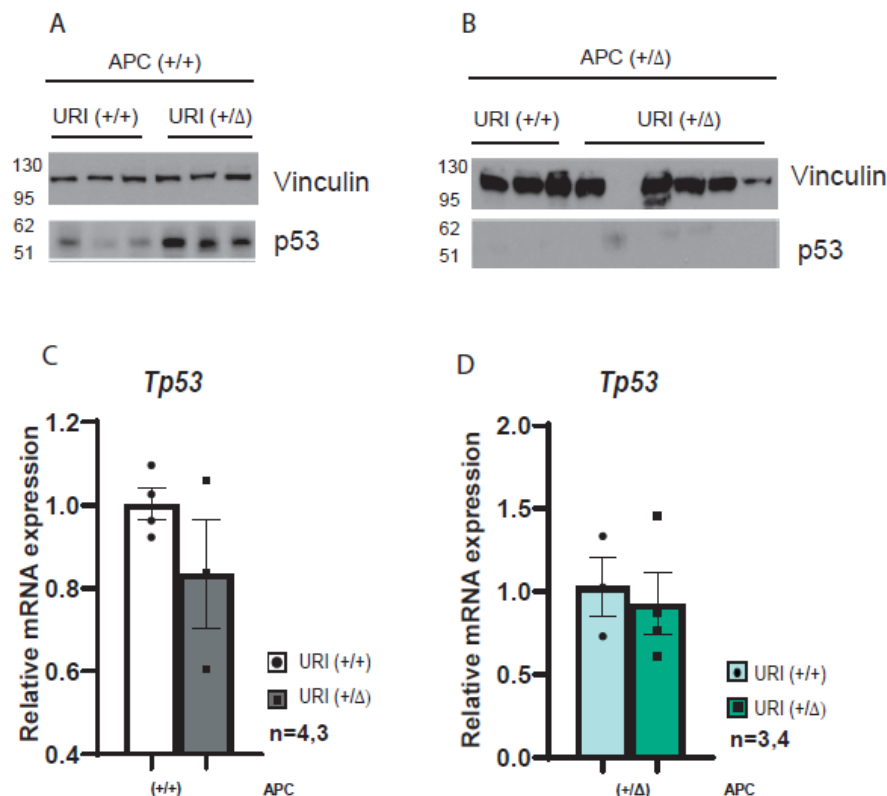


Figure 17 - URI loss promotes an increase in p53 protein levels. (A and B) Western blot for p53 protein levels analysis in *Apc*^{(+/+)int} mice (A) and *Apc*^{(+/-)int} mice (B), with vinculin as a loading control. (C and D) qPCR results, for the relative mRNA levels of *Tp53* in *Apc*^{(+/+)int} mice (C) and *Apc*^{(+/-)int} mice (D). Data are mean $\pm$ S.E.M. from n independent experiments, with n indicated on each graph.

In *Uri* wild-type mice, the protein levels of p53 increases with URI loss, while the relative *Tp53* mRNA expression decreases (Figure 17C). This further explains how URI can function as an oncogene, as loss of function of *Uri* by itself affects the expression of a tumor suppressor.

Regarding the *Apc* mutant mice, as expected, *Uri* wild-type mice do not express p53, as demonstrated in the western blot data of Figure 17B. On the other hand, URI loss reestablishes the expression of the tumor suppressor, demonstrating that there is a switch in the phenotype upon URI

ablation. In fact, these mice lose one of the hallmarks of colorectal cancer induced by mutations in the *Apc* locus. This can be used to explain the decrease in the number and size of intestinal tumors, followed by a decrease in spleen size.

As the levels of p53 protein increase (Figure 17A and Figure 17B), but the mRNA levels of *Tp53* do not follow this tendency (Figure 17C and Figure 17D), it is clear to state that this reestablishment of p53 expression occurs at a protein level.

3.3. URI loss does not affect Unfolded Protein Response

Because URI is a molecular chaperone whose expression is apparently modulated by various environmental factors, it may be that its primary function is to maintain cellular and protein homeostasis (Chaves-Pérez et al., 2018). Therefore, we further analyzed how the unfolded protein response in altered in our mice model.

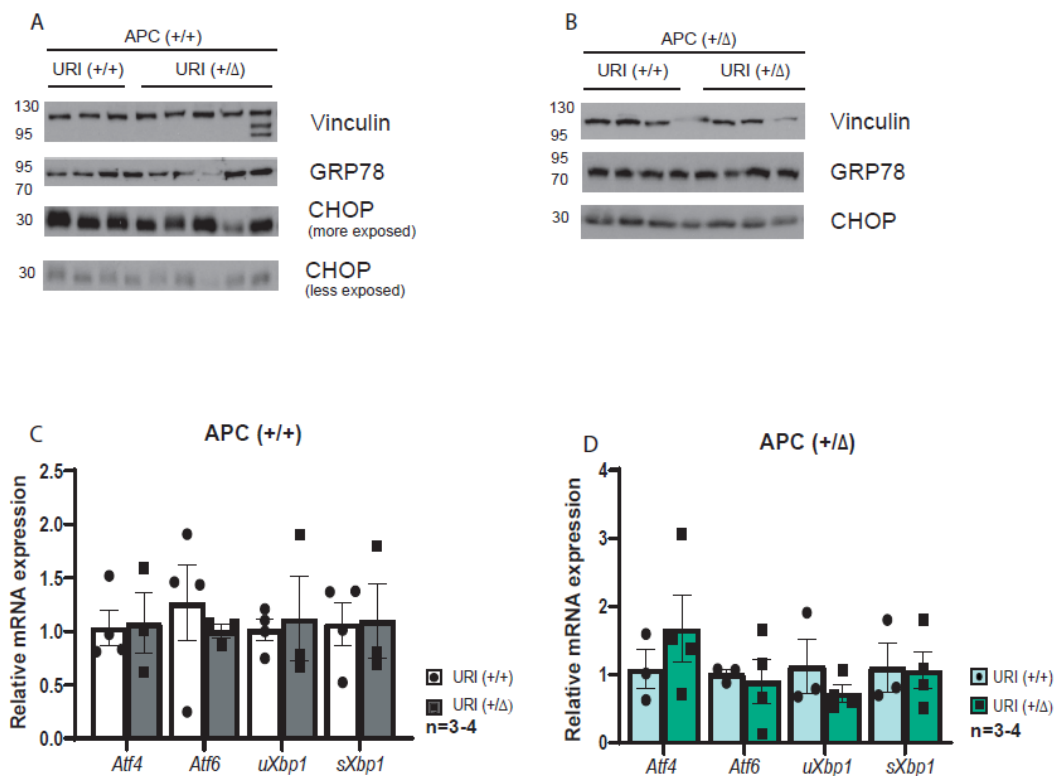


Figure 18 - URI loss effects on the UPR. (A and B) Western blot for GRP78 and CHOP protein levels analysis in *Apc*^{(+/+)int} mice (A) and *Apc*^{(+/Δ)int} mice (B), with vinculin as a loading control. (C and D) qPCR results, for the relative mRNA levels of *Atf4*, *Atf6*, *uXbp1* and *sXbp1* in *Apc*^{(+/+)int} mice (C) and *Apc*^{(+/Δ)int} mice (D). Data are mean ± S.E.M. from n independent experiments, with n indicated on each graph.

Phosphorylation of eIF2 α by PERK leads to transcriptional upregulation of several genes, including Growth Arrest and DNA Damage-inducible protein (*GADD153/CHOP*), *GADD34*, and ER oxidase-1 (Hiss & Gabriels, 2009). These nuclear transcriptional factors have been linked not only to cellular responses to ER stress, but also to apoptosis and cancer. *GADD153/CHOP* is a mediator of ERAD and the UPR. Its increased expression, following ER stress, represents a transition from an adaptive UPR response to an apoptotic or cell death response, as *CHOP* forms heterodimers with other C/EBP proteins and causes growth arrest by blocking G1 to S phase cell cycle progression (Hiss & Gabriels, 2009; Zhu et al., 2007). Therefore, the levels of GRP78/Bip and *CHOP* were evaluated by Western blot (Figure 18A and Figure 18B), while the mRNA expression of *Atf4*, *Atf6*, *uXbp1* and *sXbp1* were evaluated by qPCR (Figure 18C and Figure 18D).

In both *Apc* wild-type and mutant mice, URI loss does not significantly affect the levels of the UPR effectors, both at protein and mRNA levels.

In most cancers, *CHOP* expression may be a robust indicator of the initiation of apoptosis in response to ER stress or the UPR (Hiss & Gabriels, 2009). Therefore, as there are no differences in the levels of *CHOP* with the mutations in *Uri*, one may expect that apoptosis is not altered with URI loss.

3.4. URI loss does not affect apoptosis

Cellular stress, such as DNA damage, oncogene activation, nutrient deprivation, hypoxia, and ER stress activate sensor proteins that phosphorylate p53, leading to its stabilization and oligomerization. P53 targets the activation of checkpoint genes that promote cell cycle progression through the p53/p21waf1/cip1 pathway, initiate apoptosis, promote DNA repair, senescence, and apoptosis, as demonstrated in Figure 19 (Hernández Borrero & El-Deiry, 2021; Hiss & Gabriels, 2009).

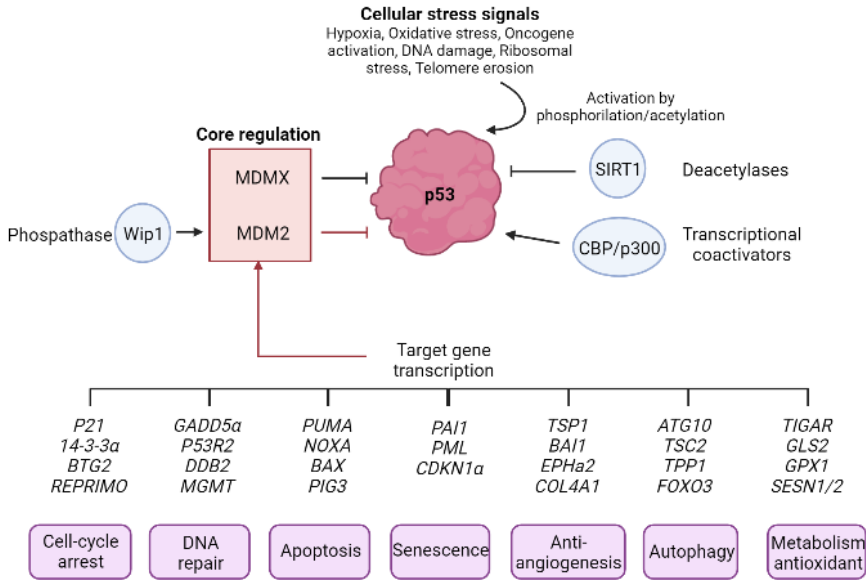


Figure 19 - The p53 pathways. p53 is activated by cellular stress signals and activates different cellular responses, as cell-cycle arrest, apoptosis, senescence, and autophagy, by controlling target gene transcription (Hernández Borrero & El-Deiry, 2021; Hiss & Gabriels, 2009). Created with Biorender.com

As p53 is predominantly associated with apoptosis, we first sought to determine how the induced mutation in *Uri* locus affects apoptosis in the small intestine. BCL2 is an anti-apoptotic protein mostly regulated through posttranscriptional regulation (Shen et al., 2004), therefore apoptosis can be examined through the expression of *Bcl2* mRNA (Figure 20A and Figure 20B). The *Apc* wild-type mice reveal a decrease in *Bcl2* with URI loss, thus mice lacking URI undergo more apoptosis than wild-type mice. The opposite effect is detected in the *Apc* mutant mice - URI^{+/-} have significantly higher levels of *Bcl2*, thus they undergo apoptosis less frequently.

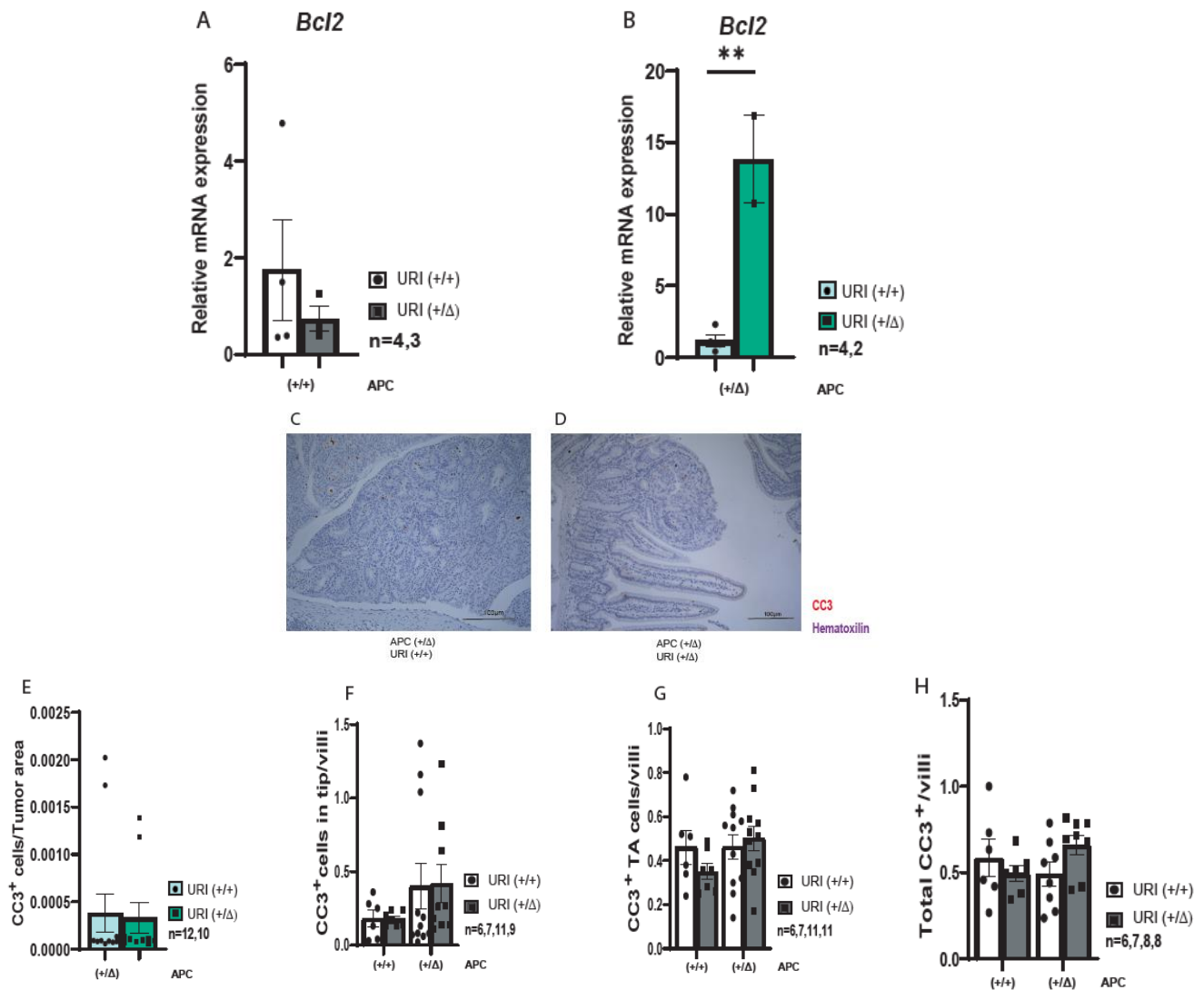


Figure 20 - URI loss decreases apoptosis in *Apc* mutant mice. (A and B) qPCR results for the relative mRNA levels of *Bcl2* in *Apc*^{(+/+)int} mice (A) and *Apc*^{(+/Δ)int} mice (B). (C and D) IHC of CC3 in intestinal tumors from *Apc*^{(+/+)int} *Uri*^{(+/+)int} mice (C) and *Apc*^{(+/Δ)int} and *Uri*^{(+/Δ)int} mice (D). (E-H) Quantification of positive CC3 cells per tumor area (E), in the tip of the villi normalized by the number of villi (F), in the TA (cells in the villi that are not on the top) cells normalized by the number of villi (G), and the total positive cells per villi (H). Data are mean±S.E.M. from n independent experiments, n is indicated on each graph. Statistical analysis by Students t-test (**=p<0.01).

Apoptosis is a form of programmed cell death regulated by the BCL-2 family, but also by the caspase family of proteins (Brentnall et al., 2013). Intrinsic apoptosis is a mitochondrion-centered cell death, mediated by mitochondrial outer membrane permeabilization, that results in apoptosome formation, activation of caspase-9 and subsequent activation of effector caspases (Brentnall et al., 2013). After activation by caspase-9, caspase-3 inhibits reactive oxygen species production and is required for efficient execution of apoptosis. Therefore, cleavage of caspase-3 is generally considered a universal marker of apoptosis, as its activity is required for the majority of morphological and biochemical events associated with apoptosis (Crowley & Waterhouse, 2016). Caspase-3 is cleaved at two sites that result in three fragments - the prodomain, the large subunit, and the small subunit - the second cleavage is the one that results in full activation.

Therefore, apoptosis in the small intestine was analyzed by the expression of cleaved caspase 3 (CC3) in the intestine, by immunohistochemistry (Figure 20C-H). In the intestine, the epithelium is renewed every four to five days, as the cells move up in the villi and undergo apoptosis (van der Flier & Clevers, 2009). Thus, the levels of CC3 in the cells of the top of the villi (Figure 20F) are not expected to change due to alterations in *Uri*, as this is a physiological procedure. However, *Apc* mutant mice, especially with URI loss, are the ones presenting the higher number of positive cells for CC3 in the entire tip (Figure 20F). This effect is not clear when analyzing the TA (cells in the villi that are not on the top) cells (Figure 20G).

The total CC3 expression in cells on both the cells on the tip and not on the tip (Figure 20H), normalized by the number of villi, decreases with *Apc* mutation, in mice expressing wild-type URI. The opposite occurs in mice with URI loss of function, with an increase in the number of total positive cells in the villus, in the *Apc* model. In general, in the *Apc* mutant mice, *Uri* mutation increases the number of cells undergoing apoptosis. This can be a sign that tumors are not being formed by an increase in the apoptosis of cells in the villi, that by proliferating less, harbor a smaller ability to form tumors.

Regarding the tumors in *Apc* mutant mice (Figure 20C-E), URI wild-type mice demonstrate a higher number of cells undergoing apoptosis, demonstrated here by the staining of cells for CC3.

3.5. URI loss promotes senescence

As p53 is not altering the number of apoptotic cells in the URI mutant mice, the protein must be involved in other pathways (Figure 19). In fact, activation of p53 can lead to cell senescence. Cellular senescence is a permanent state of cell-cycle arrest, that promotes tissue remodeling, upon development and after injury, and contributes to the decline of the regenerative potential and function of tissues, to inflammation, and to tumorigenesis in aged organisms (Hernandez-Segura et al., 2018). The senescence phenotype is characterized by activation of a chronic DNA damage response, cyclin-dependent kinase inhibitors, enhanced secretion of proinflammatory and tissue-remodeling factors, induction of antiapoptotic genes, altered metabolic rates and ER stress (Hernandez-Segura et al., 2018) (Figure 21). Although this is a well-studied state, the phenotype of senescent cells is heterogenous and dynamic, as there may be various distinct senescence programs.

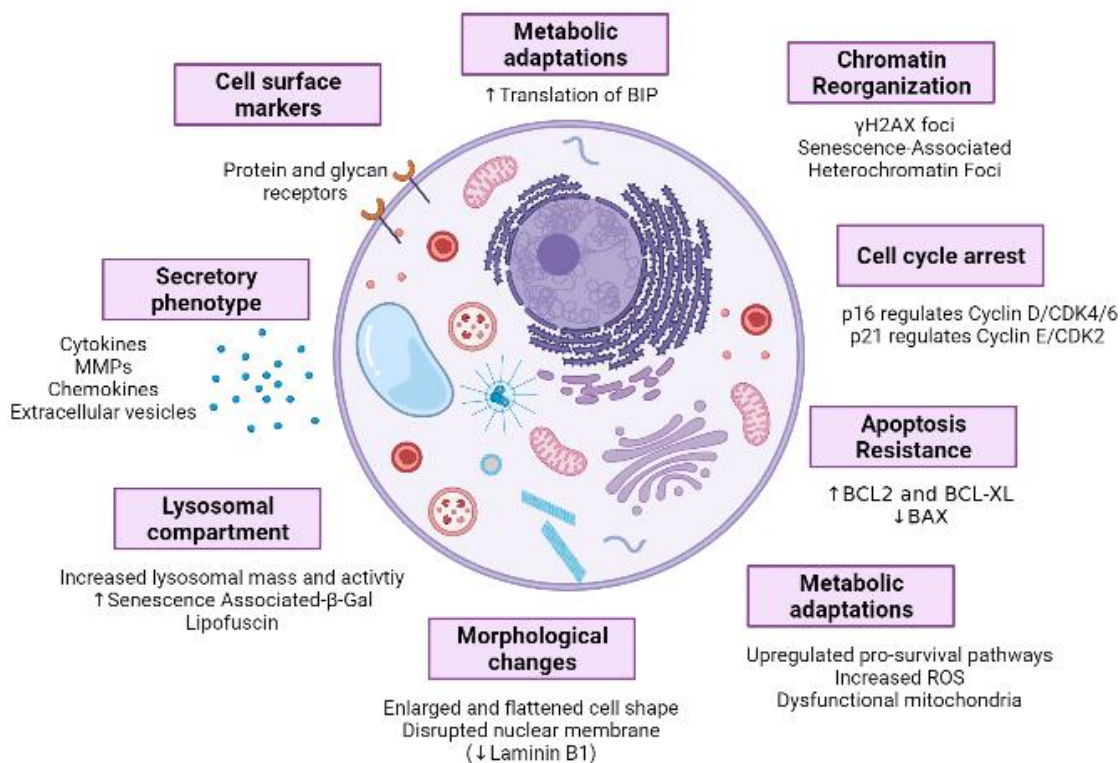


Figure 21 - Senescence phenotype. Senescent cells present different metabolic, morphological, and secretory phenotypes, while also undergoing cell cycle arrest and resisting apoptosis. Created with Biorender.com, based on (Hernandez-Segura et al., 2018).

Senescent cells are metabolically active, and increase their AMP/ATP and ADP/ATP ratios (Hernandez-Segura et al., 2018). P53 can regulate cellular metabolism by leading to inhibition of glucose uptake and glycolysis and promoting the tricarboxylic acid cycle, with the latter being essential for p53 activation in senescent cells. Many other proteins besides AMPK directly or indirectly

regulate p53, for instance via post-translational modifications or direct interactions, including MDM2 and loss of PTEN.

The Senescence-associated secretory phenotype (SASP) is, to a large extent, a transcriptional program mediated by the proinflammatory transcription factor NF- κ B. SASP leads to an increase in protein synthesis, ER stress and UPR. Indeed, senescent cells have an increased UPR (Hernandez-Segura et al., 2018). This senescence state is also characterized by the upregulation of many lysosomal proteins and increased lysosomal content. The activity of the lysosomal enzyme senescence-associated beta-galactosidase activity is used as a marker for this enhanced lysosomal content of senescent cells (Hernandez-Segura et al., 2018).

Besides, senescent cells present an enlarged and irregularly shaped body, due to a rearrangement of the cytoskeleton and ATF6 α activity (Hernandez-Segura et al., 2018).

Cell division relies on activation of cyclins, that bind to cyclin-dependent kinases (CDKs), to induce cell-cycle progression towards S phase (Coqueret, 2003). This process is highly controlled, as uncontrolled activity of CDKs is often the cause of cancer. Therefore, CDK's function is regulated by cell-cycle inhibitors, as p21 and p27, that act through similar molecular mechanisms. Indeed, senescent cells activate cyclin-dependent kinase inhibitors (Hernandez-Segura et al., 2018). Following anti-mitogenic or DNA damage, p21 and p27 bind to cyclin-CDK complexes, inhibit their catalytic activity and induce cell-cycle arrest (Coqueret, 2003). P16 directly interacts and inhibits CDK4/6 and its transcriptional activation has been used to assess the presence of senescent cells *in vivo* (Hernandez-Segura et al., 2018). The translation of *CDKN2A* mRNA, encoding for p16, can be modulated a region on its 5' untranslated region. Moreover, p16 protein is degraded by polyubiquitination and the proteasome. P16 protein is relatively stable and its expression is primary regulated by transcriptional control (KOTAKE et al., 2015).

P21 was the first p53 transcriptional target to be identified and shown to be activated by p53 (Fischer et al., 2016). It is capable of inhibiting a range of CDKs, but it is also necessary for cell cycle progression (Hernandez-Segura et al., 2018). It is upregulated in response to different senescence-inducing stimuli and is directly transactivated via p53. P21 can also be activated in a p53-independent manner. P19 binds directly to and sequesters MDM2, inhibiting the capacity of MDM2 to induce degradation of the p53 tumor suppressor protein. This loss of MDM2 function, in turn, results in the stabilization of p53 and activation of p53-mediated growth arrest or apoptosis (Lundberg et al., 2000).

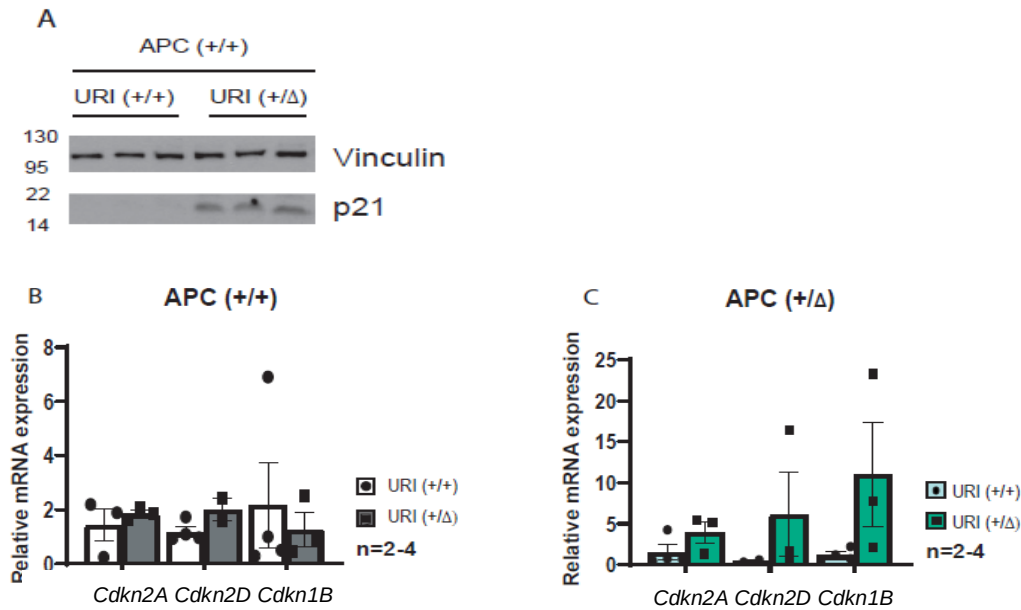


Figure 22 - URI loss promotes cell senescence. (A) Western blot for p21 protein levels analysis in *Apc*^{(+/+)int} mice, with vinculin as a loading control. (B and C) qPCR results for the relative mRNA levels of *Cdkn2A*, *Cdkn2D*, *Cdkn1B* in *Apc*^{(+/+)int} mice (B) and *Apc*^{(+/Δ)int} mice (C). Data are mean±S.E.M. from n independent experiments, with n indicated on each graph.

After a careful characterization of the senescence pathways, we analyzed the mRNA levels of several genes involved in cell senescence, including the ones related to cell cycle arrest (*Cdkn2A*, *Cdkn2D*, *Cdkn1B*), as demonstrated in Figure 22B and Figure 22C. The protein levels of p21 in *Apc* wild-type mice were also assessed and p21 protein levels are increased in URI mutant mice (Figure 22A). As p53 is under-expressed in *Apc* mutant mice, so are their downstream effectors, as p21. Therefore, it was not possible to detect p21 by Western blot in these mice. A western blot for Cyclin D was also performed, in *Apc* wild-type and mutant mice, but again no expression was revealed.

In the *Apc* mutant mice, upon URI loss, there is a clear increase in the relative mRNA expression of *Cdkn2A*, *Cdkn2D*, *Cdkn1B*, encoding for p16, p19 and p27, respectively (Figure 22B and Figure 22C).

Taken together all the results, we can conclude that, in general, the cells in the intestine are senescent. It is a fact that apoptosis-resistance is a main feature of senescent cells, therefore senescent cells reveal increase levels of antiapoptotic proteins, as BCL2, as cells with URI loss do (Figure 20B). Moreover, the protein levels of p21 and mRNA levels of *Cdkn2A*, *Cdkn2D* and *Cdkn1B* increased with URI loss of function, further contributing to the conclusion that these cells are senescent. However, senescent cells have an increased UPR (Hernandez-Segura et al., 2018), which is not consistent with our results (Figure 18).

To understand if this activation of senescence pathways occurs in the tumors of *Apc* mutant mice, the enzyme senescence-associated beta-galactosidase activity must be assessed (Hernandez-Segura et al., 2018). This staining is planned to be executed in intestine samples from our model, however it is performed in frozen samples and not in paraffin tissues, as the ones we have until now.

3.6. URI loss does not increase immune infiltration

The canonical subtype of colorectal cancer is described as immunological cold. As the mice with URI loss revealed a decrease in tumor size, we sought to determine if this could be also a consequence of an increase in the immune cell infiltration.

The tumors on *Apc* mutant mice were stained for CD3 and NK1.1. by immunohistochemistry, and mCSF by immunofluorescence. CD3 is a transmembrane glycoprotein that serves as a co-receptor for T-cell receptor, NK1.1 is expressed by natural killer (NK) cells and NK T cells in C57BL/6 mice and mCSF recognizes monocytes, lymphocytes, and macrophages. A IHC staining for CD8, present in T cells with cytotoxic activity, was also performed, but no CD8⁺ cells were detected in any of the mice.

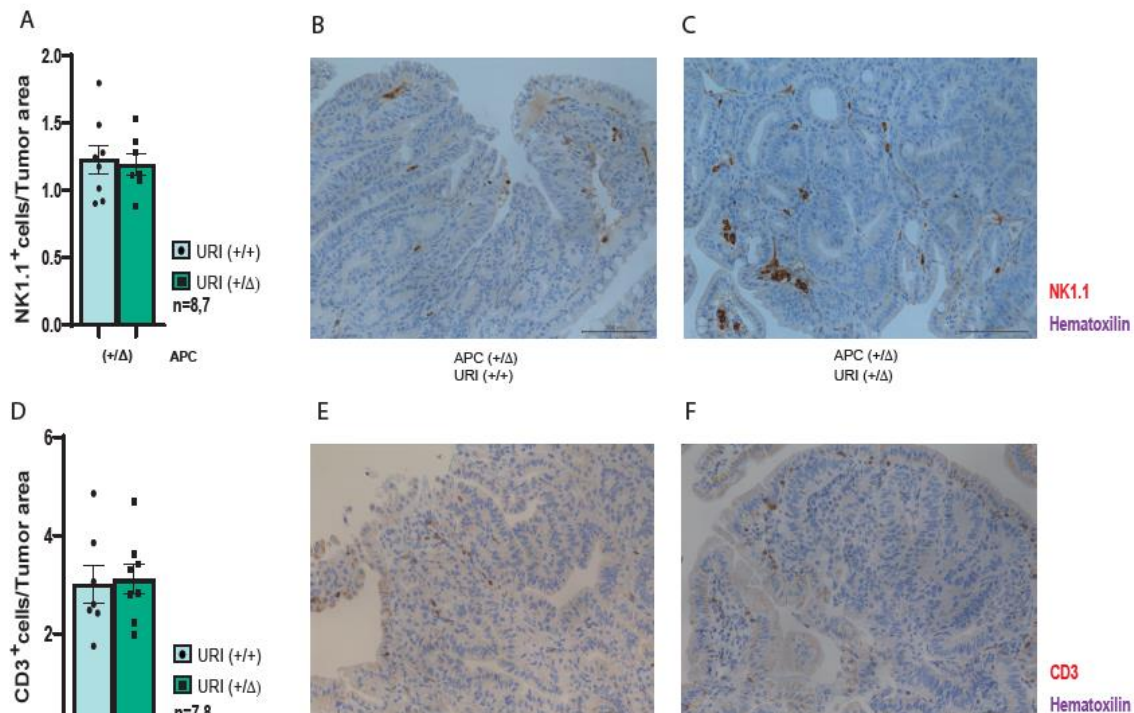


Figure 23 - URI loss does not influence immune cell infiltration within the intestine tumors. (A) Quantification of NK1.1 positive cells in intestinal tumors from *Apc*^(+/Δ) *Uri*^(+/+) mice and *Uri*^(+/Δ) mice. (B-C) IHC of NK1.1 positive cells in intestinal tumors in *Apc*^(+/Δ) *Uri*^(+/+) mice (B) and in *Apc*^(+/Δ) *Uri*^(+/Δ) mice (C). (D) Quantification of CD3 positive cells in intestinal tumors from *Apc*^(+/Δ) *Uri*^(+/+) mice and *Uri*^(+/Δ) mice. (E-F) IHC of CD3 positive cells in intestinal tumors in *Apc*^(+/Δ) *Uri*^(+/+) mice (E) and in *Apc*^(+/Δ) *Uri*^(+/Δ) mice (F). Data are mean±S.E.M. from n independent experiments, with n indicated on each graph.

Therefore, we propose to repeat this assay, completing an assessment of the immune response in the tumors.

Regarding the NK1.1. and CD3 staining, no differences in the number of positive cells between *Uri*^{+/+} and *Uri*^{+/-} mice were detected (Figure 23). Therefore, hampering URI does not alter the immune infiltration of the tumors formed by *Apc* mutation.

Unfortunately, I did not have time to quantify the results of the immunofluorescence staining for mCSF. Illustrative pictures of this staining are still represented in Figure 24.

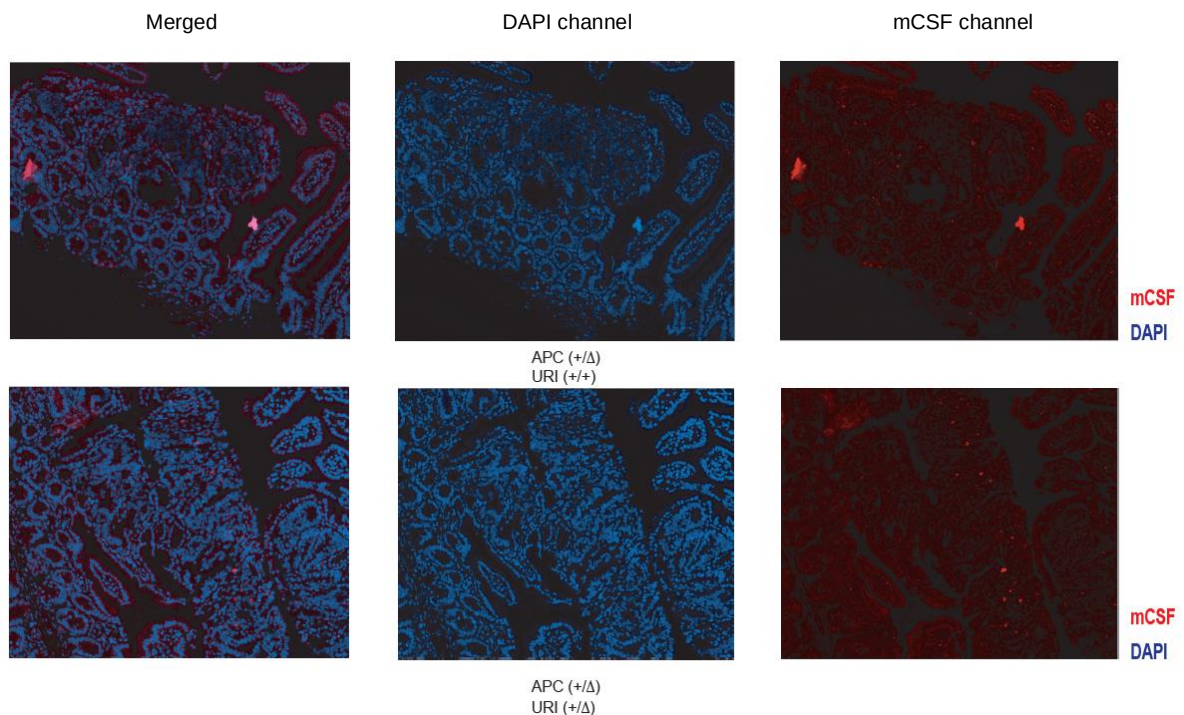


Figure 24 - IF staining of mCSF in intestine tumors. The upper pictures represent the positive mCSF cells (red) and DAPI (blue) in intestinal tumors in *Apc*^{(+/Δ)int} *Uri*^{(+/+)int} mice, while the pictures below show tumors in *Apc*^{(+/Δ)int} *Uri*^{(+/Δ)int} mice.

3.7. Human colorectal cancer cell line can incorporate different versions of mouse and human URI

As explained previously, the mutated versions of URI with deletion of 1 or 2 Aspartic Acid aminoacids, leading to the versions 12D and 11D, respectively. In order to understand why these are the most common mutation in URI in colorectal cancer patients, we studied the stability of these proteins, by transfecting cells with the wild-type version of URI (13D), the known deletions (11D and 12D), another deletion (10D) and an addition (14D), in the presence or absence of proteasome inhibitor. The expression of HA (the tag of the plasmids used) and human URI was assessed by western blot (Figure 25).

As expected, in the wild-type version of URI (13D), and in 14D, the protein expression increases with the inhibition of the proteasome, meaning that, in normal conditions, the protein is degraded by this mechanism. However, the 10D, 11D and 12D variants do not significantly change the expression of HA and URI when the proteasome is inhibited, meaning that these versions of URI are more stable, get more accumulated, predisposing for the development of tumors. pcDNA3 functions as a control - as the plasmid was empty, the cells will not express the plasmid's URI (expressed by HA), only expressing the endogenous URI of the transfected cells.

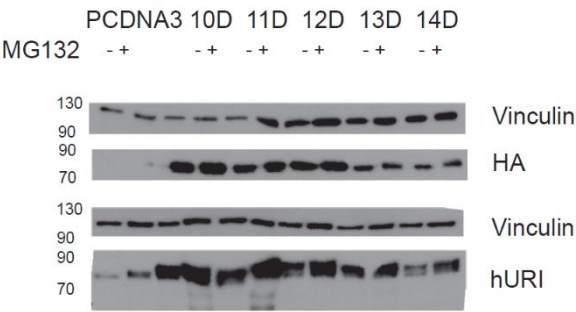


Figure 25 - The effects of proteasome inhibition in the expression of human URI. Western blot results of the tag HA and human URI, with vinculin as a loading control, in the HCT-116 transfected cells, with or without the proteasome inhibitor MG132.

As we know that in humans there are mutated versions of URI that are more common in colorectal cancer patients and that are, by our results, more stable in the cells, we want to mimic this situation in a mouse model. For that, mutated versions of URI were created, and HCT-116 cells transfected with these, in the presence or absence of a proteasome inhibitor. The results of HA expression are represented in Figure 26, however the expression of the loading control Vinculin was not achieved during my time in the lab.

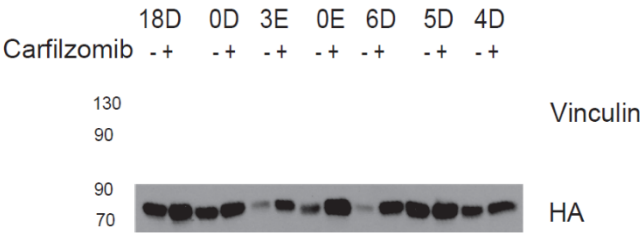


Figure 26 - The effects of proteasome inhibition in the expression of mouse URI. Western blot results of the tag HA, in the HCT-116 transfected cells, with or without the proteasome inhibitor Carfilzomib. The expression of a loading control was not achieved.

Without the proteasome inhibitor, Carfilzomib, the expression of the mutated versions 0D, 3E, 0E and 6D is smaller than the expression of the wild-type version (18D). When we add the proteasome inhibitor, the expression is recovered, revealing that these versions are getting degraded by the proteasome. The expression of 18D also increases with Carfilzomib. However, this is not detected in 5D and 4D, which may indicate that these versions are more stable, under physiological conditions.

Although these are preliminary data, we can associate the results for 5D and 4D versions of mouse URI with the results of 11D and 12D versions, known to be more oncogenic. Therefore, it is suggested that 5D and 4D versions are the most prone to be oncogenic in mice and should be used in mouse models to understand its impact.

Chapter 4

4. Conclusions and Future Work

4.1. Conclusions

APC mutations play a crucial role in the pathogenesis of colorectal cancer in humans, potentiating tumor formation and loss of p53 expression. Thus, we used an *Apc*-mutated mice model to study this pathology, although the tumors in these mice mainly develop in the small intestine, instead of in the colon.

While *Apc* wild-type mice do not develop tumors in the colon or intestine, *Apc* mutant mice present tumors in both intestines. We focused on the tumors in small intestine. Upon URI loss, the *Uri* mutant mice reveal fewer, smaller, and less aggressive tumors in the intestine. Concluding, reduction of the levels of the oncogenic protein URI inhibits tumor formation. These effects can be associated with an increase the protein levels of p53, as loss of p53 is a hallmark of *APC*-derived tumors.

We further analyzed the unfolded protein response in wild-type and mutant mice for *Apc* and *Uri*. No changes were potentiated by the ablation of URI. In accordance, *Apc* and *Uri* mutants do not increase expression of cleaved caspase 3, but increase the relative mRNA expression of *Bcl2*, an antiapoptotic protein. Thus, the tumors found in these mice are not decreasing their size due to apoptosis.

Besides leading to apoptosis, activation of p53, due to oncogene activation, hypoxia, DNA damage or oxidative stress, can also lead to cell senescence. The senescent cells phenotype is characterized by morphological changes, chromatin reorganization, cell cycle arrest, apoptosis resistance and metabolic adaptations, among other alterations. The p21 levels were studied in *Apc* and *Uri* wild-type and mutant mice, although no expression could be detected in the *Apc* mutant mice. In *Apc* wild-type mice, the result is clear - URI loss increases the expression of p21 and potentiates cell cycle arrest. Moreover, URI loss also potentiates an increase in mRNA levels of genes associated with cell cycle arrest, in both *Apc* mutants and wild-types. Concluding, URI loss of function activates cell cycle arrest. Besides, the cell cycle arrest, these animals also demonstrate apoptosis resistance, proved by an increase in the levels of *Bcl2*, which further proves the activation of senescence. Other experiments must be performed to confirm this phenotype, as the beta-galactosidase staining, that can prove alteration in the cells' lysosomal compartment, and the analysis of cell morphology.

As no alterations in the immune infiltration was detected in the tumors upon URI loss, we conclude that immune cells are not influencing tumor development.

We were able to demonstrate that URI mutant mice are capable to re-express p53, while decreasing the tumor formation potentiated by mutation in the *Apc* gene. We proved that URI loss of function induces cell senescence, although this might not be the only mechanism activated.

Although with preliminary data, we were also able to detect mutations in URI that form versions potentially more oncogenic, as we associated their expression to the expression of the 11D and 12D versions of human URI.

4.2. Future Work

The question of how URI ablation can impair tumor development in *Apc* mutant mice was not completely solved with this work. Therefore, besides making the results presented more robust, by repeating assays that did not work, as western blots to detect cell cycle arrest and immune cells detection and quantification, other animal models can be created and evaluated.

First a model with *Apc* mutation and overexpression of URI can be studied, trying to understand if the complete opposite affects are found in these mice. The second suggestion is to create mice mutant for *Apc* and *Uri* with downregulation of p53. If the opposite effects can be seen in these mice, it is proved that the effects of URI loss depend on p53 expression. On the other hand, it would be interesting to study *Apc* mutant mice with URI gain of function and p53 overexpression. This model would be useful to know if the effects attributed to URI loss are in fact all due to the gain of expression of p53.

During my time in the laboratory, we also tested the expression of mutated versions of URI by colorectal cancer cell lines. The idea behind this is that oncogenic versions of URI are more stable and not degraded by the proteasome. The idea is to further test how different mutation can affect the stabilization of the protein, as our results were very preliminary. The versions more likely to be oncogenic should be used in the different mice models where URI is studied.

Chapter 5

5. References

- Arap, M. A., Lahdenranta, J., Mintz, P. J., Hajitou, A., Sarkis, Á. S., Arap, W., & Pasqualini, R. (2004). Cell surface expression of the stress response chaperone GRP78 enables tumor targeting by circulating ligands. *Cancer Cell*, 6(3), 275-284. <https://doi.org/10.1016/j.ccr.2004.08.018>
- Barker, N., Bartfeld, S., & Clevers, H. (2010). Tissue-Resident Adult Stem Cell Populations of Rapidly Self-Renewing Organs. *Cell Stem Cell*, 7(6), 656-670. <https://doi.org/10.1016/j.stem.2010.11.016>
- Bodine, P. V. N., & Komm, B. S. (2007). Wnt signaling and osteoblastogenesis. *Reviews in Endocrine and Metabolic Disorders*, 7(1-2), 33-39. <https://doi.org/10.1007/s11154-006-9002-4>
- Brandt, M., Grazioso, T. P., Fawal, M.-A., Tummala, K. S., Torres-Ruiz, R., Rodriguez-Perales, S., Perna, C., & Djouder, N. (2018). mTORC1 Inactivation Promotes Colitis-Induced Colorectal Cancer but Protects from APC Loss-Dependent Tumorigenesis. *Cell Metabolism*, 27(1), 118-135.e118. <https://doi.org/10.1016/j.cmet.2017.11.006>
- Brenner, H., Kloor, M., & Pox, C. P. (2014). Colorectal cancer. *The Lancet*, 383(9927), 1490-1502. [https://doi.org/10.1016/s0140-6736\(13\)61649-9](https://doi.org/10.1016/s0140-6736(13)61649-9)
- Brentnall, M., Rodriguez-Menocal, L., De Guevara, R. L., Cepero, E., & Boise, L. H. (2013). Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. *BMC Cell Biology*, 14(1), 32. <https://doi.org/10.1186/1471-2121-14-32>
- The Cancer Genome Atlas*. National Cancer Institute. Retrieved 03 from <https://gdc.cancer.gov/>
- Cao, S. S., & Kaufman, R. J. (2012). Unfolded protein response. *Current Biology*, 22(16), R622-R626. <https://doi.org/10.1016/j.cub.2012.07.004>
- Chalmers, F., Mogre, S., Son, J., Blazanin, N., & Glick, A. B. (2019). The multiple roles of the unfolded protein response regulator IRE1α in cancer. *Molecular Carcinogenesis*, 58(9), 1623-1630. <https://doi.org/10.1002/mc.23031>
- Chan, D. S. M., Lau, R., Aune, D., Vieira, R., Greenwood, D. C., Kampman, E., & Norat, T. (2011). Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies. *PLoS ONE*, 6(6), e20456. <https://doi.org/10.1371/journal.pone.0020456>
- Chappell, W. H., Steelman, L. S., Long, J. M., Kempf, R. C., Abrams, S. L., Franklin, R. A., Bäsecke, J., Stivala, F., Donia, M., Fagone, P., Malaponte, G., Mazzarino, M. C., Nicoletti, F., Libra, M., Maksimovic-Ivanic, D., Mijatovic, S., Montalto, G., Cervello, M., Laidler, P., Milella, M., Tafuri, A., Bonati, A., Evangelisti, C., Cocco, L., Martelli, A. M., & McCubrey, J. A. (2011). Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR Inhibitors: Rationale and Importance to Inhibiting These Pathways in Human Health. *Oncotarget*, 2(3), 135-164. <https://doi.org/10.18632/oncotarget.240>
- Chaves-Pérez, A., Thompson, S., & Djouder, N. (2018). Roles and Functions of the Unconventional Prefoldin URI. *Adv Exp Med Biol*, 1106, 95-108. https://doi.org/10.1007/978-3-030-00737-9_7
- Chaves-Pérez, A., Yilmaz, M., Perna, C., Rosa, S. d. l., & Djouder, N. (2019). URI is required to maintain intestinal architecture during ionizing radiation. *Science*, 364(6443), eaaq1165. <https://doi.org/doi:10.1126/science.aaq1165>

Clevers, H. (2013). The Intestinal Crypt, A Prototype Stem Cell Compartment. *Cell*, 154(2), 274-284. <https://doi.org/10.1016/j.cell.2013.07.004>

Coqueret, O. (2003). New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends in Cell Biology*, 13(2), 65-70. [https://doi.org/10.1016/S0962-8924\(02\)00043-0](https://doi.org/10.1016/S0962-8924(02)00043-0)

Crowley, L. C., & Waterhouse, N. J. (2016). Detecting Cleaved Caspase-3 in Apoptotic Cells by Flow Cytometry. *Cold Spring Harb Protoc*, 2016(11). <https://doi.org/10.1101/pdb.prot087312>

Dekker, E., Tanis, P. J., Vleugels, J. L. A., Kasi, P. M., & Wallace, M. B. (2019). Colorectal cancer. *The Lancet*, 394(10207), 1467-1480. [https://doi.org/10.1016/s0140-6736\(19\)32319-0](https://doi.org/10.1016/s0140-6736(19)32319-0)

Ditonno, I., Losurdo, G., Rendina, M., Pricci, M., Girardi, B., Ierardi, E., & Di Leo, A. (2021). Estrogen Receptors in Colorectal Cancer: Facts, Novelties and Perspectives. *Current Oncology*, 28(6), 4256-4263. <https://doi.org/10.3390/curroncol28060361>

Dong, D., Ni, M., Li, J., Xiong, S., Ye, W., Virrey, J. J., Mao, C., Ye, R., Wang, M., Pen, L., Dubeau, L., Groshen, S., Hofman, F. M., & Lee, A. S. (2008). Critical Role of the Stress Chaperone GRP78/BiP in Tumor Proliferation, Survival, and Tumor Angiogenesis in Transgene-Induced Mammary Tumor Development. *Cancer Research*, 68(2), 498-505. <https://doi.org/10.1158/0008-5472.can-07-2950>

Fedirko, V., Tramacere, I., Bagnardi, V., Rota, M., Scotti, L., Islami, F., Negri, E., Straif, K., Romieu, I., La Vecchia, C., Boffetta, P., & Jenab, M. (2011). Alcohol drinking and colorectal cancer risk: an overall and dose-response meta-analysis of published studies. *Annals of Oncology*, 22(9), 1958-1972. <https://doi.org/10.1093/annonc/mdq653>

Ferlay J, E. M., Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F. (2020). *Global Cancer Observatory: Cancer Today*. International Agency for Research on Cancer. Retrieved 22/03 from <https://gco.iarc.fr/today>

Fischer, M., Quaas, M., Steiner, L., & Engeland, K. (2016). The p53-p21-DREAM-CDE/CHR pathway regulates G₂/M cell cycle genes. *Nucleic Acids Research*, 44(1), 164-174. <https://doi.org/10.1093/nar/gkv927>

Goss, K. H., & Groden, J. (2000). Biology of the Adenomatous Polyposis Coli Tumor Suppressor. *Journal of Clinical Oncology*, 18(9), 1967-1979. <https://doi.org/10.1200/jco.2000.18.9.1967>

Guinney, J., Dienstmann, R., Wang, X., De Reyniès, A., Schlicker, A., Soneson, C., Marisa, L., Roepman, P., Nyamundanda, G., Angelino, P., Bot, B. M., Morris, J. S., Simon, I. M., Gerster, S., Fessler, E., De Sousa E Melo, F., Missiaglia, E., Ramay, H., Barras, D., Homicsko, K., Maru, D., Manyam, G. C., Broom, B., Boige, V., Perez-Villamil, B., Laderas, T., Salazar, R., Gray, J. W., Hanahan, D., Tabernero, J., Bernards, R., Friend, S. H., Laurent-Puig, P., Medema, J. P., Sadanandam, A., Wessels, L., Delorenzi, M., Kopetz, S., Vermeulen, L., & Tejpar, S. (2015). The consensus molecular subtypes of colorectal cancer. *Nature Medicine*, 21(11), 1350-1356. <https://doi.org/10.1038/nm.3967>

Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., & Ron, D. (2000). Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells. *Molecular Cell*, 6(5), 1099-1108. [https://doi.org/10.1016/s1097-2765\(00\)00108-8](https://doi.org/10.1016/s1097-2765(00)00108-8)

Helander, H. F., & Fändriks, L. (2014). Surface area of the digestive tract - revisited. *Scandinavian Journal of Gastroenterology*, 49(6), 681-689. <https://doi.org/10.3109/00365521.2014.898326>

- Hernandez-Segura, A., Nehme, J., & Demaria, M. (2018). Hallmarks of Cellular Senescence. *Trends in Cell Biology*, 28(6), 436-453. <https://doi.org/https://doi.org/10.1016/j.tcb.2018.02.001>
- Hernández Borrero, L. J., & El-Deiry, W. S. (2021). Tumor suppressor p53: Biology, signaling pathways, and therapeutic targeting. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1876(1), 188556. <https://doi.org/10.1016/j.bbcan.2021.188556>
- Herranz-Montoya, I., Park, S., & Djouder, N. (2021). A comprehensive analysis of prefoldins and their implication in cancer. *iScience*, 24(11), 103273. <https://doi.org/10.1016/j.isci.2021.103273>
- Hiss, D. C., & Gabriels, G. A. (2009). Implications of endoplasmic reticulum stress, the unfolded protein response and apoptosis for molecular cancer therapy. Part I: targeting p53, Mdm2, GADD153/CHOP, GRP78/BiP and heat shock proteins. *Expert Opinion on Drug Discovery*, 4(8), 799-821. <https://doi.org/10.1517/17460440903052559>
- Huang, D., Sun, W., Zhou, Y., Li, P., Chen, F., Chen, H., Xia, D., Xu, E., Lai, M., Wu, Y., & Zhang, H. (2018). Mutations of key driver genes in colorectal cancer progression and metastasis. *Cancer and Metastasis Reviews*, 37(1), 173-187. <https://doi.org/10.1007/s10555-017-9726-5>
- Huang, J., Pan, H., Wang, J., Wang, T., Huo, X., Ma, Y., Lu, Z., Sun, B., & Jiang, H. (2021). Unfolded protein response in colorectal cancer. *Cell & Bioscience*, 11(1). <https://doi.org/10.1186/s13578-021-00538-z>
- Ijsselsteijn, M. E., Sanz-Pamplona, R., Hermitte, F., & De Miranda, N. F. C. C. (2019). Colorectal cancer: A paradigmatic model for cancer immunology and immunotherapy. *Molecular Aspects of Medicine*, 69, 123-129. <https://doi.org/10.1016/j.mam.2019.05.003>
- Jess, T., Rungoe, C., & Peyrin-Biroulet, L. (2012). Risk of Colorectal Cancer in Patients With Ulcerative Colitis: A Meta-analysis of Population-Based Cohort Studies. *Clinical Gastroenterology and Hepatology*, 10(6), 639-645. <https://doi.org/https://doi.org/10.1016/j.cgh.2012.01.010>
- Jiang, Y., Ben, Q., Shen, H., Lu, W., Zhang, Y., & Zhu, J. (2011). Diabetes mellitus and incidence and mortality of colorectal cancer: a systematic review and meta-analysis of cohort studies. *European Journal of Epidemiology*, 26(11), 863-876. <https://doi.org/10.1007/s10654-011-9617-y>
- Kostic, A. D., Gevers, D., Pedamallu, C. S., Michaud, M., Duke, F., Earl, A. M., Ojesina, A. I., Jung, J., Bass, A. J., Tabernero, J., Baselga, J., Liu, C., Shivdasani, R. A., Ogino, S., Birren, B. W., Huttenhower, C., Garrett, W. S., & Meyerson, M. (2012). Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. *Genome Research*, 22(2), 292-298. <https://doi.org/10.1101/gr.126573.111>
- KOTAKE, Y., NAEMURA, M., MURASAKI, C., INOUE, Y., & OKAMOTO, H. (2015). Transcriptional Regulation of the p16 Tumor Suppressor Gene. *Anticancer Research*, 35(8), 4397-4401. <https://ar.iijournals.org/content/anticancer/35/8/4397.full.pdf>
- Lee, B.-R., Chang, S.-Y., Hong, E.-H., Kwon, B.-E., Kim, H. M., Kim, Y.-J., Lee, J., Cho, H.-J., Cheon, J.-H., & Ko, H.-J. (2014). Elevated endoplasmic reticulum stress reinforced immunosuppression in the tumor microenvironment <i>via</i> myeloid-derived suppressor cells. *Oncotarget*, 5(23), 12331-12345. <https://doi.org/10.18632/oncotarget.2589>
- Lee, C. S., Perreault, N., Brestelli, J. E., & Kaestner, K. H. (2002). <i>Neurogenin 3</i> is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity. *Genes & Development*, 16(12), 1488-1497. <https://doi.org/10.1101/gad.985002>

- Leslie, A., Carey, F. A., Pratt, N. R., & Steele, R. J. C. (2002). The colorectal adenoma-carcinoma sequence. *British Journal of Surgery*, 89(7), 845-860. <https://doi.org/10.1046/j.1365-2168.2002.02120.x>
- Liang, P. S., Chen, T.-Y., & Giovannucci, E. (2009). Cigarette smoking and colorectal cancer incidence and mortality: Systematic review and meta-analysis. *International Journal of Cancer*, 124(10), 2406-2415. <https://doi.org/10.1002/ijc.24191>
- Lundberg, A. S., Hahn, W. C., Gupta, P., & Weinberg, R. A. (2000). Genes involved in senescence and immortalization. *Current Opinion in Cell Biology*, 12(6), 705-709. [https://doi.org/https://doi.org/10.1016/S0955-0674\(00\)00155-1](https://doi.org/https://doi.org/10.1016/S0955-0674(00)00155-1)
- Ma, H., Brosens, L. A. A., Offerhaus, G. J. A., Giardiello, F. M., De Leng, W. W. J., & Montgomery, E. A. (2018). Pathology and genetics of hereditary colorectal cancer. *Pathology*, 50(1), 49-59. <https://doi.org/10.1016/j.pathol.2017.09.004>
- Ma, Y., Yang, Y., Wang, F., Zhang, P., Shi, C., Zou, Y., & Qin, H. (2013). Obesity and Risk of Colorectal Cancer: A Systematic Review of Prospective Studies. *PLoS ONE*, 8(1), e53916. <https://doi.org/10.1371/journal.pone.0053916>
- Markowitz, S. D., & Bertagnolli, M. M. (2009). Molecular Basis of Colorectal Cancer. *New England Journal of Medicine*, 361(25), 2449-2460. <https://doi.org/10.1056/nejmra0804588>
- Maurel, M., McGrath, E. P., Mnich, K., Healy, S., Chevet, E., & Samali, A. (2015). Controlling the unfolded protein response-mediated life and death decisions in cancer. *Seminars in Cancer Biology*, 33, 57-66. <https://doi.org/10.1016/j.semcancer.2015.03.003>
- Mhaidat, N. M., Alzoubi, K. H., & Abushbak, A. (2015). X-box binding protein 1 (XBP-1) enhances colorectal cancer cell invasion. *Journal of Chemotherapy*, 27(3), 167-173. <https://doi.org/10.1179/1973947815Y.0000000006>
- Mhaidat, N. M., Alzoubi, K. H., Khabour, O. F., Banihani, M. N., Al-Balas, Q. A., & Swaidan, S. (2016). GRP78 regulates sensitivity of human colorectal cancer cells to DNA targeting agents. *Cytotechnology*, 68(3), 459-467. <https://doi.org/10.1007/s10616-014-9799-8>
- Moor, A. E., Harnik, Y., Ben-Moshe, S., Massasa, E. E., Rozenberg, M., Eilam, R., Bahar Halpern, K., & Itzkovitz, S. (2018). Spatial Reconstruction of Single Enterocytes Uncovers Broad Zonation along the Intestinal Villus Axis. *Cell*, 175(4), 1156-1167.e1115. <https://doi.org/10.1016/j.cell.2018.08.063>
- Navas, L. E., & Carnero, A. (2021). NAD⁺ metabolism, stemness, the immune response, and cancer. *Signal Transduction and Targeted Therapy*, 6(1). <https://doi.org/10.1038/s41392-020-00354-w>
- Paduch, R. (2016). The role of lymphangiogenesis and angiogenesis in tumor metastasis. *Cellular Oncology*, 39(5), 397-410. <https://doi.org/10.1007/s13402-016-0281-9>
- Pelaseyed, T., Bergström, J. H., Gustafsson, J. K., Ermund, A., Birchenough, G. M. H., Schütte, A., Van Der Post, S., Svensson, F., Rodríguez-Piñero, A. M., Nyström, E. E. L., Wising, C., Johansson, M. E. V., & Hansson, G. C. (2014). The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunological Reviews*, 260(1), 8-20. <https://doi.org/10.1111/imr.12182>
- Pelengaris, S., Khan, M., & Evan, G. I. (2002). Suppression of Myc-Induced Apoptosis in B Cells Exposes Multiple Oncogenic Properties of Myc and Triggers Carcinogenic Progression. *Cell*, 109(3), 321-334. [https://doi.org/10.1016/s0092-8674\(02\)00738-9](https://doi.org/10.1016/s0092-8674(02)00738-9)

- Robanus-Maandag, E. C., Koelink, P. J., Breukel, C., Salvatori, D. C. F., Jagmohan-Changur, S. C., Bosch, C. A. J., Verspaget, H. W., Devilee, P., Fodde, R., & Smits, R. (2010). A new conditional Apc-mutant mouse model for colorectal cancer. *Carcinogenesis*, 31(5), 946-952. <https://doi.org/10.1093/carcin/bgq046>
- Sansom, O. J., Reed, K. R., Hayes, A. J., Ireland, H., Brinkmann, H., Newton, I. P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I. S., Clarke, A. R., & Winton, D. J. (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes & Development*, 18(12), 1385-1390. <https://doi.org/10.1101/gad.287404>
- Sato, T., Van Es, J. H., Snippert, H. J., Stange, D. E., Vries, R. G., Van Den Born, M., Barker, N., Shroyer, N. F., Van De Wetering, M., & Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature*, 469(7330), 415-418. <https://doi.org/10.1038/nature09637>
- Shen, Y., Iqbal, J., Huang, J. Z., Zhou, G., & Chan, W. C. (2004). BCL2 protein expression parallels its mRNA level in normal and malignant B cells. *Blood*, 104(9), 2936-2939. <https://doi.org/10.1182/blood-2004-01-0243>
- Simons, B. D., & Clevers, H. (2011). Stem cell self-renewal in intestinal crypt. *Experimental Cell Research*, 317(19), 2719-2724. <https://doi.org/10.1016/j.yexcr.2011.07.010>
- Simpson, L. O. (1975). Spleen and liver weight changes in NZB mice with haemolytic anaemia. *Laboratory Animals*, 9(4), 261-273. <https://doi.org/10.1258/002367775780957232>
- Sonnenberg, A., & Genta, R. M. (2013). Helicobacter pylori is a Risk Factor for Colonic Neoplasms. *Official journal of the American College of Gastroenterology | ACG*, 108(2), 208-215. <https://doi.org/10.1038/ajg.2012.407>
- Sullivan, K. D., Galbraith, M. D., Andrysik, Z., & Espinosa, J. M. (2018). Mechanisms of transcriptional regulation by p53. *Cell Death & Differentiation*, 25(1), 133-143. <https://doi.org/10.1038/cdd.2017.174>
- Taylor, D. P., Burt, R. W., Williams, M. S., Haug, P. J., & Cannon-Albright, L. A. (2010). Population-Based Family History-Specific Risks for Colorectal Cancer: A Constellation Approach. *Gastroenterology*, 138(3), 877-885. <https://doi.org/10.1053/j.gastro.2009.11.044>
- Teske, B. F., Wek, S. A., Bunpo, P., Cundiff, J. K., McClintick, J. N., Anthony, T. G., & Wek, R. C. (2011). The eIF2 kinase PERK and the integrated stress response facilitate activation of ATF6 during endoplasmic reticulum stress. *Molecular Biology of the Cell*, 22(22), 4390-4405. <https://doi.org/10.1091/mbc.e11-06-0510>
- Thuerauf, D. J., Morrison, L., & Glembotski, C. C. (2004). Opposing Roles for ATF6 α and ATF6 β in Endoplasmic Reticulum Stress Response Gene Induction. *Journal of Biological Chemistry*, 279(20), 21078-21084. <https://doi.org/10.1074/jbc.m400713200>
- Tummala, K. S., Gomes, A. L., Yilmaz, M., Graña, O., Bakiri, L., Ruppen, I., Ximénez-Embún, P., Sheshappanavar, V., Rodríguez-Justo, M., Pisano, D. G., Wagner, E. F., & Djouder, N. (2014). Inhibition of de novo NAD(+) synthesis by oncogenic URI causes liver tumorigenesis through DNA damage. *Cancer Cell*, 26(6), 826-839. <https://doi.org/10.1016/j.ccell.2014.10.002>
- van der Flier, L. G., & Clevers, H. (2009). Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu Rev Physiol*, 71, 241-260. <https://doi.org/10.1146/annurev.physiol.010908.163145>

Wang, C., & Fakih, M. (2021). Targeting KRAS in Colorectal Cancer. *Current Oncology Reports*, 23(3). <https://doi.org/10.1007/s11912-021-01022-0>

Wang, Y., Alam, G. N., Ning, Y., Visioli, F., Dong, Z., Nör, J. E., & Polverini, P. J. (2012). The Unfolded Protein Response Induces the Angiogenic Switch in Human Tumor Cells through the PERK/ATF4 Pathway. *Cancer Research*, 72(20), 5396-5406. <https://doi.org/10.1158/0008-5472.can-12-0474>

West, A. B., Isaac, C. A., Carboni, J. M., Morrow, J. S., Mooseker, M. S., & Barwick, K. W. (1988). Localization of villin, a cytoskeletal protein specific to microvilli, in human ileum and colon and in colonic neoplasms. *Gastroenterology*, 94(2), 343-352. [https://doi.org/10.1016/0016-5085\(88\)90421-0](https://doi.org/10.1016/0016-5085(88)90421-0)

Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., Harada, A., & Mori, K. (2007). Transcriptional Induction of Mammalian ER Quality Control Proteins Is Mediated by Single or Combined Action of ATF6 α and XBP1. *Developmental Cell*, 13(3), 365-376. <https://doi.org/10.1016/j.devcel.2007.07.018>

YIU, A. J., & YIU, C. Y. (2016). Biomarkers in Colorectal Cancer. *Anticancer Research*, 36(3), 1093-1102. <https://ar.iijournals.org/content/anticancer/36/3/1093.full.pdf>

Young, M., Ordonez, L., & Clarke, A. R. (2013). What are the best routes to effectively model human colorectal cancer? *Molecular Oncology*, 7(2), 178-189. <https://doi.org/10.1016/j.molonc.2013.02.006>

Zhu, Q., Wani, G., Yao, J., Patnaik, S., Wang, Q. E., El-Mahdy, M. A., Prætorius-Ibba, M., & Wani, A. A. (2007). The ubiquitin-proteasome system regulates p53-mediated transcription at p21waf1 promoter. *Oncogene*, 26(29), 4199-4208. <https://doi.org/10.1038/sj.onc.1210191>

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