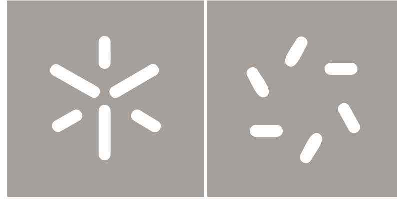

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The role of *KRAS* mutations signalling in autophagy control in Colorectal Carcinomas

Tese de Mestrado
Genética Molecular

Trabalho efectuado sob a orientação da
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ABSTRACT

Colorectal carcinoma (CRC) is one of the most common types of cancer, leading to a high rate of mortality in Europe. Current therapies for CRC patients present poor outcomes, being mandatory new therapeutic approaches. Autophagy is an essential process for protein turn-over and cellular homeostasis being tightly implicated in cancer. This process has been described as fundamental in CRC cells chemoresistance emerging as a crucial target to anticancer therapy. One of the most frequent events found in CRC is the presence of activating mutations in *KRAS* (*KRAS*^{G13D}, *KRAS*^{G12D} and *KRAS*^{G12V}) which have a role in the genesis and progression of these tumours. Several studies have already demonstrated the importance of KRAS^{G12V} in autophagy induction either in fibroblasts and epithelial cells. However, little is known concerning the precise role of the different *KRAS* mutations in autophagy regulation in CRC cells.

This project aimed to determine the role of *KRAS* mutations signalling in cellular survival/proliferation and autophagy regulation in CRC. For such purpose, a CRC derived cell line harbouring *KRAS*^{G12V} mutation (SW480) and a kidney derived cell line (HEK 293) stably expressing Flag-KRAS^{WT}, Flag-KRAS^{G13D}, Flag-KRAS^{G12D}, and Flag-KRAS^{G12V} were used as *in vitro* models. Down-regulation of KRAS was performed by RNA interference (RNAi); the levels of autophagic markers were accessed by Western-blot analysis; cellular survival and proliferation were evaluated by cell cycle analysis and colony formation assay technique.

Our preliminary results showed that in SW480 cells, upon *KRAS* inhibition by RNAi, there was a decrease in autophagic markers levels namely LC3 II, Atg5 and p62 as well as a decrease in ERK phosphorylation. Down-regulation of *KRAS* led to a decrease in S-phase and an increase of sub-G1 phase of the cell cycle. Moreover, treatment with the autophagy inhibitor, Bafilomycin A1, also led to an increase in cell death. In contrast, the results observed in HEK 293 cells do not support the obtained in SW480 cells, which might indicate that HEK 293 cells are not a suitable model to study the role of typical CRC *KRAS* mutations in autophagy regulation.

Altogether the results indicate that KRAS^{G12V} protein may play a crucial role in autophagy regulation, survival and cell proliferation, possibly involving RAS–RAF–MEK–ERK signalling pathway, though further studies are required. The results also showed that CRC cells harbouring *KRAS*^{G12V} mutation seem to be dependent on autophagy for survival which can have important implications in CRC therapy.

RESUMO

O carcinoma colo-rectal (CCR) é um dos tipos de cancro mais comuns, apresentando uma elevada taxa de mortalidade na Europa. Uma vez que as terapias para pacientes com CCR apresentam maus resultados, torna-se fundamental encontrar novas abordagens terapêuticas. A autofagia, um processo de renovação de proteínas e manutenção da homeostasia celular, tem sido descrita como fundamental na quimio-resistência das células do CCR, sendo um alvo importante para a terapia anti-cancro. A presença de mutações ativantes do *KRAS* (*KRAS*^{G13D}, *KRAS*^{G12D} e *KRAS*^{G12V}) é um dos eventos mais frequentes no CCR, estando implicada na sua formação e progressão. Inúmeros estudos demonstraram a importância do *KRAS*^{G12V} na indução da autofagia em fibroblastos bem como em células epiteliais. No entanto, o papel específico das diferentes mutações do *KRAS* na regulação da autofagia no CCR é ainda pouco claro.

Este projeto teve como objetivo a determinação da função das mutações do *KRAS* na sobrevivência/proliferação celular bem como na regulação da autofagia no CCR. Para isso, uma linha celular derivada de CCR com a mutação *KRAS*^{G12V} (SW480) e uma linha celular derivada de rim (HEK 293) que expressa Flag-KRAS^{WT}, Flag-KRAS^{G13D}, Flag-KRAS^{G12D} e Flag-KRAS^{G12V} foram usadas como modelos *in vitro*. O silenciamento do *KRAS* foi conseguido através da técnica de RNA de interferência (RNAi); os níveis de expressão dos marcadores autofágicos foram analisados por “Western-blot”; a sobrevivência e proliferação celulares foram avaliadas por análise do ciclo celular e pela técnica de “colony formation assay”.

Os nossos resultados preliminares mostraram que, nas células SW480, após inibição do *KRAS*, a expressão de LC3 II, Atg5 e p62 diminuiu, assim como a fosforilação das ERK. O silenciamento do *KRAS* levou a uma diminuição de células em fase S e um aumento em fase sub-G1 do ciclo celular. Além disso, o tratamento com um inibidor da autofagia, Bafilomicina A1, promoveu o aumento da morte celular. Os resultados observados nas células HEK 293 não estão de acordo com os obtidos nas células SW480, sugerindo que as células HEK 293 não são um bom modelo no estudo da regulação da autofagia pelas mutações do *KRAS* típicas do CCR.

Os resultados indicam que o KRAS^{G12V} desempenha um papel fundamental na regulação da autofagia, sobrevivência e proliferação celulares, envolvendo possivelmente a via RAS–RAF–MEK–ERK, porém mais estudos são necessários. Os resultados demonstraram também que as células de CCR com a mutação *KRAS*^{G12V} parecem estar dependentes da autofagia para a sua sobrevivência, o que pode ter importantes implicações no tratamento dos CCR.

TABLE OF CONTENTS

ABBREVIATIONS ix - xii

1. INTRODUCTION 1

1.1 Cancer Epidemiology 3

1.2 Colorectal Carcinoma 6

1.2.1 Hereditary or Familial Colorectal Carcinoma 7

1.2.2 Sporadic Colorectal Carcinoma: CIN and MIS 8

1.3 MAPK pathway and RAS proteins 9

1.3.1 Oncogenic activation in Colorectal Carcinoma: KRAS Mutations 12

1.4 Autophagy 13

1.4.1 Autophagy Process 14

1.4.2 Autophagy Regulation 17

1.5 RAS and Autophagy 19

1.6 *Rationale* and aim 21

2. MATERIAL AND METHODS 23

2.1 Cell lines and culture conditions 25

2.2 Optimization of sIRNA transfection in SW480 cell line 25

2.2.1 Forward Transfection 26

2.2.2 Reverse Transfection 27

2.2.3 Assessment of siRNA-AF internalization using Fluorescence Microscopy and Flow Cytometer 27

2.3 Western-blot analysis 28

2.3.1 Total protein extraction 28

2.3.2 Western blotting 28

2.4 Trypan Blue exclusion Assay 30

2.5 Cell Cycle analysis 30

2.6 Colony Formation Assay 31

2.7 Statistical analysis 31

3. RESULTS 33

3.1 Optimization of RNA interference conditions 35

3.1.1 LipoGen Transfection Reagent 35

3.1.2 RNAi-MAX Transfection Reagent 38

3.1.3 Lipofectamine 2000 Transfection Reagent 40

3.2 Inhibition of KRAS expression in SW480 cells using RNA interference 45

3.2.1 Effect of KRAS inhibition in autophagy of SW480 cells 45

3.2.2 Effect of KRAS inhibition in ERK signalling of SW480 cells 49

3.2.3 Effect of KRAS inhibition in the cell cycle and cell death of SW480 cells 50

3.2.4 Effect of autophagy inhibition in cell survival of SW480 cells 52

3.3 The cellular effect of *KRAS* mutations in HEK 293 cell line 54

3.3.1 Effect of *KRAS* mutations in autophagy induction in HEK 293 cells 54

3.3.2 Effect of expression of *KRAS* mutations on Colony Formation Ability of HEK 293 cells 57

4. DISCUSSION 61

5. CONCLUSIONS AND FUTURE PERSPECTIVES 67

5.1 Conclusions 69

5.2 Future Perspectives 70

6. REFERENCES 71

ABBREVIATIONS

3-MA	3-METHYLADENINE
AIF2	APOPTOSIS INDUCING FACTOR
AMP	ADENOSINE MONOPHOSPHATE
AMPK	AMP-ACTIVATED PROTEIN KINASE
APC	ADENOMATOUS POLYPOSIS COLI
APS	AMMONIUM PERSULFATE
ASK	APOPTOSIS SIGNAL REGULATION KINASE
ATG	AUTOPHAGY-RELATED GENES
ATP	ADENOSINE TRIPHOSPHATE
BAF A1	BAFILOMYCIN A1
BCL-2	B-CELL CLL/LYMPHOMA 2
BNIP3	BCL- 2/ADENOVIRUS E1B 19 KDA PROTEIN – INTERACTING PROTEIN 3
BSA	BOVINE SERUM ALBUMIN
CDK4	CYCLIN-DEPENDENT KINASE 4
CIN	CHROMOSOMAL INSTABILITY
CMA	CHAPERONE-MEDIATED AUTOPHAGY
CRC	COLORECTAL CARCINOMA
CRE	CYCLIC AMP/CALCIUM RESPONSE ELEMENT
DAPK	DEATH-ASSOCIATED PROTEIN KINASE
DNA	DEOXYRIBONUCLEIC ACID
DMEM	DULBECCO´S MODIFIED EAGLE MEDIUM
DRP1	DYNAMIN-RELATED PROTEIN 1
EGFR	EPIDERMAL GROWTH FACTOR RECEPTOR
ELK	MIXED LINEAGE KINASE
ERKP	ERK PHOSPHORYLATED
ERKT	ERK TOTAL
FAP	FAMILIAL ADENOMATOUS POLYPOSIS
FBS	FETAL BOVINE SERUM
GDP	GUANOSINE DIPHOSPHATE
GTP	GUANOSINE TRIPHOSPHATE
GRB	GROWTH FACTOR RECEPTOR – BOUND

HBSS	Hank´s Balanced Salt Solution
HCl	Hidrochloric acid
HEK	Human Embryonic Kidney
hHLM	Human mutL Homolog
HNPCC	Hereditary NonPolyposis Colorectal Carcinoma
HPP	HyperPlastic Polyosis
HRAS	Harvey Rat Sarcoma viral oncogene homolog
HSPIN1	Human homologue of the Drosophila Spin gene product
hVps34	Vacuolar protein sorting 34 homologue
IARC	International Agency for Research on Cancer
iBMK	immortalized Baby Mouse Kidney
JNK	c-Junk N-terminal Kinase
JPS	Juvenile Polyposis Syndrome
KRAS	Kirsten rat sarcoma 2 viral oncogene homolog
LC3	microtubule-associated protein 1 Light Chain 3 (MAP1LC3)
MAP	MUTYH -Associated Polyposis
MAPK	Mitogen-Activated Protein Kinase
MEF2	Myocyte Enhancer Factor 2
miRNA	micro RNA
MSI	Microsatellite Instability
mTOR	Mammalian Target Of Rapamycin
NaF	Sodium Fluoride
Na3VO4	Sodium Orthovanadate
NF1	NeuroFibromin 1
NRAS	Neuroblastoma Rat Sarcoma viral oncogene homolog
Opti-MEM	Reduced serum medium
PBS	Phosphate Buffered saline
PBS-T	Phosphate Buffer saline – Tween
PE	PhosphatidylEthanolamine
PenStrep	Penicillin-Streptomycin
pERK	phosphorilated ERK protein
PETACC-3	Pan European Trial Adjuvant Colon Cancer 3

PFA	Paraformaldehyde
PH	Pleckstrin Homology
PI	Propidium Iodide
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PI3K	PhosphatidylInositol-3 Kinase
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PJS	Peutz-Jeghers Syndrome
PKB	Protein Kinase B
PKC	Protein Kinase C
PLCε	Phospholipase Cε
PLD	Phospholipase D
PTEN	Phosphatase and Tensin homologue
PVDF	Polyvinylidene Fluoride
RalGDS	Ral guanine nucleotide-dissociation stimulator
RAS	Rat Sarcoma
Rb	Retinoblastoma tumour suppressor protein
RGL	RalGDS - like gene
RNAi	RNA interference
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
rpm	Rotations per minute
RT	Room Temperature
SAPK	Stress-Activated Protein kinase
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate - polyacrylamide Gel Electrophoresis
siRNA	small interfering RNA
siRNA-AF	Small interfing RNA – Alexa Fluor
SOS	son of sevenless
SQSTM1	Sequestrome 1
STAT 1	Signal Transducer and Activator of Trascription factor 1
TBS-T	Tris Buffered Saline – Tween
TEMED	tetramethylethylenediamine

tERK	TOTAL ERK PROTEIN
TIAM1	T-CELL LYMPHOMA INVASION AND METASTASIS-INDUCING PROTEIN 1
TGN	TRANS-GOLGI-NETWORK
TSC	TUBEROUS SCLEROSIS COMPLEX
UVRAG	UV RADIATION RESISTANCE-ASSOCIATED GENE

1 . INTRODUCTION

1.1 Cancer Epidemiology

Cancer remains a worldwide major public health problem. Despite consistent development medical research, the global burden of cancer has more than doubled during the last 30 years, being nowadays a major cause of death. In the past years, cancer was generally considered to be a disease of high-resource and industrialised western countries manly because of lifespan increased and population lifestyle. However, nowadays, the situation has dramatically changed, and many cancer cases appeared in low- and medium-resource countries. In the year of 2008, the International Agency for Research on Cancer (IARC) estimated that cancer affected 28 million persons (within 5 years from initial diagnosis) and over than 12 million new cases were diagnosed. In this same year, cancer was responsible for 7.6 million deaths (about 13% of all deaths worldwide) [1].

The most commonly diagnosed cancers worldwide are lung (1.61 million cases, corresponding to 12.7% of the total), breast (1.38 million, 10.9% of the total) and colorectal carcinomas (CRC) (1.23 million, 9.7% of the total). The most abundant causes of cancer related deaths are lung cancer (1.38 million, 18.2% of the total), stomach cancer (738 000 deaths, 9.7% of the total) and liver cancer (696 000 deaths, 9.2% of the total) [2].

In Europe, the most common cancers were colorectal (13.6% of the total), followed by breast (13.1%), lung (12.2%) and prostate cancer (11.9%). The major cause of cancer related deaths in Europe during 2008 was lung cancer (20%) followed by colorectal carcinoma (12.3%), breast cancer (7.5%) and stomach cancer (6.8%) [3] (Figure 1).

The continued growth and ageing of the world's population will significantly affect the cancer burden so it could be expected that, by the year of 2030, 27 million new cases of cancer and 17 million cancer related deaths would occur [1].

Due to the high levels of incidence and worldwide mortality, enormous efforts are being developed in cancer areas of research to better understand this disease and to discover more efficient treatments and/or diagnostic tests.

Much of the research on the cancer area demonstrated that transformation of a normal cell into a cancer cell requires very few molecular, biochemical and cellular changes, despite all potential causes of cancer and carcinogenic pathways [4]. Overall, these changes occur due to accumulation of genetic mutations in normal cells affecting their proliferation, differentiation and development [5].

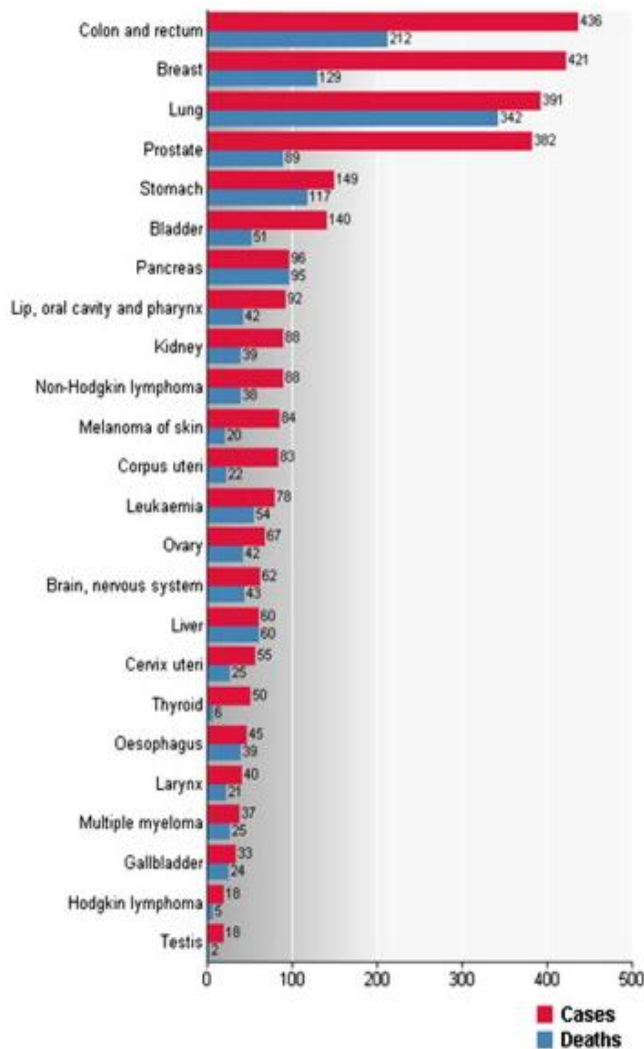


Figure 1: Estimated numbers (in thousands) of cancer cases and cancer deaths in Europe, during the year of 2008 (Adapted from [3])

Carcinogenesis is the process where cells develop general alterations responsible for its malignant transformation, and it comprise limitless replicative potential, self-sufficiency in growth signals, insensitivity to growth inhibitors, sustained angiogenesis and evasion of apoptosis. Moreover, cancer cells can invade other tissues by activation of invasion and metastasis [6]. Recently, other characteristics were added to these 6 classical hallmarks, such as the instability of genome, leading to an increase of mutations in cancer cells, inflammatory microenvironment around these cells, reprogramming of cellular energy metabolism and finally, the ability of escape to immune destruction. The last two characteristics mentioned, the emerging hallmarks, are very important to the metabolic program, allowing a continuous sustention of cell growth and

proliferation. On the other hand, the others are considered facilitating characteristics that can orchestrate all of hallmark capabilities (Figure 2) [7, 8].

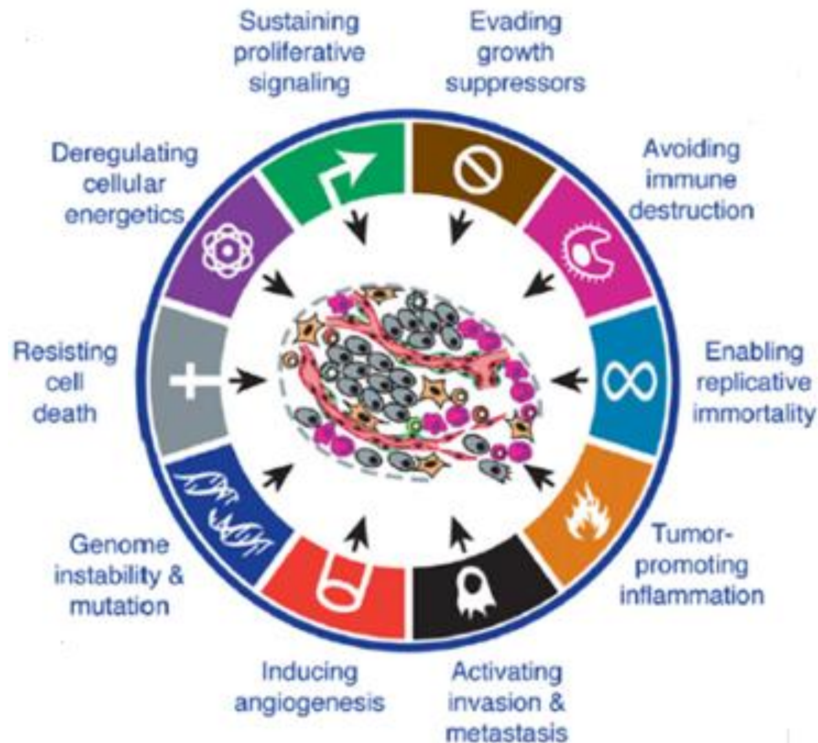


Figure 2: The ten hallmarks of cancer: acquired capabilities of cancer cells during carcinogenesis. Despite being considered a complex disease, cancer can be defined by these alterations (Adapted from [7]).

Cancer cells and therefore cancer itself, result from a set of favourable conditions, that result in a breakdown of cellular homeostasis, which can lead to a deregulation of cell division, proliferation and apoptosis mechanisms, causing, ultimately, the disease state. The development of a tumour or the carcinogenic process requires several steps during a long period of time, and can be divided into three distinct phases: initiation, promotion and progression [9]. The initial phase is a rapid event that affects the genome of the cells, giving them the ability to grow faster and to avoid normal cellular growth control mechanisms. If the damage is not repaired by the cell, a mass of abnormal cells is formed, and a progression to a malignant phenotype occurs. Fortunately, this promotion step is much longer and sometimes the disease may not even

manifest during lifetime of the individual. The progression step is associated to the ability of cancer cells to invade surrounding tissues and to metastasize [4] (Figure 3).

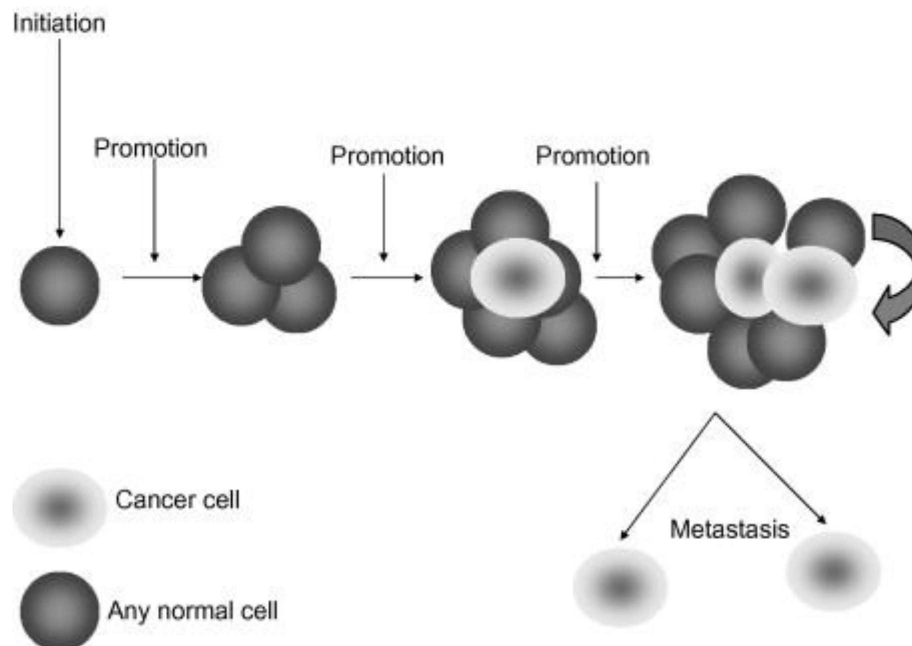


Figure 3: The carcinogenic process. Cancer formation is the result of several changes in any cell type of the body that occurs in a long period of time. After the initiation step, a period of tumour promotion occurs. Each stage of multi-step carcinogenesis reflects genetic changes in the cell with a selection advantage that drives the progression towards a highly malignant cell [4].

1.2 Colorectal Carcinoma

According to the most recent data (2008), colorectal carcinoma is the third most commonly diagnosed cancer worldwide reaching 1 233 711 CRC cases accounting for 9.7% of total cancer cases and one of the most abundant causes of cancer related deaths with approximately 608 000 deaths (8% of total cancer deaths). Incidence and mortality rates of CRC are very similar in both sexes, being the incidence in men 10% and 9.4% in women, while mortality is 7.6% in men and 8.6% in women [3]. In Portugal, CRC is the third most common form of cancer, with 695 200 cancer diagnoses, about 16.1% of all diagnoses and 369 100 deaths (Globocan project, 2008, http://www-dep.iarc.fr/).

CRC is originated as a result of pathologic transformations of apparently normal mucosa (epithelium) into a benign adenomatous polyp that can, ultimately, progress to an invasive

tumour. This progression requires mutations in many genes, activation of proto-oncogenes and/or inactivation of tumour-suppressor genes, that confer a proliferative advantaged to cells and contribute for a malignant phenotype, which takes years or even decades to cause the carcinoma. In Figure 4 is represented the multistep progression of the CRC. Although these genetic alterations often occur in a favourite order, the total accumulation of changes appears to be a critical factor of the biological properties of the carcinoma [10].

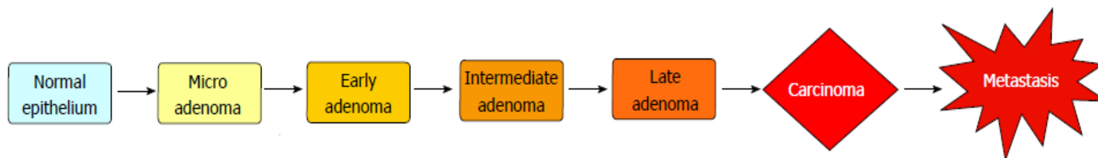


Figure 4: A multistep genetic model for colorectal carcinogenesis. The arrows represent possible mutations. Among others, mutations in *APC* (Adenomatous polyposis coli), activation of *KRAS* (Kirsten rat sarcoma viral oncogene homolog) and inactivation of *p53* are the most frequent events in this carcinogenic process (Adapted from [11]).

Colorectal carcinomas can have two different origins, so it can be divided into two distinct groups: the hereditary CRC group that represent about 20% of total CRC cases, and the sporadic CRC group that have a bigger impact with an incidence of 80% [11].

1.2.1 Hereditary or familial Colorectal Carcinoma

In hereditary or familial CRC, a little part of the total percentage is very well known and defined, being associated with well-characterized syndromes, such as hereditary nonpolyposis colorectal carcinoma (HNPCC), familial adenomatous polyposis (FAP), attenuated FAP, and MUTYH-associated polyposis (MAP), that correspond to conditions expressing adenomatous polyps. Other examples are the conditions with hamartomatous polyps like Peutz-Jeghers syndrome (PJS) and juvenile polyposis syndrome (JPS). The last condition is the hyperplastic polyposis (HPP), which is rare but has a substantial cancer risk. All of these conditions are inherited, autosomal dominant disorders, except MAP, which is autosomal recessive, and HPP, which is rarely inherited [12]. The remainder percentage of this class of CRC is still not

completely understood. However, it is though that they could correspond to alterations in single genes that are less penetrant but more common, leading to the high-risk familial, non-syndromic colon cancers and common familial-risk colon cancers [12]. A better understanding of the genetics of this group is very important to improve preventions strategies, as well as the efficiency of diagnosis and treatments.

1.2.2 Sporadic Colorectal Carcinoma: CIN and MSI

The sporadic group of CRC can be subdivided in two types of colorectal tumours that can be distinguished by their carcinogenesis process. One of them is the traditional pathway, also called the "suppressor" or chromosomal instability pathway (CIN) and the second colorectal carcinogenesis pathway is the "mutator" pathway or the microsatellite instability (MSI) [11, 13].

The CIN group, which represents more than 2/3 of CRCs, is characterized by the accumulation of structural or numerical abnormalities, preferentially in chromosome 5q, 17p and 18q [14-16]. This carcinogenesis pathway shows mutations in classical proto-oncogenes like *KRAS* (Kirsten rat sarcoma viral oncogene homolog) and tumour-suppressor genes such as *p53*, *APC* (adenomatous polyposis coli) and *SMAD4*, besides having alterations in the Wnt pathway [17]. However, just a small group of CIN has the complete set of these molecular abnormalities [18].

The MSI pathway represents about 15 - 20% of all cases of sporadic colorectal carcinoma [19] and it is characterized by the existence of alterations/mutations in the microsatellite sequences [17]. Microsatellites are nucleotide repeat sequences that exist naturally throughout the genome with two to nine nucleotides. In a situation of MSI the number of nucleotide repeats, in these microsatellite regions, is different when compared with germline DNA. The alterations in the microsatellites sequences in MSI are caused by dysfunctions of DNA mismatch repair genes, during DNA replication, typically in hMLH1 (MutS Homolog 1) or hMSH2 (MutS Homolog 2) [11].

The two different pathways previously referred (CIN and MSI) are mutually exclusive, so it is reasonable to think that the presence of genomic instability is essential and that either pathway is sufficient to drive colorectal carcinogenesis. More recently, epigenetic factors have been considered as important factors in the development of some subsets of cancers and polyps [20].

1.3 MAPK pathway and RAS proteins

The MAPK (mitogen-activated protein kinase) pathway is involved in very important cellular processes, such as proliferation, differentiation, cell survival, senescence and apoptosis [21] being the key signalling pathways for cell proliferation from the cell surface to the nucleus [22] (Figure 5). There are three major subfamilies of mitogen-activated protein kinases: the c-junk N-terminal or stress-activated protein kinases (JNK or SAPK); the MAPK14 and finally the extracellular-signal-regulated kinases (ERK MAPK, RAS – RAF – MEK – ERK) having the last one a large involvement in the pathogenesis, progression and oncogenic behaviour of CRC [23]. It is thought that at least 50% of colorectal carcinomas have a deregulation of the MAPK pathway [24].

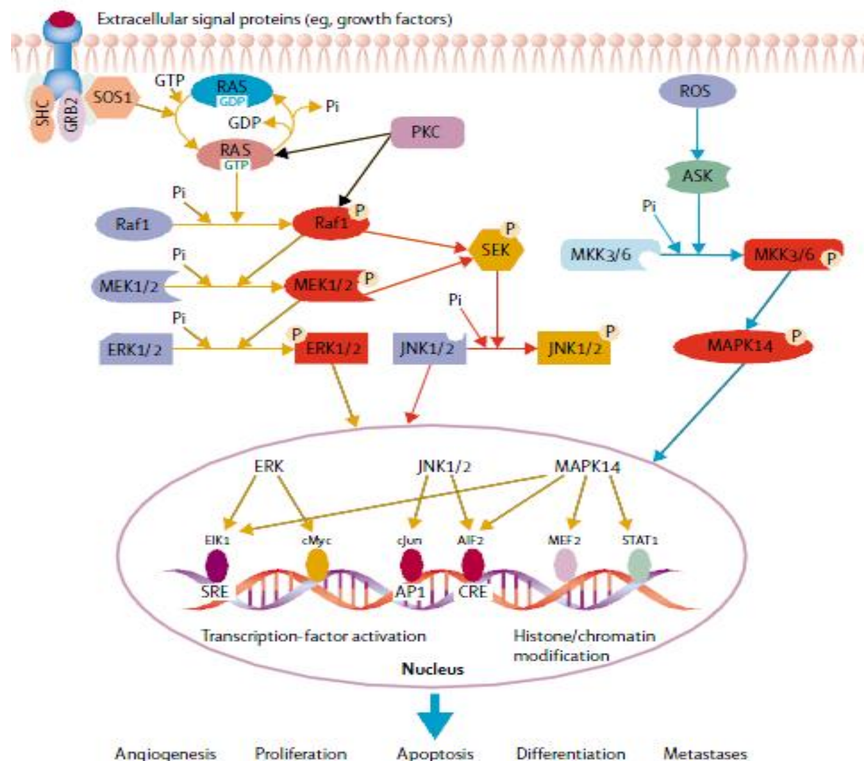


Figure 5: The MAPK signalling in pathogenesis. Three related signal transduction cascades activated through growth factors, stress or inflammation. (AIF2=apoptosis inducing factor. ASK= apoptosis signal regulating kinase. CRE=cyclic AMP/calcium response element. ELK=mixed lineage kinase. MKK=MAPK kinase. MEF2=myocyte enhancer factor 2. PKC=protein kinase C.

ROS=reactive oxygen species. SOS=son of sevenless. STAT1=signal transducer and activator of transcription factor 1) (Adapted from [24]).

RAS (rat sarcoma) proteins, encoded by three different *RAS* genes, are small GTPases (guanosine triphosphatases) consisting of 188 or 189 amino acids, involved in intracellular signalling [25]. These proteins change between an active state, linked to a guanosine triphosphate (RAS-GTP), and an inactive guanosine diphosphate (GDP) – bound state (RAS-GDP) [26] (Figure 6).

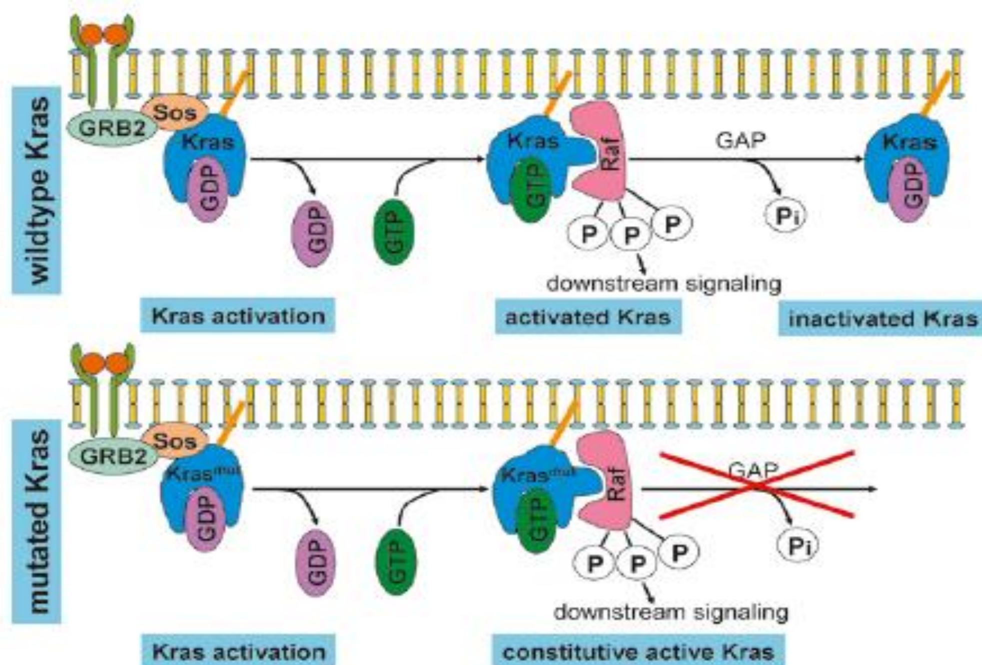


Figure 6: The mechanism of KRAS activation. This activation occurs as a result of the formation of a complex, GRB2 (Growth factor receptor-bound protein 2) and SOS, that leads to a conformational change mediating the exchange of GDP (inactive state) for GTP (active state). In wild type KRAS, this activation can be reversed by hydrolyses of GTP however, mutated KRAS remains constitutive active (Adapted from [27]).

There are four highly homologous RAS proteins, the HRAS (Harvey rat sarcoma viral oncogene homolog), NRAS (neuroblastoma rat sarcoma viral oncogene homolog) and KRAS (4A and 4B). KRAS is activated in response to receptors activation, and this response is usually transient as result to its intrinsic GTPase activity. However, when KRAS is mutated, it is constitutively active which results in an activation of downstream signalling pathways and

probably oncogenesis [28]. The activation of RAS regulates a complex signalling network that modulates the cellular behaviour, such as cytoskeletal organization, cell survival and proliferation, vesicle trafficking and calcium signalling, through the interaction with several effectors such as TIAM1 (T-cell lymphoma invasion and metastasis-inducing protein 1), PI3K (PhosphatidylInositol-3 Kinase), RAF, RalGDS (Ral guanine nucleotide-dissociation stimulator) and PLCε (Phospholipase Cε) (reviewed by [28]). The signalling pathways regulated by RAS protein are schematized in Figure 7.

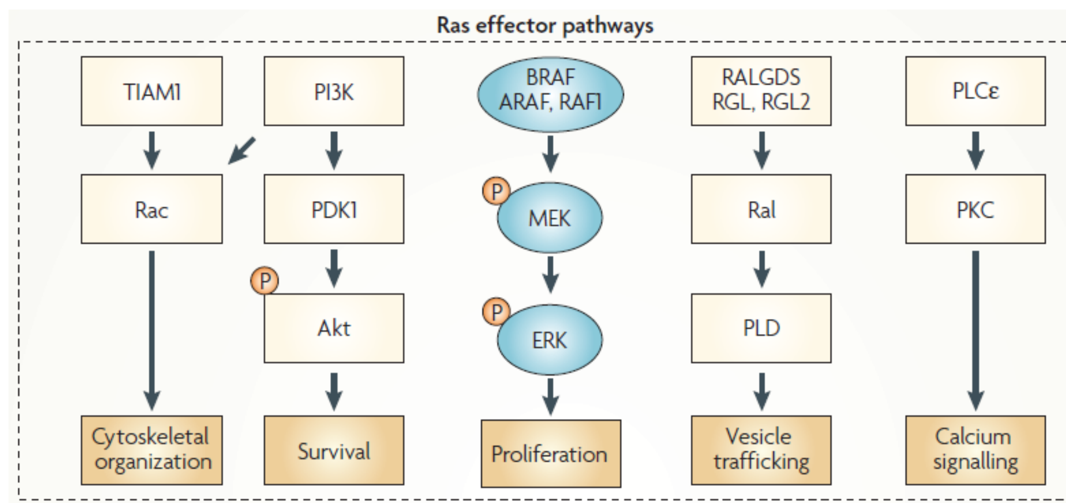


Figure 7: The RAS signalling pathway. RAS protein activates the BRAF–mitogen-activated and extracellular-signal regulated kinase kinase (MEK) – extracellular signal-regulated kinase (ERK) cascade which often determines proliferation and becomes deregulated in certain cancers and in developmental disorders. RAS also activates the phosphatidylinositol 3-kinase (PI3K) – 3-phosphoinositide-dependent protein kinase 1 (PDK1)–Akt pathway that frequently determines cellular survival. RalGDS (Ral guanine nucleotide-dissociation stimulator), RALGDS-like gene (RGL), RGL2 and TIAM1 (T-cell lymphoma invasion and metastasis-inducing protein 1) are exchange factors of Ral and Rac, respectively. Phospholipase D (PLD), an effector of Ral, is an enzyme that regulates vesicle trafficking. Rac regulates actin dynamics and, therefore, the cytoskeleton. RAS also binds and activates the enzyme phospholipase Cε (PLCε), the hydrolytic products of which regulate calcium signalling and the protein kinase C (PKC) family. P, phosphate. (Adapted from [28]).

1.3.1 Oncogenic activation in Colorectal Carcinoma: KRAS Mutations

KRAS is one of the initial members of the MAPK signalling pathway and it is mutated in 21% of all human sporadic cancers and in about 30 - 60% of CRCs cases [24, 29, 30].

In human tumours, the *KRAS* mutations can be somatic mutations of the *KRAS* gene (*KRAS*^{G12D}, *KRAS*^{G12V} and *KRAS*^{G13D}), upstream activation of receptor tyrosine kinases [31]. In CRC this mutations result in amino-acid substitutions, usually in exon 2, at codons 12 or 13 (about 85% of all *KRAS* mutations) and in exons 3 and 4 (about 15%) at codons 61, 117 and 146 [32, 33]. Activating *KRAS* mutations are very common in CRC and correspond to an early event in this type of cancer, noting it greater occurrence in adenomas larger than one centimetre [34].

Whereas the relevance of *KRAS* mutations in the pathogenesis of colorectal carcinoma is undisputed, the data regarding the role of this protein in tumour progression are conflicting, however the incidence of *KRAS* mutations have been reported to be identical throughout all the tumour stages [34]. Mutant RAS is capable of stimulating tumour cells proliferation through ERK MAPK signalling pathway [35] and can also stimulate angiogenesis by the activation of interleukin-8 synthesis [36]. Moreover, RAS activation can inhibit DNA repair genes [37]. Thus, the combination of these characteristics: tumour cell proliferation, induction of angiogenesis and DNA repair inhibition, seems to be a potential mechanism for enhancement of tumour progression and metastasis namely in CRC, although further clinical studies are needed in order to confirm it.

KRAS mutations have been implicated in the response to anti-cancer drugs in CRC, being established predictors of absence of response to epidermal growth factor receptor (EGFR) – targeted agents [38]. However, the utility of *KRAS* mutation as a prognostic marker in CRCs is unclear and more studies are still needed. In fact, recent studies demonstrated contradictory results of the importance of *KRAS* mutation as prognostic marker, being considered a negative prognostic indicator [39], and reported without a prognostic value in other studies, such as the large PETACC-3 (Pan European Trial Adjuvant Colon Cancer 3) translational trial [40].

In the current year, Valentino and co-workers showed that either KRAS small interfering RNA (siRNA) alone or combined PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) and KRAS siRNA treatments increased apoptosis in HCT116 cells. They also showed that siRNA treatment combined with 5-fluorouracil further inhibited CRC cells proliferation. As main conclusions, the authors claimed that the combination of PIK3CA and

KRAS siRNA treatments offer an effective therapy against colorectal carcinoma cells with coexisting mutations in PI3K/AKT/mTOR and RAS pathways, and that siRNA directed to PIK3CA and KRAS can be used to enhance the effects of current chemotherapy [41].

1.4 Autophagy

Autophagy or type II programmed cell death is a catabolic process of self-degradation of intercellular compounds, such as organelles or proteins, at lysosomal compartments [42, 43]. This is a genetically controlled and evolutionarily conserved process present in all eukaryotic cells [44]. This process occurs in tumour cells as well as in normal cells, particularly when the first ones have apoptotic defects, allowing autophagy to sustained cell survival for a long period in conditions of nutrients deprivation [45-48]. Autophagy is important to regulate normal turnover of organelles and to remove those with compromised function. This process can be activated during various types of stress, resulting in protection of cells. Thus, this cellular response can be activated by nutrient limitation, oxidative stress, hypoxia and heat in order to maintain homeostasis [49]. Autophagy is also important in cellular development and differentiation [50], as well as in innate and adaptive immunity [51]. In addition, it is known that this process is implicated in a large number of diseases like cancers, mainly localized in the centre of the tumour mass where the hypoxic stress is bigger due the lack of blood vessels [52], and neurodegenerative disorders, including Huntington', Alzheimer' and Parkinson's diseases [43, 53-55].

There are three major types of autophagy in eukaryotic cells that can be differentiated by their different machineries and physiological functions: the macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) [56, 57] (Figure 8), being the first one the most extensively studied.

Both macroautophagy and microautophagy involve dynamic membrane rearrangement that allows the engulfment of portions of cytoplasm containing large structures, like organelles. Microautophagy process involves the direct engulfment of small volumes of cytosol at the lysosome surface by invagination, protrusion and septation of the lysosome membrane [58]. While, macroautophagy is a more complex process that needs a special and double-membrane organelle, the autophagosome, which is formed by the elongation of phagophore and

subsequently annexation of a portion of cytoplasm. In the next step, the autophagosome fuses with the lysosome (forming the autolysosome) and there is the release, into the lumen, of the inner-single-membrane vesicle [59, 60]. In both cases, after formation of autophagic bodies they are lysed and their contents (the macromolecules) are transported back into the cytosol through membrane permeases for reuse [61]. Finally, the CMA method does not involve a membrane rearrangement; it involves a direct translocation of selective and soluble proteins across the membrane of the lysosome, instead. This translocation requires protein unfolding by chaperone proteins [62].

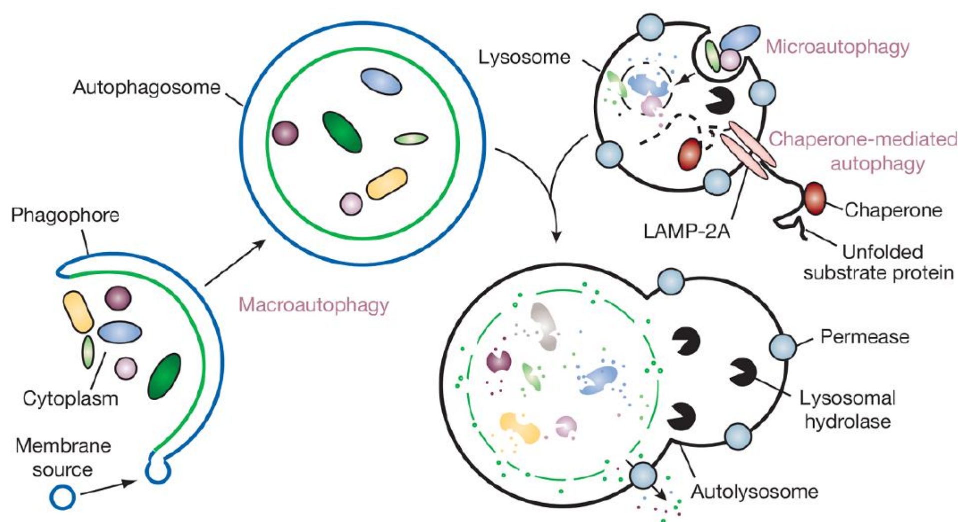


Figure 8: Schematic representation of the three main types of autophagy: Macroautophagy (engulfment of biggest cytosolic components and long-lived active proteins through the formation of autophagosome); Microautophagy (engulfment of small volumes of cytosolic components through the action of lysosome) and chaperone-mediated autophagy - CMA (translocation of soluble substrates into the lysosome with help of lysosomal chaperone proteins). Adapted from [43]).

1.4.1 Autophagy Process

Macroautophagy, hereafter termed autophagy, is a process by which cellular components are sequestered within vesicular structures (autophagosomes) and delivered to lysosomes for degradation. This process can be divided into distinct mechanistic steps: the initial induction

step, followed by nucleation, vesicle elongation, completion of autophagosome, fusion between autophagosome and lysosome and finally, degradation inside the acidic autolysosome with consequent release of the degraded products for its recycling (Figure 9).

The execution of autophagy involves a group of evolutionary conserved genes. Until now, 31 autophagy-related genes (*ATG*) are known in yeast and the majority of them have mammalian homologues [53, 63]. According to respective ATG proteins functions, in the autophagic process, they are separated in several different groups.

The induction phase of autophagy is regulated by two main complexes: the (mTOR)/Atg1/Atg13/Atg17 complex and the Beclin1/hVps34/p150/Atg14 complex also known as type III PI3K complex [64-66]. Thus, the autophagic process can be initiated by the inhibition of mTOR (mammalian Target Of Rapamycin) and consequent activation of mammalian homologues of Atg1 and Atg13 responsible for conformational alterations essential for this initial step [64, 67]. The induction of autophagy could also be promoted by the type III PI3K complex which comprises Beclin1. *Beclin 1* was the first tumour suppressor gene related with autophagy. This protein is a very important autophagic regulator that participates either in induction phase of autophagy, through interactions with vacuolar protein sorting 34 (hVps34) and mAtg14 [66, 68-70], and in autophagosomal maturation into autolysosome through the complex with hVps34 and *UVRAG* (UV radiation resistance-associated gene) [66, 68, 71]. Beclin 1 function in autophagy is mostly regulated by an anti-apoptotic Bcl-2 (B-Cell CLL/Lymphoma 2) protein [72, 73], that could inhibit the autophagic process by binding and sequestering Beclin 1 [64]. Dissociation of Beclin 1 from Bcl-2 is necessary to occur induction of autophagy and, consequently, there is a relationship between the induction of autophagy and increased expression of Beclin 1 [74, 75].

Phagophore nucleation and elongation, that leads to autophagosome formation, is possible thanks to the coordinated action of type III PI3K complex and several proteins such as Atg5-Atg12-Atg16 and pro-LC3 and Atg9 [76-78]. Microtubule-associated protein light chain 3 (LC3) is a major constituent of the autophagosomes [79]. This protein is synthesized as a pro-LC3 that is cleaved at the glycine residue by mAtg4, forming the cytosolic LC3 I [80]. The C-terminal glycine of LC3 I is then conjugated with phosphatidylethanolamine (PE) through an ubiquitin-like conjugation reaction, becoming LC3 II [81, 82]. This LC3 II is associated with both the surfaces of the double autophagosomal membrane in contrast with Atg5-Atg12-Atg16 complex, which is found only on the cytosolic surface [76, 83]. Despite all this knowledge, the origin of autophagosome membrane is not clearly understood. Several studies suggest that a

range of organelles such as endoplasmic reticulum, mitochondria and Golgi complex can provide the required membrane components for autophagosome formation [84]. The final autophagosome could be detected by the presence of LC3 II on the internal surface of autophagosome.

Autophagosome-lysosome fusion is the following step of the autophagic process. The hydrolytic enzymes of the lysosome are essential for the acidification of the autophagosome environment allowing the complete digestion and destruction of nonfunctional proteins and organelles. After this fusion, the LC3 II is degraded in LC3 I leading to the decrease of LC3 II content in autolysosome [60, 85]. Thus, LC3 II is considered a good autophagic flux marker [86]. Another important marker for the autophagic process is the p62 protein. This protein coupled with sequestrome 1 (SQSTM1) binds directly to LC3 II being transported into the autophagosome and degraded in autolysosome [81]. Both LC3 II and p62 proteins allow the autophagic flow study once there is degradation or an accumulation of these proteins in response to an autophagy induction or inhibition, respectively. The inhibition of this fusion between autophagosome and lysosome through Bafilomycin A1 (Baf A1) leads to accumulation of the LC3 II and p62 proteins [87].

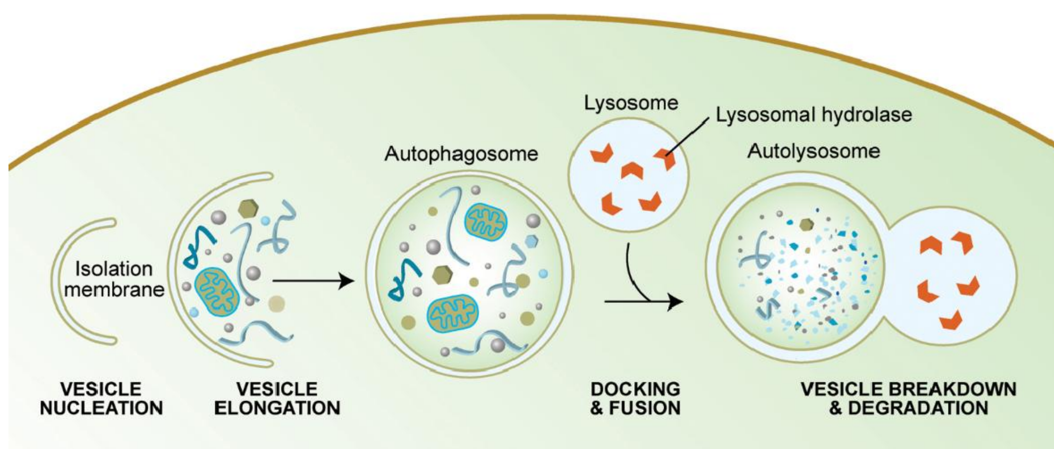


Figure 9: Schematic representation of the autophagic process: autophagy begins with the induction step, followed by nucleation, vesicle elongation, completion of autophagosome, fusion between autophagosome and lysosome and finally, degradation inside the acidic autolysosome with consequent release of the degraded products (adapted from [65]).

1.4.2 Autophagy Regulation

Autophagy is an important process for the maintenance of cellular homeostasis. This process occurs at basal levels in non-stress situations, allowing the recycling of nutrients and removal of unnecessary proteins and organelles to the cell [61]. In stress conditions such as nutrient and energy deprivation and hypoxia, the autophagy levels increase as a pro-survival action [42, 54, 88]. The regulation of autophagy process and the associated molecular machinery is very complex and involves several well characterized signalling pathways that are responsible for its induction or inhibition (Figure 10). mTOR have a crucial position in autophagy regulation under stress condition, as a major negative regulator, since the majority of signaling pathways converge in this kinase [89].

Several studies indicates that class I PI3K – AKT – mTOR pathway is very important in autophagy regulation process under starvation conditions [54, 90, 91]. The classes I PI3K is often activated in response to growth factors (insulin) and once activated, the generated PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) binds to the pleckstrin homology (PH) domains of PDK 1 (phosphoinositide-dependent protein kinase 1) and AKT leading to the translocation of both proteins to the cell membrane and their consequent activation [92, 93]. The activation of the serine/threonine kinase AKT, also named protein kinase B (PKB) occurs in two different steps: an initial phosphorylation at threonine 308 in the catalytic domain by PDK 1 and a subsequent phosphorylation at serine 473 mediated by AKT itself, PDK 1 or mTORC2 complex [94-97]. AKT activation leads to the activation of the subsequent kinase of PI3K – AKT – mTOR pathway, the mTOR, and to the inhibition of the RAS protein. While mTOR activation leads to the inhibition of autophagy, the RAS activation has a dual effect in this process. When RAS activates class I PI3K there is an autophagy inhibition, but on the other hand when this protein selectively activates the kinase cascade RAF1 – MEK1/MEK2 – ERK1/ERK2, autophagy is stimulated [98]. The phosphatase and tensin homologue (PTEN) is also able to regulate autophagy. PTEN reverses PIP3 production by class I PI3K dephosphorylation and inhibition, which suppresses the downstream AKT signaling and thus positively regulates autophagy [99].

Although mTOR is considered central key in autophagy regulation, mTOR independent pathways have also been reported to regulate autophagy. These pathways converge on Beclin1/hVps34/p150/Atg14 complex also known as type III PI3K complex (Figure 10). This complex promotes the sequestration of cytoplasmic material and consequently the autophagic

process [100]. Beclin 1 can be activated by the stress-responsive c-Jun amino-terminal kinase 1 and by the death-associated protein kinase (DAPK) and the dynamin-related protein 1 (DRP 1) [101, 102]. JNK1 and DAPK proteins phosphorylate and disrupt the association of anti-apoptotic proteins, Bcl-2 and Bcl- XL, with Beclin 1, leading to the activation of the Beclin 1-associated to class III PI3K complex, which localizes in the Trans-Golgi-Network (TGN), and stimulation of autophagy. The upregulation of the BCL- 2/adenovirus E1B 19 kDa protein – interacting protein 3 (BNIP3) or HSPIN1 (human homologue of the Drosophila spin gene product) at the mitochondria also induces the autophagic process [103].

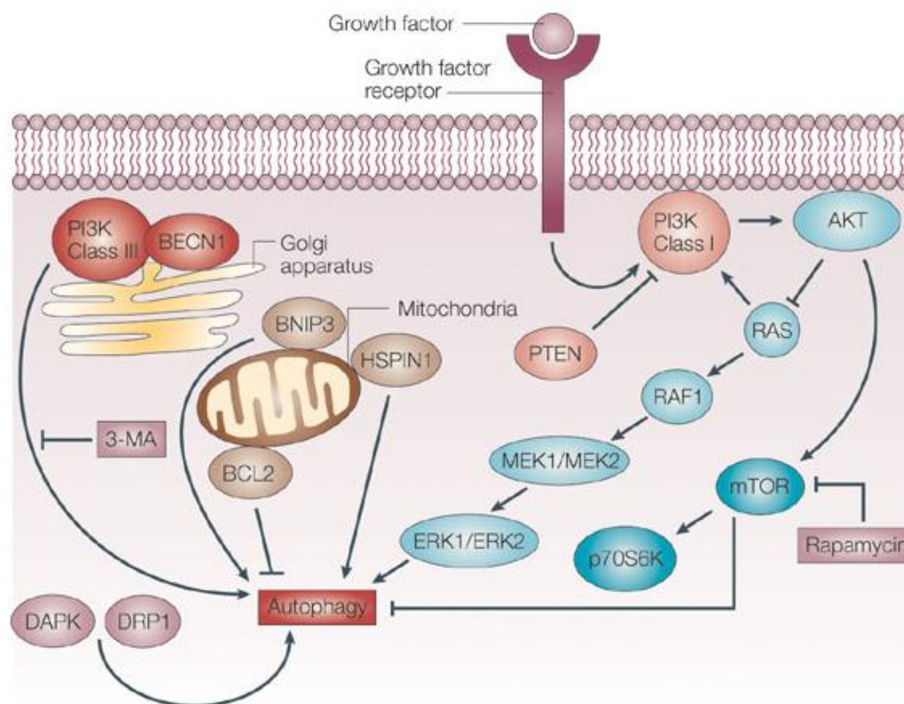


Figure 10: Schematic representation of autophagy regulation. In nutrients limiting conditions, mTOR is inhibited, de-repressing autophagy. Activation of growth factor receptor (insulin receptors) stimulates the class I PI3K. This stimulation generates PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) leading to the phosphorylation and activation of AKT. AKT activation leads to the activation of the mammalian target of rapamycin (mTOR) and to the inhibition of the RAS protein. mTOR activation leads to the inhibition of autophagy but the RAS activation has a dual effect in this process. When RAS activates class I PI3K there is an autophagy inhibition, but on the other hand when this protein selectively activates the kinase cascade RAF1 – MEK1/MEK2 – ERK1/ERK2, autophagy is stimulated. The phosphatase and tensin homologue (PTEN) is also able to positively regulate autophagy once PTEN reverses PIP3 production by class I PI3K

dephosphorylation and inhibition, which suppresses the downstream AKT signalling. Another important regulator of autophagy is the type III PI3K – Beclin 1 (BECN1) complex. This complex which is inhibited by 3-methyladenine (3-MA) promotes the sequestration of cytoplasmic material and activates the autophagic process. Beclin 1 can be activated by the death-associated protein kinase (DAPK) and the death-associated related protein kinase 1 (DRP 1). Downregulation of BCL2, or upregulation of the BCL- 2/adenovirus E1B 19 kDa protein – interacting protein 3 (BNIP3) or HSPIN1 (human homologue of the Drosophila spin gene product) at the mitochondria also induces the autophagic process. (adapted from [104])

Another important regulator of the autophagy is the level of ATP (Adenosine Trisphosphate) in cells. The detection of lower ATP levels (due to glucose starvation for example) leads to the phosphorylation and activation of AMP-activated protein Kinase (AMPK) [105]. AMPK, which senses changes in the intracellular ATP/AMP ratio phosphorylates and activates tuberous sclerosis complex 1/2 (TSC1–TSC2), leading to inactivation of mTOR and autophagy induction [106, 107].

1.5 RAS and Autophagy

In about 30% of human cancers, mutations in *RAS* proto oncogene occurs, leading to the formation of an oncogenic form of RAS. This data suggests that RAS plays important roles in tumour development. In colorectal carcinoma mutated *KRAS* has been frequently reported [108-110].

As previously mentioned, autophagy has important functions for the survival of cells in unfavourable conditions and, in other hand allows the repair or, in more severe cases, the destruction of damage cells. Recently, the autophagic process has been implicated in cancer prevention, suppressing cancer initiation; and cancer progression, enabling growth of aggressive cancers [42, 45, 111].

Very recent publications suggest that cell lines harbouring oncogenes such as *HRAS*^{V12} or *KRAS*^{V12} (*RAS*) strongly increase basal autophagy, being dependent on this process to survive to starvation. Guo and colleagues suggest that, in iBMK (immortalized baby mouse kidney) cells,

RAS-induced high basal autophagy must result from an mTOR-independent mechanism [112]. Their study demonstrated that in activated-*RAS* expressing cells autophagy facilitates tumourigenesis and cancer cell survival through the maintenance of mitochondrial metabolic function, essential for the cell viability [112]. Another study demonstrates, for the first time, that autophagy is essential to *KRAS*^{V12} - induced malignant transformation in human breast cells (MCF10A). In *KRAS*^{V12}- overexpressed cells was observed an increase of ATG proteins (Atg5 and Atg7) and an activation of MAPK pathway. The JNK was also involved in the induction of malignant transformation, in an indirect-way. Thus, the activation of JNK by ROS (Reactive Oxygen Species) was able to induce autophagy through upregulation of Atg5 and Atg7, leading to the malignant transformation of cells [113]. In both studies, the implication of autophagy in initiation and progression of cancers was demonstrated. The relation between RAS and autophagic process was also shown in ovarian HOSE epithelial cells by Elgendy and co-workers. They demonstrated that depending on the intensity of oncogene expression of *HRAS*^{V12}, in the absence of other co-transforming genes, caspase-independent cell death, autophagy, could occur [114]. This study reveals that *RAS*-induced autophagy is Noxa- and Beclin 1 –dependent, observing an increase of their expression. Noxa (BH3-only protein), which has a great affinity to Mcl-1 (Bcl-2 family member) [115], promotes autophagy through the displacement of Mcl-1 from Beclin 1.

As conclusion, depending on the degree of oncogenic *RAS* activation, the consequences in cell are different. This mutation can be crucial for the survival of tumour cells in aggressive cancers, or contrarily, to the destruction or repair of damage cell, preventing tumour development, both through autophagy.

In colorectal carcinoma harbouring *KRAS* mutations, there are indirect evidences that KRAS might regulate autophagy. In HCT116 cell line, a human CRC cell line with *KRAS*^{G13D} activation, the basal levels of autophagy increased compared with a cell line without *KRAS* mutations, which has important consequences for cell survival and proliferation [112]. Another recent study showed that activated *KRAS* also increase the autophagic response after an extracelular matrix detachment stimulus, protecting cells from detachment-induced cell death (anoikis) [116].

1.6 *Rationale* and aim

According to the most recent data, colorectal carcinoma is the third most commonly diagnosed cancer worldwide and one of the most abundant causes of cancer related deaths (Globocan project, 2008, http://www-dep.iarc.fr/). KRAS, one of the four highly homologous RAS proteins, is one of the initial members of the MAPK signalling pathway and is mutated in about 30% of human cancers [24, 29, 30].

Autophagy is an important process for the maintenance of cellular homeostasis. This process occurs at basal levels in non-stress situations, allowing the recycling of nutrients and removal of unnecessary proteins and organelles to the cell [61]. In stress conditions such as nutrient and energy deprivation and hypoxia, the autophagy levels increase as a pro-survival action [42, 54, 88]. The regulation of autophagy process and the associated molecular machinery is very complex and involves several well characterized signalling pathways.

When *KRAS* is mutated, it is constitutively active which results in an activation of downstream signalling pathways and probably oncogenesis. KRAS protein can modulate cell survival through interaction with class I PI3K, inhibiting autophagy, or through RAS – RAF – MEK - ERK pathway that stimulate autophagy [98]. Despite the association between RAS proteins and autophagic process, the precise role of *KRAS* mutations in the regulation of cellular survival through autophagy process in CRC, is not well understood.

The major aim of this work was to understand the precise contribution of KRAS in autophagy regulation in CRC. We also aimed to study the role of KRAS in cell proliferation and to understand the role of autophagy in the survival of CRC cells harbouring *KRAS*^{G12V} mutations. This study might have important consequences in the identification of new therapeutic approaches for CRC.

For that purpose, a CRC cell line was used. The SW480 cell line, with a *KRAS*^{G12V} mutation, was transfected with specific siRNA for KRAS protein in order to inhibit KRAS and the autophagic phenotype was studied. Thus the initial task of this work was to optimize the transfection conditions for the SW480 cell line. Moreover HEK 293 (Human Embryonic Kidney 293) cells stably expressing Flag-KRAS^{WT}, Flag-KRAS^{G13D}, Flag-KRAS^{G12D} and Flag-KRAS^{G12V} were used in order to study the influence of the different genetic mutations of *KRAS* in autophagy. All cell lines were subjected to HBSS (Hank´s Balanced Salt Solution) treatment, a starvation-autophagic inducer and to Bafilomycin A1 treatment, an autophagy inhibitor, and the autophagy

related proteins were monitored (Beclin 1, Atg5, p62, LC3 (I/II)) by Western-blot analysis. Moreover, we also analyzed cell survival and proliferation by cell cycle analysis and colony formation assay.

2. Material and Methods

2.1 Cell lines and culture conditions

SW480 cell line and stable cell lines derived from HEK 293 cells were used as study models. SW480, a human CRC-derived cell line (CCL-228, ATCC), harbouring a *KRAS*^{G12V} mutation was kindly provided by Professor Raquel Seruca from IPATIMUP. Stable cell lines derived from HEK 293 cells (CRL-15t3, ATCC), infected with *Flag-KRAS*^{WT} and *Flag-KRAS*^{G13D}, *Flag-KRAS*^{G12D}, and *Flag-KRAS*^{G12V} mutations were produced by PhD student Sara Alves from CBMA/ Department of Biology.

SW480 cells, used between 5 and 25 passages, were grown in RPMI (Roswell Park Memorial Institute) 1640 medium with Stable Glutamine (PAA, Austria) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS) (SIGMA, USA) and 1% (v/v) Penicillin-Streptomycin (PenStrep) (GIBCO, USA). HEK 293 derived cell lines, used between 13 and 20 passages, were grown in DMEM (Dulbecco's Modified Eagle Medium) with L-Glutamine (PAA, Austria) supplemented with 2% (v/v) Sodium Bicarbonate (GIBCO, USA), 1% (v/v) Sodium Pyruvate (GIBCO, USA) and also 10% (v/v) of inactive FBS (SIGMA, USA) and 1% (v/v) of PenStrep (GIBCO, USA). All cell lines were maintained in 25 cm^2 polystyrene flasks (TPP, USA), under a humidified, 5% CO_2, 37°C atmosphere and sub-cultured whenever the cultures became confluent (approximately 80% of confluence) using 0.05% Trypsin/ 0.02% EDTA solution (GIBCO, USA).

All cell lines were subjected to an autophagy induction by nutrient deprivation with HBSS (Hank´s Balanced Salt Solution) (GIBCO) for 6 hours, and to an autophagy inhibition by Bafilomycin A1 (Baf A1). Bafilomycin A1 is an inhibitor of the vacuolar ATPase, which blocks the fusion of autophagosomes with lysosomes, leading to an accumulation of autophagosomal structures [117]. Bafilomycin A1 was mixed with HBSS or complete RPMI or DMEM medium at a final concentration of 20nM.

2.2 Optimization of sIRNA transfection in SW480 cell line

In order to optimize small interfering RNA (siRNA) transfection conditions in the SW480 cell line, we used two different approaches, the forward and the reverse transfection. The first one is the most common technique for delivering siRNA into cells for gene silencing and involves preplating cells one day before siRNA and transfection reagent treatment. In the other hand, in

reverse transfection, siRNAs are plated at the same time as the cell suspension followed by addition of the transfection reagent, being a less-time consumer technique.

Furthermore, we also used a fluorescent control siRNA linked to Alexa Fluor 488 (all stars negative siRNA – AF 488, from Qiagen), which allowed a faster and easier result achievement since the analysis is very direct and simple and does not need Western-blot analysis. The efficacy of siRNA - AF uptake was evaluated by two different methods: Fluorescence microscopy (Leica - DM5000B + CTR5000 + ebq100) and Cytometer analysis (Beckman Coulter – Epics XL).

The specific siRNAs target sequences used in this study, were the siRNA KRAS, specifically Hs_KRAS2_8, with the following target sequence: 5`-AAGGAGAATTTAATAAAGATA -3`, and a negative control siRNA with the following target sequence: 5`-AATTCTCCGAACGTGTCACGT-3´that were purchased from Qiagen. The transfection reagents tested were Lipofectamine 2000 (Invitrogen), Lipofectamine RNAiMAX (Invitrogen) and LipoGen (Invivogen).

2.2.1 Forward Transfection

In the forward transfection, SW480 cells were plated in 6-well plates at $2x10^5$cells/well (20% of confluence) in RPMI complete medium and were incubated at 37°C for 24 hours allowing a correct attachment. The cells were then washed twice with phosphate buffer saline 1x (PBS) for FBS removal and incubated with 750µl of Opti-MEM (Reduced serum medium) at 37°C, while the transfection mix was prepared. For the transfection mix, the desired volume of transfection reagent was diluted in Opti-MEM at a final volume of 125µl (Mix 1) and incubated 5 minutes at room temperature (RT). Then, the desired volume of siRNA was also diluted in Opti-MEM at a final volume of 125µl (Mix 2) and Mix 1 was gently added to Mix 2. After 20 minutes at RT, the transfection mix was added to each well. In the control condition, without transfection reagent or siRNA, it was added 250µl of Opti-MEM. Cells were incubated at 37°C and 14 hours later, the medium was replaced for RPMI complete medium and the cells were allowed to grow for another 48 hours. All procedures were performed under RNAse free conditions.

2.2.2 Reverse Transfection

In the reverse transfection method, the desired volume of siRNA was diluted in Opti-MEM and the transfection reagent was gently added to the mixture for a total volume of 250µl. After 20 minutes at RT, the transfection mix and 750µl of cell suspension ($2x10^5$cells/well) were added into the respective well. Cell suspension was composed by RPMI medium without antibiotics and supplemented with 10% of inactive FBS. In order to mix the cells and the transfection mix, we gently shake the plate back and forth. After approximately 24 hours at 37°C, the medium was replaced by RPMI complete medium and cells were allowed to grow for 48 hours, at 37°C. All procedures were performed under RNAse free conditions.

2.2.3 Assessment of siRNA-AF internalization using Fluorescence Microscopy and Flow Cytometer

After transfection, $2x10^5$ SW480 cells were plated in 6-multiwell culture plates for fluorescent microscopy internalization analysis (Leica - DM5000B+CTR5000+ebq100) and $2x10^5$ SW480 cells were seeded in fisherbrand cover slips (12mm) for cytometer analysis (Beckman Coulter – Epics XL). For fluorescent microscopy analysis siRNA- transfected SW480 cells were washed three times with PBS 1x and fixed with 4% paraformaldehyde (PFA) for 30 minutes. Moreover, these cells were incubated with a fluorescent DNA - binding dye, using 3µl of Vectashield Mounting Medium with DAPI. Images were analyzed with an appropriated protocol in LAS AF software. siRNA - AF transfected - SW480 cells were represented as FITC - labelled cells and thus the percentage of uptake efficiency was determined by [FITC - labelled cells/total of cells * 100]. The uptake efficiency by SW480 cells were determined through manual counting of at least 500 cells for each condition.

For the assessment of siRNA-AF internalization using cytometer, siRNA-AF transfected - SW480 cells were trypsinized (500µl) and collected by centrifugation (1000 rpm for 5 minutes). The cells were then washed by centrifugation (14000 rpm, 5 minutes) three times and ressuspended in 500µl of PBS 1x. The RNAi-Alexa Fluor fluorescence was detected in a Beckman Coulter – Epics XL flow cytometer using Fl-1 (488/525 nm).

2.3 Western-blot analysis

Western-blot technique was performed to assess the expression of several proteins involved in autophagy pathway, namely KRAS, Beclin 1, LC3 I and LC3 II, Atg5, p62 and ERK: pERK (phosphorylated ERK) and tERK (total ERK). β -Actin, a constitutively expressed protein in human cells encoded by a housekeeping gene, was used as a loading control.

2.3.1 Total protein extraction

SW480 cells were plated in 6-multiwell culture plates (TPP, USA) at $4x10^5$ cells/well and 48 hours after treatment the total proteins were extracted. Cells were washed with PBS 1x, trypsinized and twice washed (5084R - EPPENDORF) at 2000 rpm for 10 minutes at 4°C. Then the pellet was vigorously resuspended in ice-cold RIPA buffer containing 1mM PMSF, phosphatase inhibitors (20mM NaF, 20mM Na_3VO_4) and 40mM of protease inhibitor cocktail, and the cell suspension was kept on ice for 20 minutes to promote cell lysis. Finally, a last centrifugation (Sigma - 2K15) was performed (14000 rpm, for 15 minutes at 4°C) allowing the recovery of total proteins in the supernatant fraction.

The protein concentration was quantified using the Bio-Rad DC protein assay (Bio-Rad Laboratories, USA) and BSA (Bovine Serum Albumin) was used as protein standard.

2.3.2 Western blotting

25µg of proteins were separated by SDS - Polyacrylamide gel electrophoresis (12.5% bis-acrylamide, 375mM Tris-HCl, 0.4% SDS, 15% Glycerol, 0.1% TEMED and 10% APS) in running buffer during approximately 1h30 at 20mA. The samples were mixed with laemmli buffer 4x (Tris – HCl1M, 40% glycerol, 10% SDS, β-Mercaptoethanol and Bromophenol Blue) and 15µl was loaded into each well. Besides, 2µl of protein molecular weight ladder (PAGE Ruller Unstained Protein Ladder – Thermo Scientific), was loaded in other well.

Proteins were transferred to a Polyvinylidene Fluoride (PVDF) membrane (ThermoScientific, USA) that was previously hydrated with methanol. Transference occurred in a mini transfer system at 54mA during 1 hour using transfer buffer (0,25M Tris - Base, 1,92M

Glycine and Methanol). Membranes were blocked for 1 hour at RT in PBS-T (PBS with 0.5% Tween-20 (Bio-Rad laboratories)) containing 5% skim milk (Molico) or in PBS-T containing 3% of BSA for phosphorylated proteins.

After blocking, the membranes were incubated with the primary antibodies overnight at 4°C, washed with PBS-T or TBS-T and incubated with the secondary antibodies for 1 hour at RT. The primary antibodies used for immunoblotting were: anti-KRAS (Santa-Cruz), anti-Beclin 1 (Cell Signalling), anti-LC3 I/II (Sigma-Aldrich), anti-Atg5 (Sigma-Aldrich), anti-p62 (Santa-Cruz), anti-pERK, anti-tERK and anti-β-Actin (all from Cell Signalling). Secondary antibodies used were goat anti-mouse or goat anti-rabbit from Jackson Laboratories. All dilutions are represented in table 1.

For ERK proteins immunodetection, an additional step was done in order to reprobe the membranes. Thus after pERK immunodetection, the membranes were striped with stripping solution (Tris-HCl1M, β-Mercaptoethanol and 10% SDS) for 30 minutes at 50°C and washed with PBS-T and TBS -T (three times, 10 minutes each solution). Membranes were then blocked again and incubated with the desired primary and secondary antibodies as previously described.

Finally, the substrate - antibodies reaction was detected with ECL reagents (Thermo Scientific) under a chemiluminescence system, the ChemiDoc XRS (Bio-Rad). Band intensity was quantified using the Quantity One software from Bio-Rad.

Table 1: Primary and secondary antibodies used and their dilutions.

Primary Antibody	***Dilution***	***Secondary Antibody***	***Dilution***
Anti – KRAS	1:100	Goat anti-mouse	1:3000
Anti – Beclin1	1:750	Goat anti-rabbit	1:3000
Anti – LC3 (I/II)	1:3000	Goat anti-rabbit	1:2000
Anti – Atg5	1:1000	Goat anti-rabbit	1:5000
Anti – p62	1:1000	Goat anti-mouse	1:2000
Anti – pERK	1:1000	Goat anti-rabbit	1:2000
Anti – tERK	1:1000	Goat anti-rabbit	1:2000
Anti – β-Actin	1:5000	Goat anti-mouse	1:5000

2.4 Trypan Blue exclusion Assay

Trypan blue exclusion assay is a simple and quick method to study cell viability. This dye exclusion stain allows distinguishing viable cells, with an intact membrane, from death cells since living cells are able to exclude the dye while cells without an intact membrane take up the colouring agent, presenting a blue coloration.

For cellular viability study, a cell suspension of SW480 cell line after treatment was prepared and mixed with trypan blue solution in a 1:1 dilution. After 5 minutes at 37°C, 10µl of the mixture were loaded in counting chambers of a haemocytometer, and the number of stained and total cells was counted. The percentage of cell death is the result of the formula: [(blue cells/total of cells)*100].

2.5 Cell Cycle analysis

The cell cycle is the ordered series of events required for cell division which allow the continuity of life. The stages of the cell cycle include interphase (which is made up of three stages: G1, S and G2), mitosis and cytokinesis. The interphase is composed for two "gap" phases (G1 and G2) responsible for cell preparation and growth and for the S phase, where DNA duplication occurs. After interphase, the duplicated chromosomes are separated through a set of well characterized steps (prophase, anaphase, metaphase and telophase) culminating in cytoplasm division, the cytokinesis [118].

Cell cycle analysis of SW480 cell line was performed by flow cytometry that allows the study of the amount of cells in interphase cell cycle phases. The cytometer is able to distinguish the different phases according to DNA content present in each cell. In this work G1, S and G2 phases were analyzed as well as a sub-G1 peak, correspondent to apoptotic cells. Apoptotic cells present reduced DNA content due the DNA fragmentation. Therefore this group is represented by a sub-G1 population seen to the left of the G1 peak.

For cell cycle analysis, cells were collected by centrifugation (500g for 3 minutes) and ressuspended in 500µl. After 15 minutes on ice, the cell suspension was rapidly pipeted into 1.5ml of ice-ethanol (stored at -20°C) and incubated on ice for more 15 minutes for cells fixation. After ethanol fixation cells were centrifuged at 3000rpm during 3 minutes at 4°C (EPPENDORF –

5804R) and the resulting pellet was washed in 2ml of PBS 1x. Two more washes were done and after the last wash, cells were ressuspended in 500μl of PBS 1x. 50μl of RNAse A solution (200μg/ml in sodium citrate (1% w/v)) was added into cells and after vortex, cells were incubated at 37°C in a water bath for 15 minutes. In the end, PI (Propidium Iodide) stain solution (0.5mg/ml in sodium citrate (1% w/v)), which is light sensitive, was added to cells and after vortex were incubated in darkness at RT for at least 30 minutes.

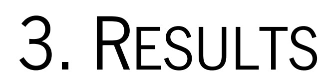
The cells were analysed in Beckman Coulter- Epics XL flow cytometer and the data obtained was treated using FlowJo 7.6 software.

2.6 Colony Formation Assay

Colony formation assay is a method where the ability of one single cell to form a colony is tested. For that purpose, 400 and 800 HEK 293 cells were plated (per well) using DMEM complete medium. Cells were maintained at 37°C in a humidified atmosphere with 5% of CO_2 during three weeks, allowing their growth. The DMEM medium was replaced once a week in order to restore all the nutrients needed. At the end of three weeks, cells were fixed and stained using a 6% glutaraldehyde/0.5% crystal violet solution. Firstly DMEM medium was carefully removed and cells were washed twice with PBS 1x. Then, 2-3ml of fixing and staining solution were added to cells, for at least 30 minutes at RT. Finally, after careful removal of glutaraldehyde/crystal violet solution and thorough rinsing with water, cells were allowed to dry at RT. Colonies of HEK 293 derived cell lines were then photographed and counted.

2.7 Statistical analysis

Data is reported as the mean ± standard error of the mean (SEM). Statistical analysis was performed by the one-way ANOVA test, following by the Tukey post test, using the GraphPad Prism 5.0 software (San Diego, CA, USA).



3. RESULTS

3.1 Optimization of RNA interference conditions

In order to determine the precise role of mutated KRAS protein in autophagy regulation on CRC cells, we silenced this protein by RNA interference (RNAi) in the SW480 cell line that harbours a RKAS^{G12V} mutation. For that purpose, we began to optimize the RNAi conditions using two different approaches of siRNA transfection (forward and reverse transfection) and several transfection reagents such as LipoGen, RNAi-MAX, and Lipofectamine 2000.

LipoGen, RNAi-MAX, and Lipofectamine 2000 were formulated for nucleic acids (DNA, RNA and mRNA) transfection on a broad range of cell types. All these three transfection reagents interact with the nucleic acids, forming a compact complex that allows a very efficient transfection.

3.1.1 LipoGen Transfection Reagent

Using the forward transfection approach, we tested 3µl and 6µl of LipoGen transfection reagent combined with 50nM of siRNA – AF (Figure 11). The fluorescent images of Figure 11A shown a bigger uptake of siRNA – AF, 49.15%, using 3µl of lipoGen compared with the 33.33% of siRNA – AF uptake using 6µl of lipoGen. Besides the siRNA - AF uptake, we also studied the toxicity of lipoGen transfection reagent by trypan blue exclusion assay (Figure 11C). The 3µl of lipoGen condition presented a lower percentage of toxicity to the SW480 cells (10.15%) when compared with 6µl of lipoGen with 12.64% of cellular death (Figure 11C).

Even though the previous results indicated that the combination of 3µl of lipoGen transfection reagent with 50nM of siRNA presented the better condition for further KRAS silencing, the Western-blot analysis using specific siRNA for KRAS proved that lipoGen was not indicated for siRNA transfection in SW480 cell line. Thus, in western-blot analysis we used 2µl and 4µl, instead of 3µl of lipoGen, and two different concentrations of siRNA KRAS (50nM and 100nM). The Western-blot for KRAS protein and its normalization to β-Actin protein in all conditions tested are shown in Figure 12. We could not observe a decreased in the expression levels of KRAS after 2µl of LipoGen, revealing inefficient silencing of KRAS (Figure 12A). Better results were obtained when 4µl of lipoGen were used, since we could observe a decrease of KRAS expression. However, in siRNA negative control conditions and in the transfection control condition, it was also observed the some effect on KRAS protein expression (Figure 12B).

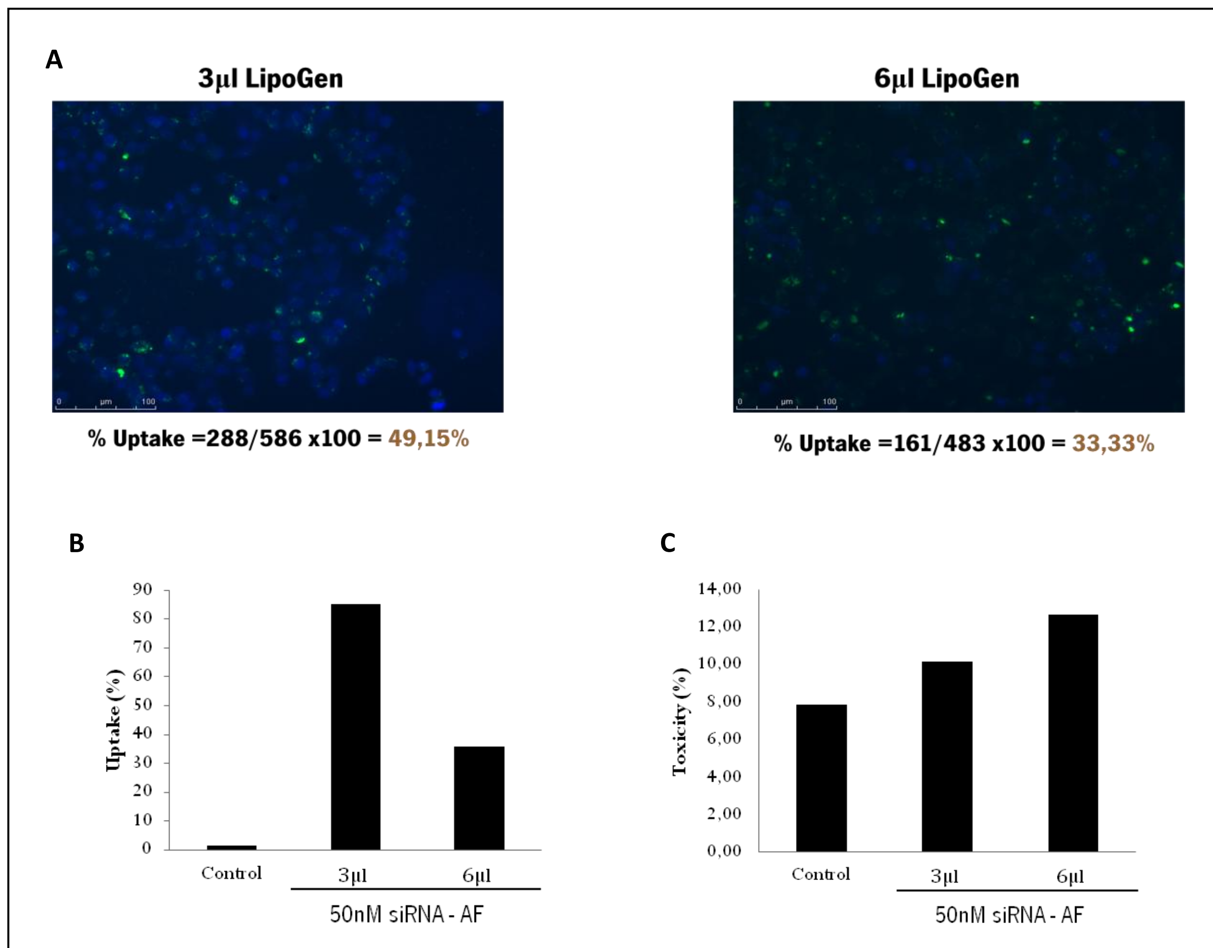


Figure 11: Optimization of siRNA silencing in SW480 cell line using siRNA - AF (50nM) and LipoGen transfection reagent (3µl and 6µl) by forward transfection. (A) Fluorescent images of SW480 nuclei stained with DAPI (blue) and siRNA - AF (green). The percentage of uptake was calculated through the formula: [FITC - labelled cells/total of cells * 100] (20x); (B) Cytometry results of siRNA - AF uptake by SW480 cell line; (C) Toxicity of LipoGen transfection reagent measured through trypan blue exclusion assay. The toxicity percentage was calculated through the formula: [(blue cells/total of cells)*100].

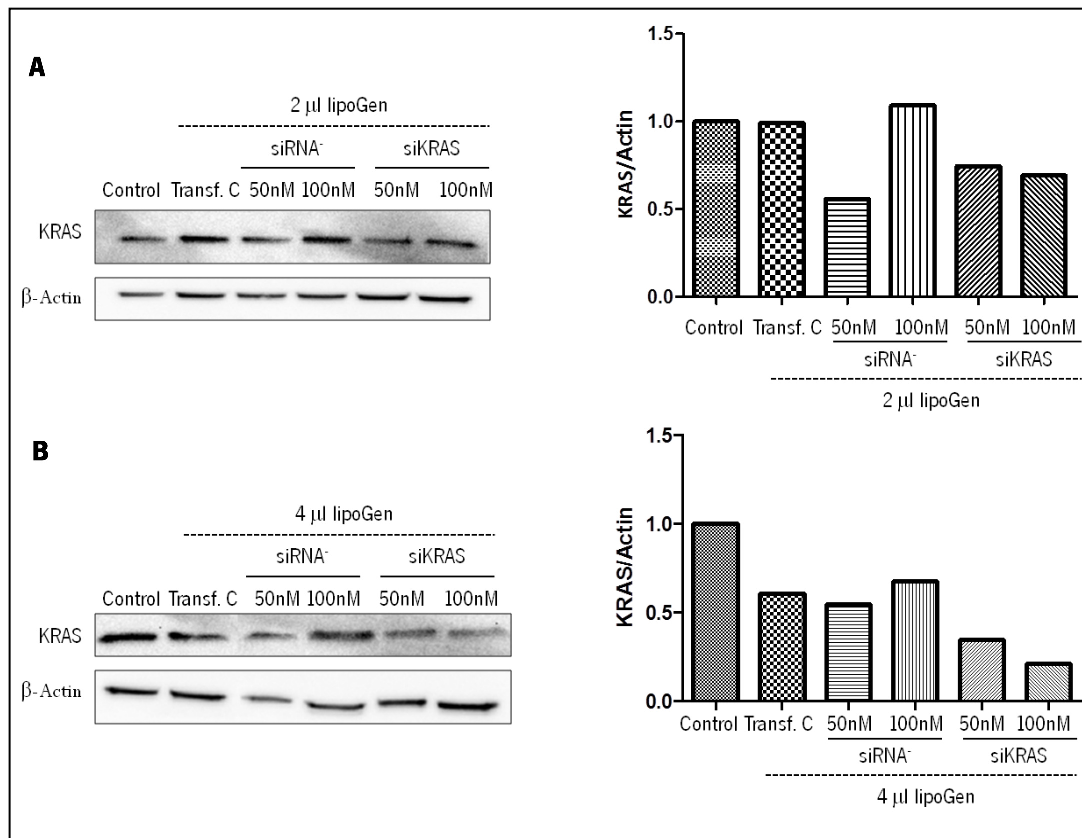


Figure 12: Western-blot analysis of KRAS protein and respective quantification relative to β - Actin in SW480 cell line. Transfection control (Transf C.) condition corresponds to the situation using only LipoGen; siRNA negative conditions (siRNA-) correspond to an unspecific siRNA; and siKRAS conditions correspond to the specific siRNA for KRAS. Forward transfection approach was used. (A) Conditions tested with 2μl of LipoGen; (B) Conditions tested with 4μl of LipoGen. Quantification of KRAS was performed using the *Quantity One* software.

We also tested the reverse transfection method for SW480 cell line transfection using 4μl and 5μl of LipoGen transfect reagent. In this experiment, the siRNA optimization conditions was not performed using the siRNA – AF, instead we directly tested siRNA for KRAS inhibition using 4μl and 5μl of LipoGen and 150nM of siRNA KRAS. In Figure 13, that shown the Western-blot for the expression levels of KRAS protein in all conditions, we could see that the LipoGen reagent lead to KRAS silencing, both in specific siRNA KRAS (siKRAS) conditions and in siRNA negative condition as already observed with the forward RNAi protocol.

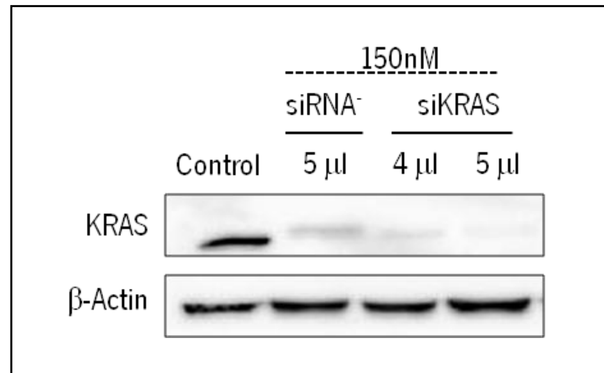


Figure 13: Western-blot analysis of KRAS protein in SW480 cell line. siRNA negative conditions (siRNA-) correspond to an unspecific siRNA; and siKRAS conditions correspond to the specific siRNA for KRAS. The siRNA was used at 150nM and were tested 4µl and 5µl of LipoGen transfection reagent. The reverse transfection approach was used.

In the reverse transfection as well as in the forward transfection, the LipoGen reagent revealed to be an inefficient transfection reagent for SW480 cell line, since it did not allow the specific silencing of the KRAS protein.

3.1.2 RNAi-MAX Transfection Reagent

SW480 cell line was transfected using 4µl, 6µl and 7µl of RNAi-MAX combined with 50nM of siRNA – AF by forward transfection approach. As demonstrated in Figure 14, the siRNA - AF uptake in the conditions mentioned above, was very low, being the highest uptake value of 3.9%. In Figure 14A, was possible to see, by the fluorescent images, that green fluorescence (correspondent to siRNA – AF), is not inside the cytoplasm of SW480 cells. The arrangement of cells, in aggregates, might decreased the transfection efficiency. Moreover, as demonstrated in Figure 14C, the RNAi-MAX transfection reagent presented a higher cellular toxicity (increased cellular death percentage), comparing to untreated cells.

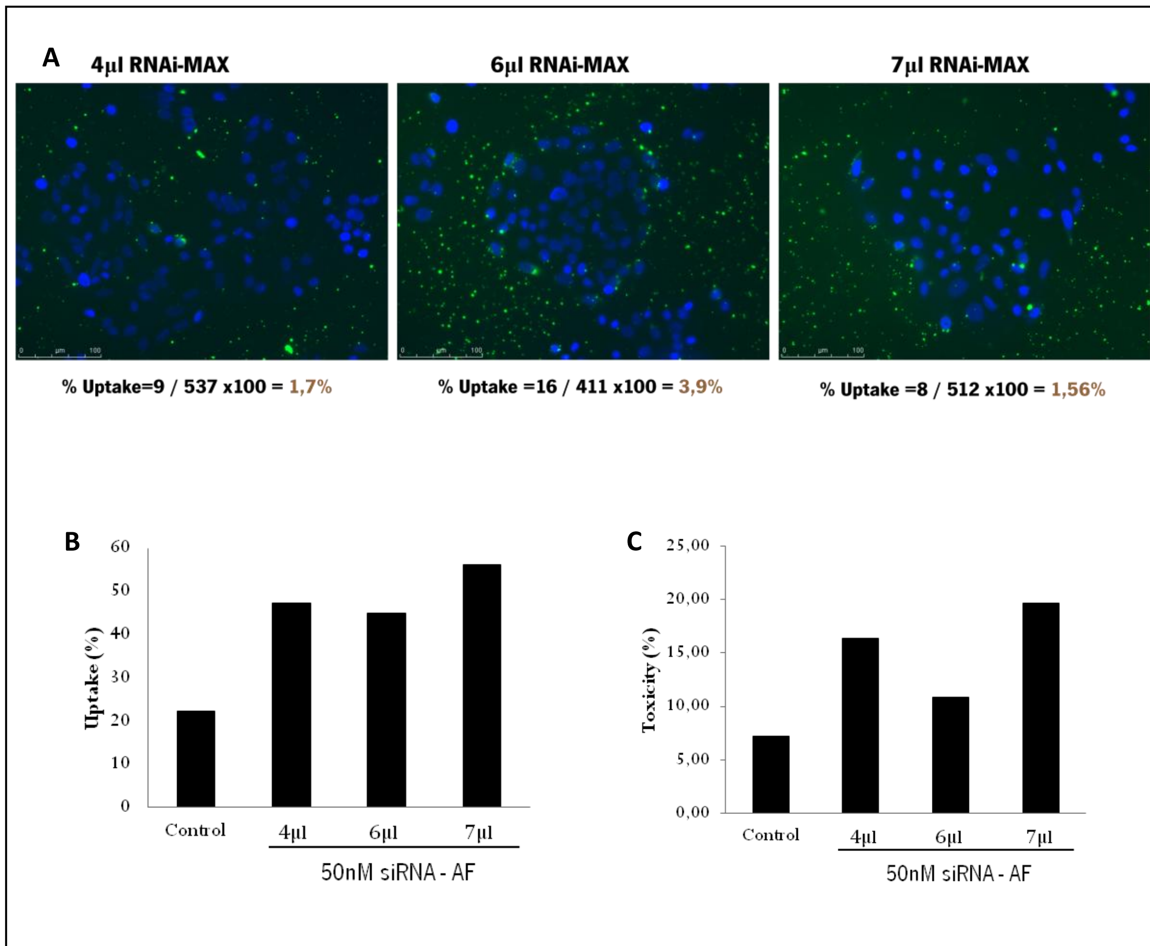


Figure 14: Optimization of siRNA silencing in SW480 cell line using siRNA - AF (50nM) and RNAi-MAX transfection reagent (4µl, 6µl and 7µl) by forward transfection. (A) Fluorescent images of SW480 nuclei stained with DAPI (blue) and siRNA - AF (green). The percentage of uptake was calculated through the formula: [FITC - labelled cells/total of cells * 100] (20x). (B) Cytometry results of siRNA - AF uptake by SW480 cell line; (C) Toxicity of RNAi-MAX transfection reagent measured through trypan blue exclusion assay. The toxicity percentage was calculated through the formula: [(blue cells/total of cells)*100].

We also tested the reverse transfection approach using 3µl and 6µl of RNAi-MAX transfection reagent and we test directly 50nM and 100nM of siRNA for KRAS inhibition (Figure 15). The Western-blot analysis for these conditions showed that KRAS inhibition was almost complete for all conditions tested. Moreover, the siRNA negative condition (6µl of RNAi-MAX and

150nM of siRNA-) did not affect the KRAS expression, proving that RNAi-MAX is a good transfection reagent for SW480 cell line, using the reverse transfection technique.

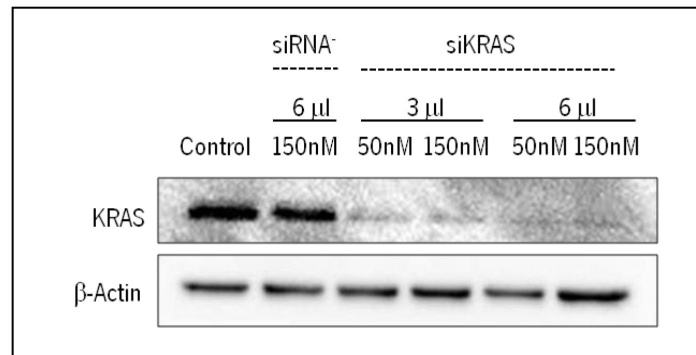


Figure 15: Western-blot analysis of KRAS protein in SW480 cell line. siRNA negative conditions (siRNA-) correspond to an unspecific siRNA; and siKRAS conditions correspond to the specific siRNA for KRAS. The siRNA was used at 50nM and 150nM and were tested 3µl and 6µl of RNAi-MAX transfection reagent. The reverse transfection approach was used.

3.1.3 Lipofectamine 2000 Transfection Reagent

For the optimization of RNA interfering conditions using Lipofectamine 2000 we tested both forward and reverse transfection approaches using the fluorescent siRNA – AF.

For the forward transfection approach we used 100nM of siRNA – AF combined with 4µl and 6µl of Lipofectamine 2000 (Figure 16). According to our results, although the siRNA – AF uptake was higher than in RNAi-MAX treatments, the Lipofectamine 2000 decreased the siRNA – AF uptake comparatively with LipoGen, obtaining 22.4% and 12.3% of uptake with 4µl and 6µl of Lipofectamine 2000, respectively (Figure 16A). The percentages of siRNA - AF uptake were calculated using both, fluorescence microscopy and flow cytometry methods (Figure 16). We saw different uptake results, being higher in the cytometry method (approximately 90 and 80% of uptake). However the criteria followed was the results obtained with the microscopy fluorescent images. Moreover, our results also showed that 4µl and 6µl of Lipofectamine 2000 transfection reagent led to a high toxicity in SW480 cells (Figure 16C). In this way, these results suggested that Lipofectamine 2000 was not the better transfection reagent for the forward transfection in SW480 cells.

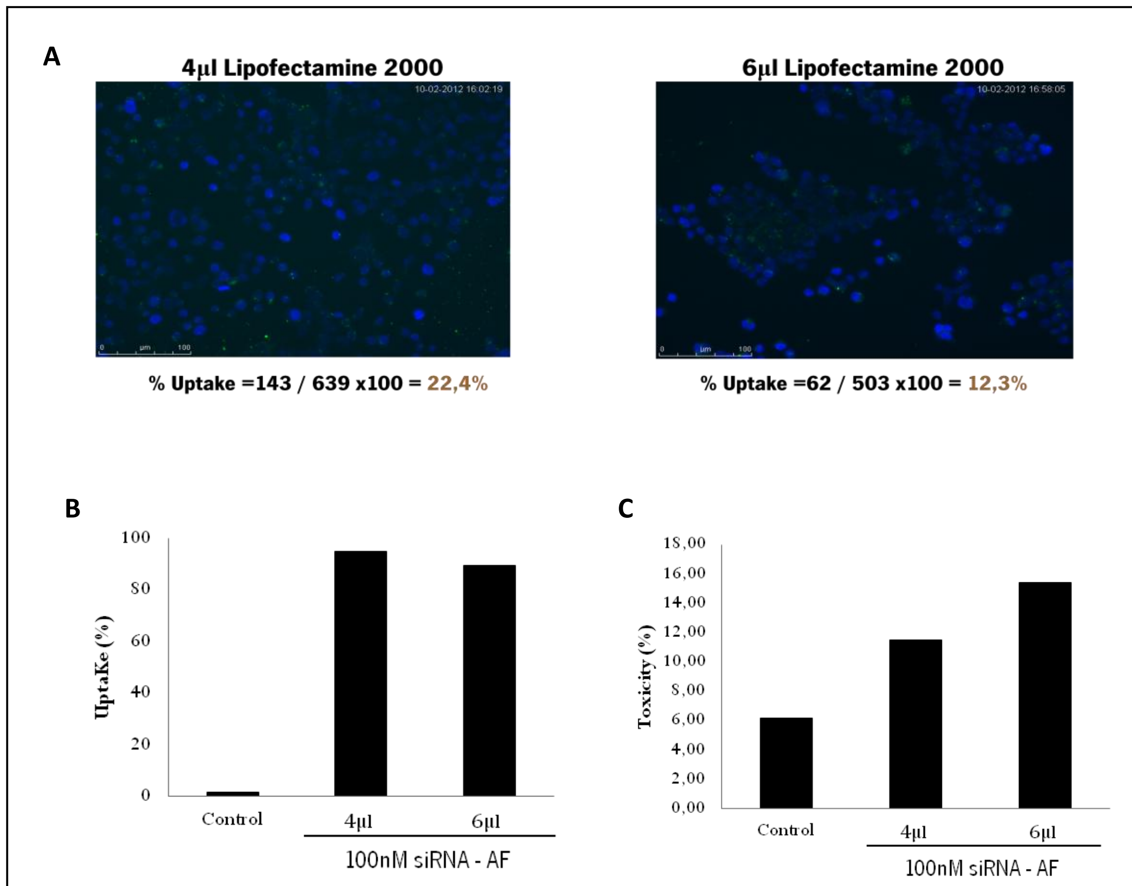


Figure 16: Optimization of siRNA silencing in SW480 cell line using siRNA - AF (100nM) and Lipofectamine 2000 transfection reagent (4µl and 6µl) by forward transfection. (A) Fluorescent images of SW480 nuclei stained with DAPI (blue) and siRNA - AF (green). The percentage of uptake was calculated through the formula: [FITC - labelled cells/total of cells * 100] (20x); (B) Cytometry results of siRNA - AF uptake by SW480 cell line; (C) Toxicity of Lipofectamine 2000 transfection reagent measured through trypan blue exclusion assay. The toxicity percentage was calculated through the formula: [(blue cells/total of cells)*100].

The reverse transfection method using Lipofectamine 2000 transfection reagent was also tested, and for that we used 2µl and 3µl of Lipofectamine 2000 combined with 150nM of siRNA – AF. Moreover, we decided to optimize the appropriate number of plating cells for the reverse transfection method, testing $3x10^5$ SW480 cells/well and $4x10^5$ SW480 cells/well.

In Figure 17 are represented the results of the uptake conditions optimization using $3x10^5$ SW480 cells/well. These results showed high uptake efficiency of siRNA - AF, since we obtained 43.3% and 45% of uptake with 2µl and 3µl of Lipofectamine 2000, respectively. Furthermore we confirmed the effectiveness of siRNA - AF uptake, by the cytometry results (Figure 17B), which demonstrated an uptake efficiency greater than 90% for both conditions. In relation to the toxicity evaluation, we observed that Lipofectamine 2000 was not very toxic and that 3µl of Lipofectamine 2000 was the best condition, with only 4.51% of cellular death (Figure 17C).

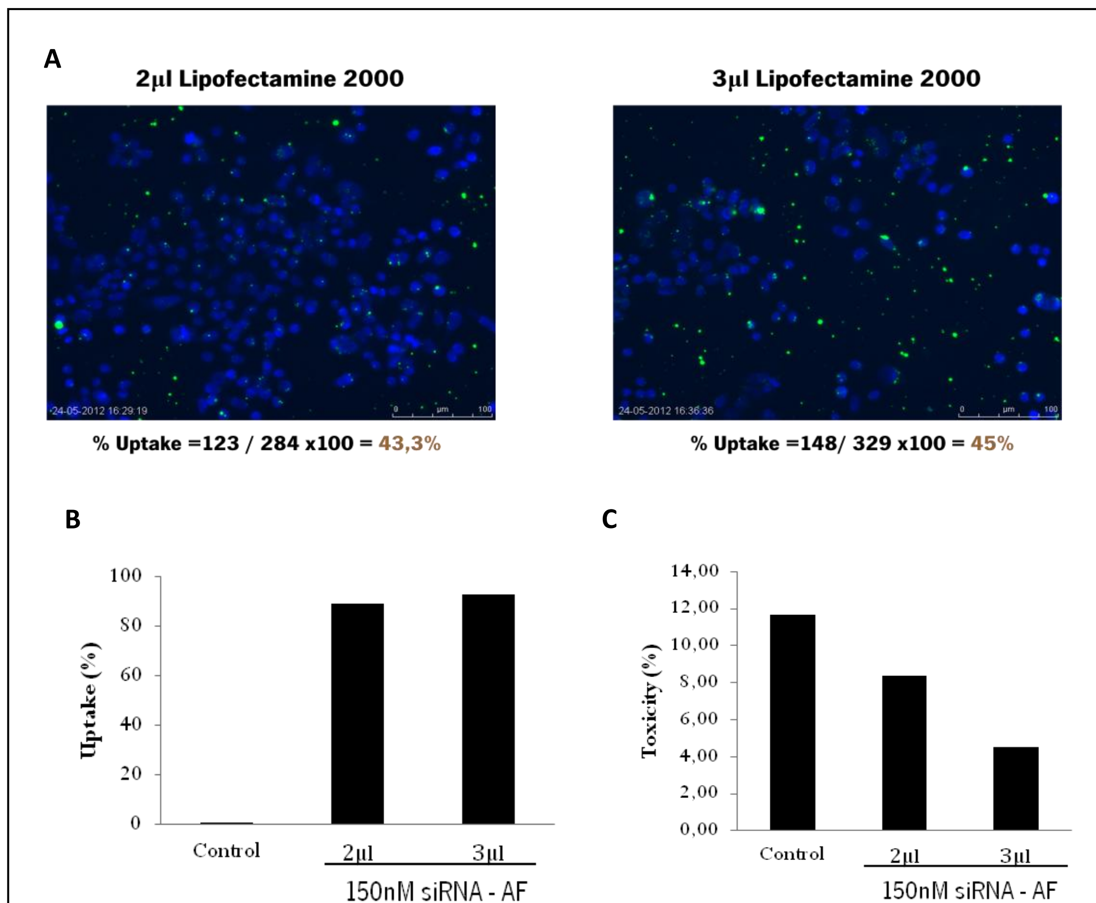


Figure 17: Optimization of siRNA silencing in SW480 cell line at a cellular concentration of $3x10^5$ cells /well. Test of 2µl and 3µl of Lipofectamine 2000 transfection reagent and 150nM of siRNA - AF using the reverse transfection protocol. (A) Fluorescent images of SW480 nuclei stained with DAPI (blue) and siRNA - AF (green). The percentage of uptake was calculated through the formula: [FITC - labelled cells/total of cells * 100] (20x). (B) Cytometry results of siRNA - AF uptake by SW480. (C) Toxicity of Lipofectamine 2000 transfection reagent measured through trypan blue exclusion assay. The toxicity percentage was calculated through the formula: [(blue cells/total of cells)*100].

Additionally, using the same reverse transfection approach, we observed that the siRNA – AF uptake by $4x10^5$ SW480 cells/well was higher compared with the uptake in $3x10^5$ cells/well experiment. These results allowed us to conclude that higher number of SW480 cells contributes to the improvement of the uptake. We also concluded that 3µl of Lipofectamine 2000 was the best uptake condition, since we obtained more than 50% of siRNA - AF uptake, 58.7%, and the lowest percentage of cellular death, 4.46% (Figure 18A and 18C). Regarding the flow cytometry results we also obtained a great uptake efficacy for 3µl of Lipofectamine 2000 condition (Figure 18C).

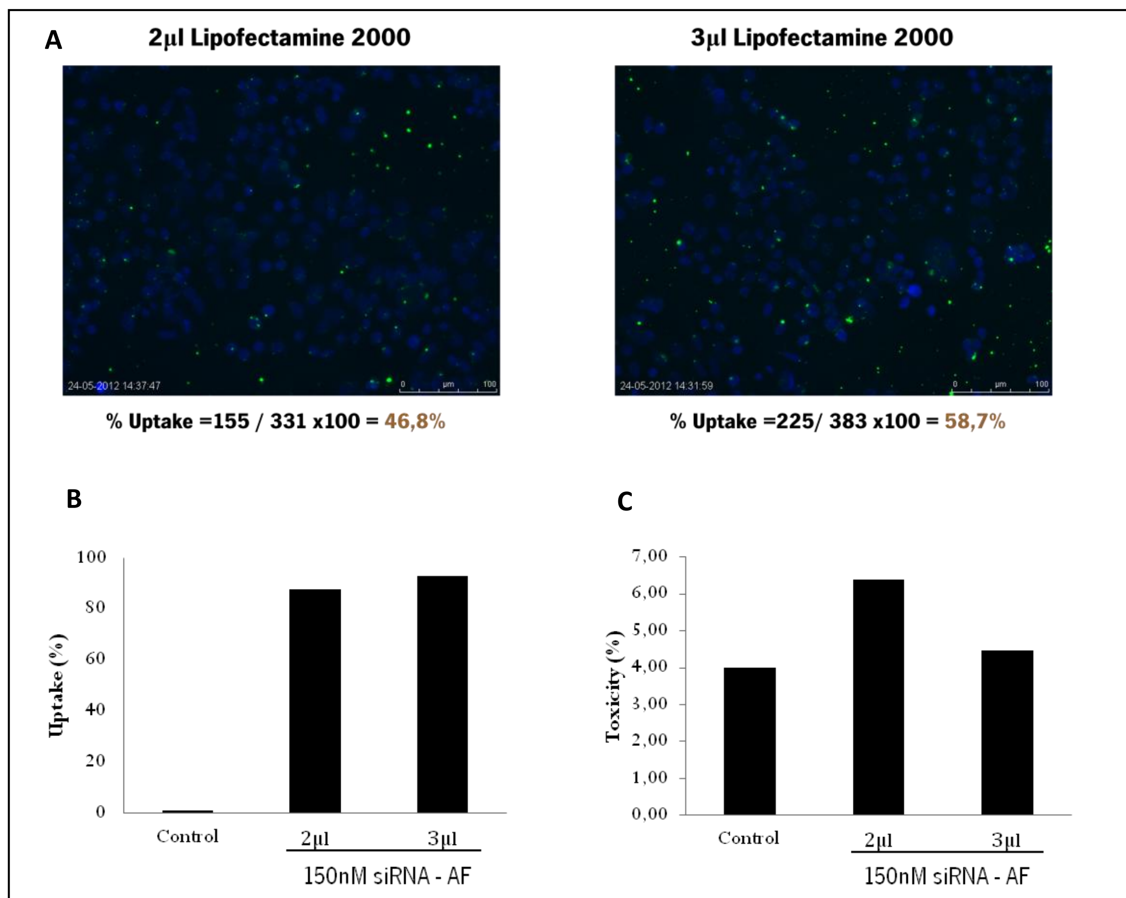


Figure 18: Optimization of siRNA silencing in SW480 cell line at a cellular concentration of $4x10^5$ cells /well. Test of 2µl and 3µl of Lipofectamine 2000 transfection reagent and 150nM of siRNA - AF using the reverse transfection protocol. (A) Fluorescent images of SW480 nuclei stained with DAPI (blue) and siRNA – AF (green). The percentage of uptake was calculated through the formula: [FITC - labelled cells/total of cells * 100] (20x); (B) Cytometer results of siRNA - AF uptake by SW480 cell line; (C) Toxicity of Lipofectamine 2000 transfection reagent measured through trypan blue exclusion assay. The toxicity percentage was calculated through the formula: [(blue cells/total of cells)*100].

Take into account the previous results we evaluated the specific siRNA for KRAS transfection using the higher cellular concentration of SW480 cell line (4x10^{5} cells/well). In Figure 19 is represented the Western-blot for KRAS protein and its normalization relative to β-Actin protein in all conditions (150nM of siRNA combined with 2µl and 3µl of Lipofectamine 2000). Both conditions (2µl of Lipofectamine 2000 and 3µl of Lipofectamine 2000) showed a considerable decrease of KRAS expression in the siRNA KRAS conditions, which demonstrated the efficient silencing of this protein. Furthermore, no alterations in KRAS expression on siRNA negative (siRNA^{-}) condition were observed.

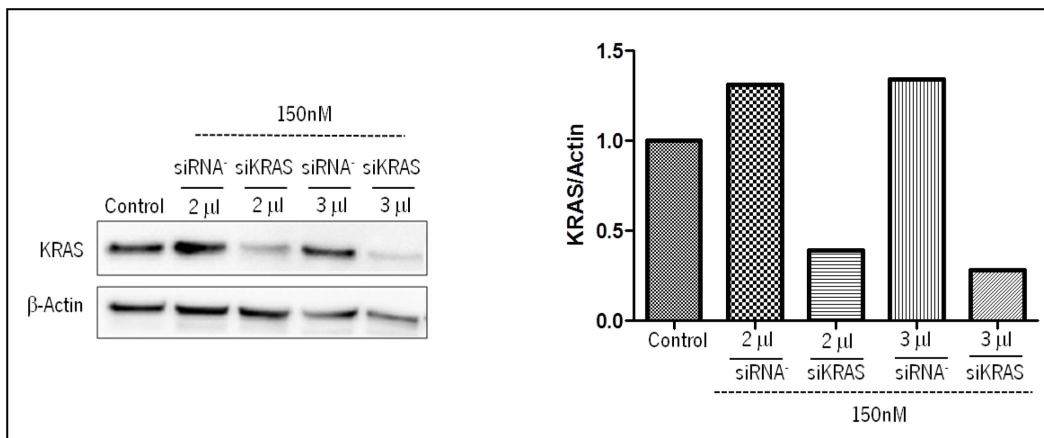


Figure 19: Western-blot analysis of KRAS protein and respective quantification relative to β-Actin in SW480 cell line (4x10^{5} cells/well). siRNA negative conditions (siRNA^{-}) correspond to an unspecific siRNA; and siKRAS conditions correspond to the specific siRNA for KRAS. The siRNA was used at 150nM and were tested 2µl and 3µl of Lipofectamine 2000 transfection reagent. The reverse transfection approach was used. Quantification of KRAS was performed using the *Quantity One* software.

Summing up, after all the optimizations using different transfection reagents and different transfection approaches (forward and reverse) we decided to use SW480 cells at a concentration of 4x10^{5} cells/well and the RNAi reverse transfection method. With this protocol cells were plated directly with the siRNA silencing cocktail, increasing the cell membrane contact with the siRNA and thereby increasing their uptake by SW480 cells.

Both RNAi-MAX and Lipofectamine 2000 transfection reagents gave good results for siRNA transfection in SW480 cell line, but we decided to select Lipofectamine 2000 for all our

future experiments. In fact, we decided to use 150nM of siRNA KRAS and 3μl of Lipofectamine 2000 for further silencing KRAS studies.

3.2 Inhibition of KRAS expression in SW480 cells using RNA interference

3.2.1 Effect of KRAS inhibition in autophagy of SW480 cells

SW480 cell line was selected to study the role of KRAS in autophagic process on CRC cells. For this purpose, we inhibited KRAS expression by RNAi, according to the parameters previously optimized, induced autophagy by serum deprivation and monitored autophagic markers. We also subjected cells to Bafilomycin A1 treatment as a control for the study of some autophagic proteins. In Figure 20 was possible to see that SW480 cells phenotype after all the treatments (KRAS silencing, induction of autophagy with HBSS and treatment with Bafilomycin A1) remained very similar to the control.

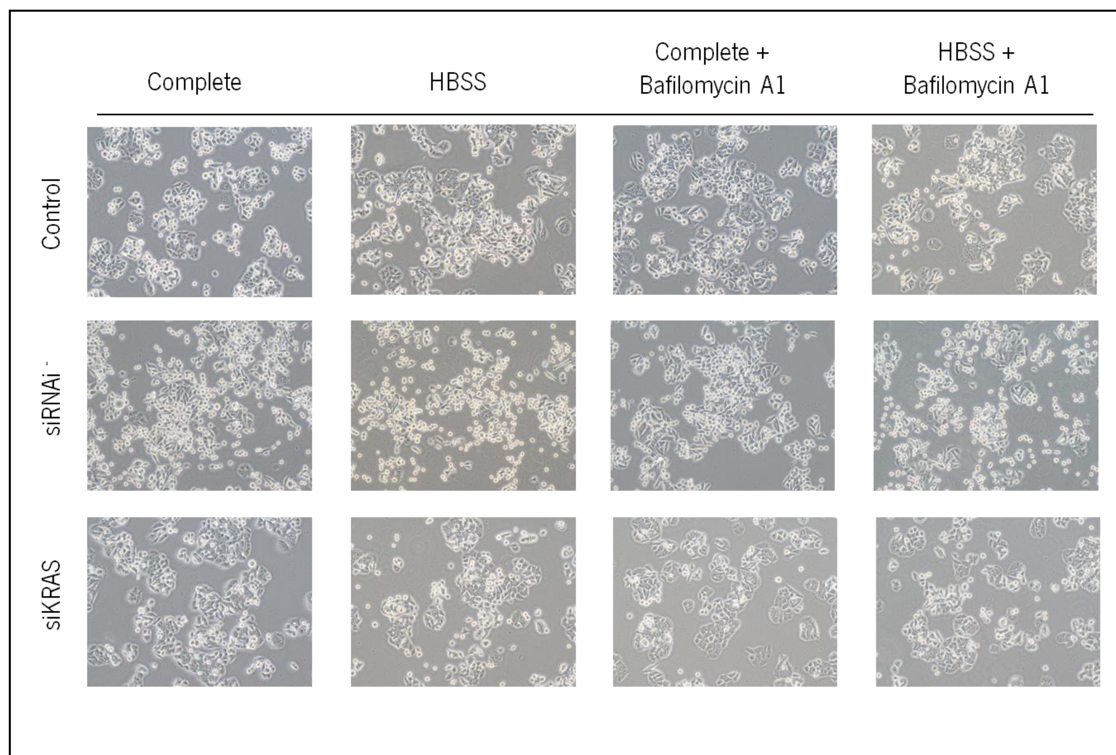


Figure 20: SW480 cell line phenotype for all treatments (KRAS silencing, induction of autophagy with HBSS and treatment with Bafilomycin A1). siRNA negative (siRNA-) correspond to an unspecific siRNA; siKRAS correspond to the specific siRNA for KRAS silencing. Images were obtained in a phase contrast microscope (10x).

The effect of KRAS silencing in autophagy was studied by the analysis of different proteins related to distinct steps of this process: Beclin 1, Atg5, LC3 (I/II) and p62. Beclin 1, combined with PI3 kinase class III, is important for phagophore expansion/nucleation. Atg5 and pro-LC3 are recruited to phagophore membrane elongation. Pro-LC3 is processed by Atg4 and become LC3 I, that is then conjugated with phosphatidylethanolamine to become LC3 II, present in fusion of autophagosome and lysosome. Finally, p62 is a protein that binds to LC3 II and is required for the formation and degradation of polyubiquitin-containing bodies by autophagy.

The induction of starvation by incubation in HBSS medium led to a decrease on Beclin 1 expression in all conditions tested (control, siRNA- and siKRAS) (Figure 21). Upon KRAS inhibition, Beclin 1 protein expression was lower comparing to the control in HBSS treatment.

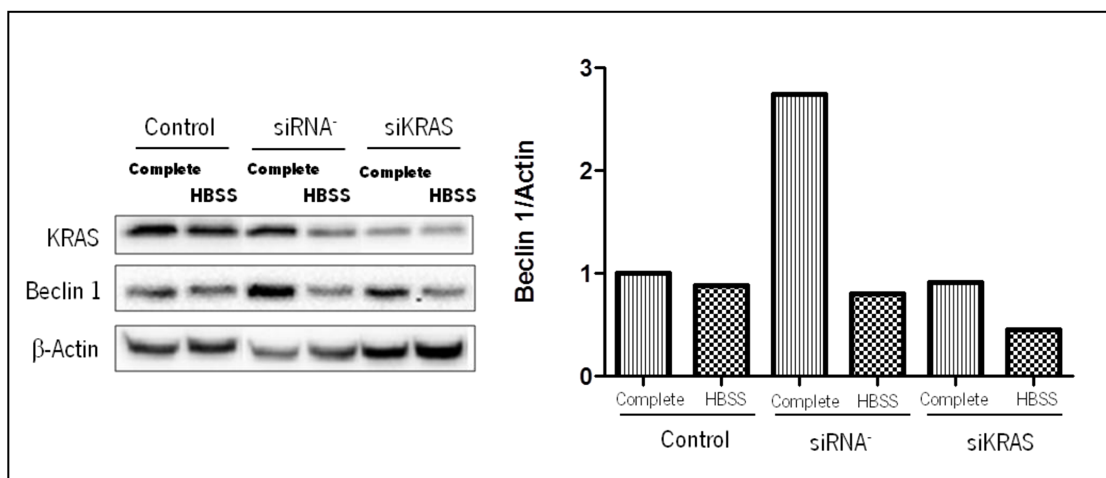


Figure 21: Western-blot analysis of Beclin 1 protein and respective normalization relative to β-Actin in SW480 cell line. Cells were incubated 48 hours in RPMI complete medium and then switched to HBSS during 6 hours or maintained in complete medium. siRNA negative conditions (siRNA-) correspond to an unspecific siRNA; siKRAS conditions correspond to the specific siRNA for KRAS. β-Actin levels were used as an internal control to show equal protein loading. Quantification of Beclin 1 was performed using the *Quantity One* software.

LC3 is an important autophagic protein, as aforementioned. Pro-LC3 is processed to LC3 I and conjugated to a lipid, phosphatidyl-thanolamine, forming LC3 II (or LC3-PE). Nevertheless, as LC3 II is degraded in the autolysosome, the amount of this protein would not provide accurate information about alterations on autophagy process. Therefore, Bafilomycin A1 was used to

inhibit the fusion between autophagosomes and lysosomes and consequently to avoid LC3 II degradation, allowing its accumulation. In SW480 cells treated with HBSS + Bafilomycin A1 was possible to see a greater accumulation of LC3 II when compared with complete medium + Bafilomycin A1 situations (Figure 22). Moreover, expression levels of LC3 II on siRNA KRAS conditions were lower compared with control, suggesting that KRAS protein plays an important role in autophagy regulation. The differences on LC3 II expression, between siRNA negative control and siRNA KRAS conditions are not statistically significant in the two experiments performed.

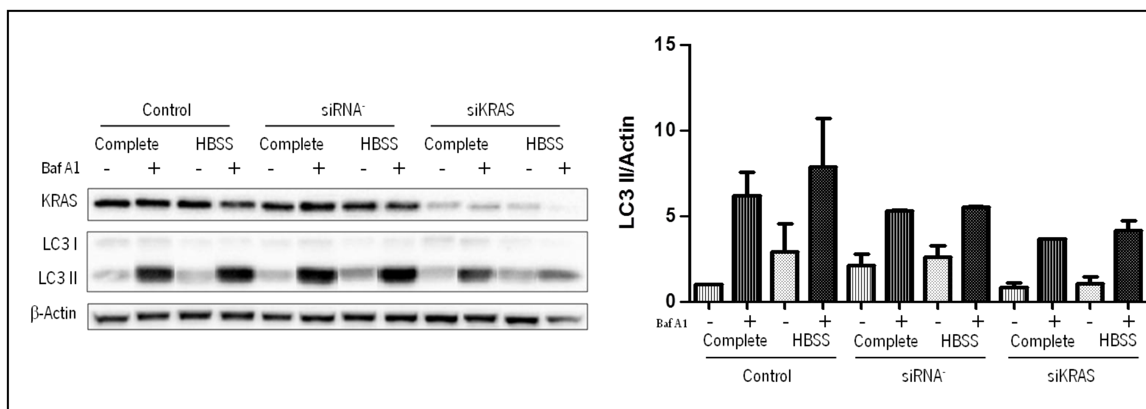


Figure 22: Western-blot analysis of LC3 protein and respective normalization relative to β-Actin in SW480 cell line. Cells were incubated 48 hours in RPMI complete medium and then switched to HBSS during 6 hours or maintained in complete medium with or without 20nM of Bafilomycin A1 (Baf A1). siRNA negative conditions (siRNA-) correspond to an unspecific siRNA; siKRAS conditions correspond to the specific siRNA for KRAS. β-Actin levels were used as an internal control to show equal protein loading. Data are shown as means +/- SEM (n=2). Quantification of LC3 was performed using the *Quantity One* software.

Atg5 protein, involved in initials steps of autophagy, namely in phagophore membrane elongation, was another protein analyzed. The results of Atg5 levels obtained by Western-blot analysis were concordant with the results of LC3 II analysis. In SW480 cells harbouring *KRAS*^{G12V} mutation, autophagy induction by HBSS increased Atg5 protein expression. Moreover, we could observe a decrease in Atg5 expression after KRAS inhibition (Figure 23) suggesting an important role of KRAS protein in the autophagic process. Although there was a strong trend for a decrease,

the differences on Atg5 expression, between siRNA negative control and siRNA KRAS conditions were not statistically significant.

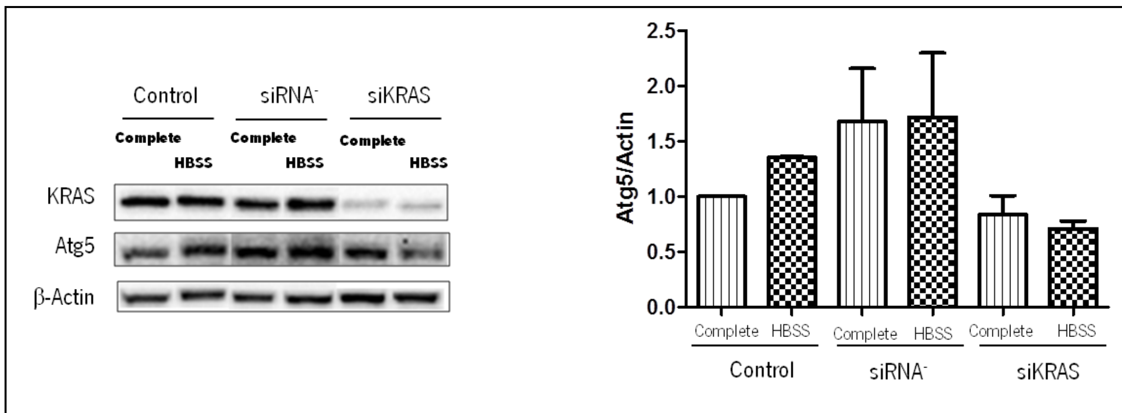


Figure 23: Western-blot analysis of Atg5 protein and respective normalization relative to β-Actin in SW480 cell line. Cells were incubated 48 hours in RPMI complete medium and then switched to HBSS during 6h or maintained in complete medium. siRNA negative conditions ($siRNA_{-}$) correspond to an unspecific siRNA; siKRAS conditions correspond to the specific siRNA for KRAS. β-Actin levels were used as an internal control to show equal protein loading. Data are shown as means +/- SEM (n=2). Quantification of Atg5 was performed using the *Quantity One* software.

Regarding the p62 protein, which is degraded during autophagy, like LC3 II, the properly study of this protein was also done with Bafilomycin A1 treatment. Western-blot analysis of p62 protein presented in Figure 24, demonstrated that the levels of p62 protein were very high (Complete medium + Baf. A1), compared to the conditions of starvation induction (HBSS + Baf. A1). Although the visible increase of p62 protein expression, in control and $siRNA_{-}$ Bafilomycin A1 positive conditions, p62 protein decreased after KRAS silencing. Moreover, after HBSS treatment, the p62 accumulation in KRAS inhibited condition decreased compared to control situation. These facts suggest that inhibition of KRAS regulates p62 and consequently the autophagic process.

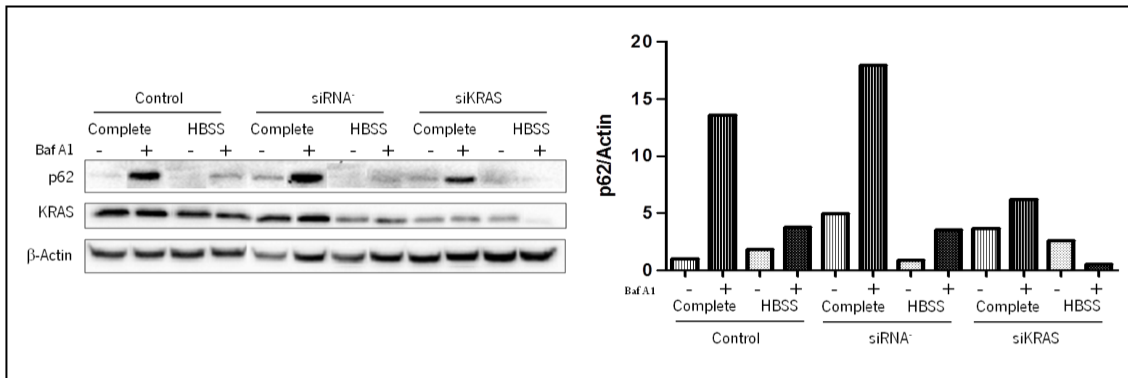


Figure 24: Western-blot analysis of p62 protein and respective normalization relative to β-Actin in SW480 cell line. Cells were incubated 48 hours in RPMI complete medium and then switched to HBSS during 6 hours or maintained in complete medium with or without 20nM of Bafilomycin A1 (Baf A1). siRNA negative conditions (siRNA-) correspond to an unspecific siRNA; siKRAS conditions correspond to the specific siRNA for KRAS. β-Actin levels were used as an internal control to show equal protein loading. Quantification of p62 was performed using the *Quantity One* software.

3.2.2 Effect of KRAS inhibition in ERK signalling of SW480 cells

The results previously obtained showed that KRAS regulates autophagy although the signalling pathways implicated are not known. Thus, we decided to study if inhibition of KRAS interfered with the levels of the RAS – RAF – MEK – ERK signalling pathway. We studied the levels of ERK activation after KRAS silencing conditions and upon autophagy induction by HBSS. The results obtained are presented in Figure 25.

Our preliminary results showed that induction of autophagy, by starvation with HBSS, may have lead to an activation of ERK proteins, increasing pERK expression. We could observe that starvation induced by HBSS after inhibition of KRAS led to a decrease in the levels of both pERK and of tERK. We performed an analysis of pERK/β-Actin (without influence from tERK) which allowed us to verify that upon KRAS silencing there was a decrease in pERK protein expression. Moreover, we saw that this decrease was even higher under starvation conditions (HBSS treatment), despite the decrease of the tERK. All together these results suggest that KRAS protein might regulates autophagy process through RAS – RAF – MEK – ERK signalling pathway. However, it is important to notice that these results were from a single experience and needed to be confirmed.

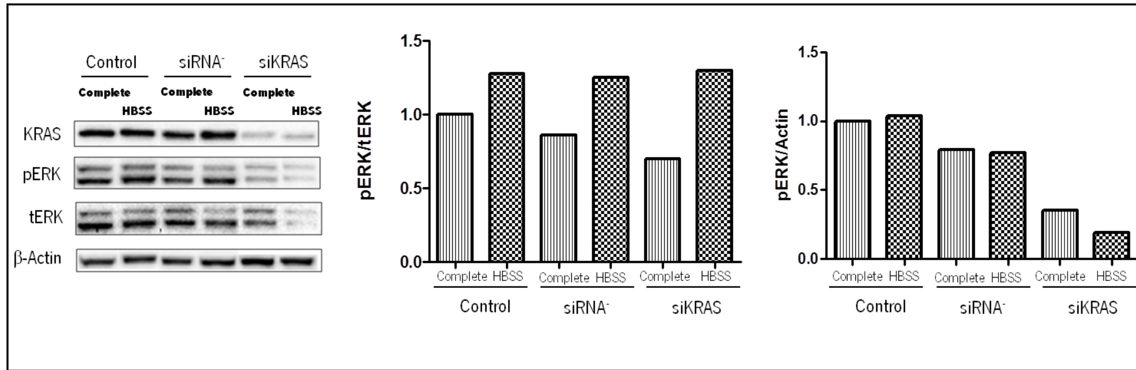


Figure 25: Western-blot analysis of pERK protein and respective quantification relative to tERK and β-Actin in SW480 cell line Cells were incubated 48 hours in RPMI complete medium and then switched to HBSS during 6h or maintained in complete medium. siRNA negative conditions ($siRNA^-$) correspond to an unspecific siRNA; siKRAS conditions correspond to the specific siRNA for KRAS. β-Actin levels were used as an internal control to show equal protein loading. Quantification of ERK was performed using the *Quantity One* software.

3.2.3 Effect of KRAS inhibition in the cell cycle and cell death of SW480 cells

The effect of KRAS inhibition in proliferation and cellular death in SW480 cell line was performed by flow cytometry and trypan blue exclusion assay. The results are presented in Figure 26. The cell cycle analysis of SW480 cell line expressing KRAS protein (control and $siRNA^-$ conditions) showed that most cells were in the initial phases of cell cycle - interphase (G1, S and G2 phases). In fact, KRAS- expressed SW480 cells presented a high number of proliferative cells (in S-Phase), 32.78% and 32.03% in control and $siRNA^-$ conditions, respectively (Figure 26A and 26B). Moreover, we observed a very small percentage of death cells (sub-G1 phase) in these conditions, only 7.9% and 6.01%, respectively. However, after KRAS inhibition (siRNA KRAS condition) the percentage of cells in S phase decreases to 26.58% and the levels of cellular death, represented by the sub-G1 peak, increased, for 13.69%. These results suggested the importance of KRAS protein expression on the cellular proliferation and survival of CRC cells harbouring *KRAS*^{G12V} mutation.

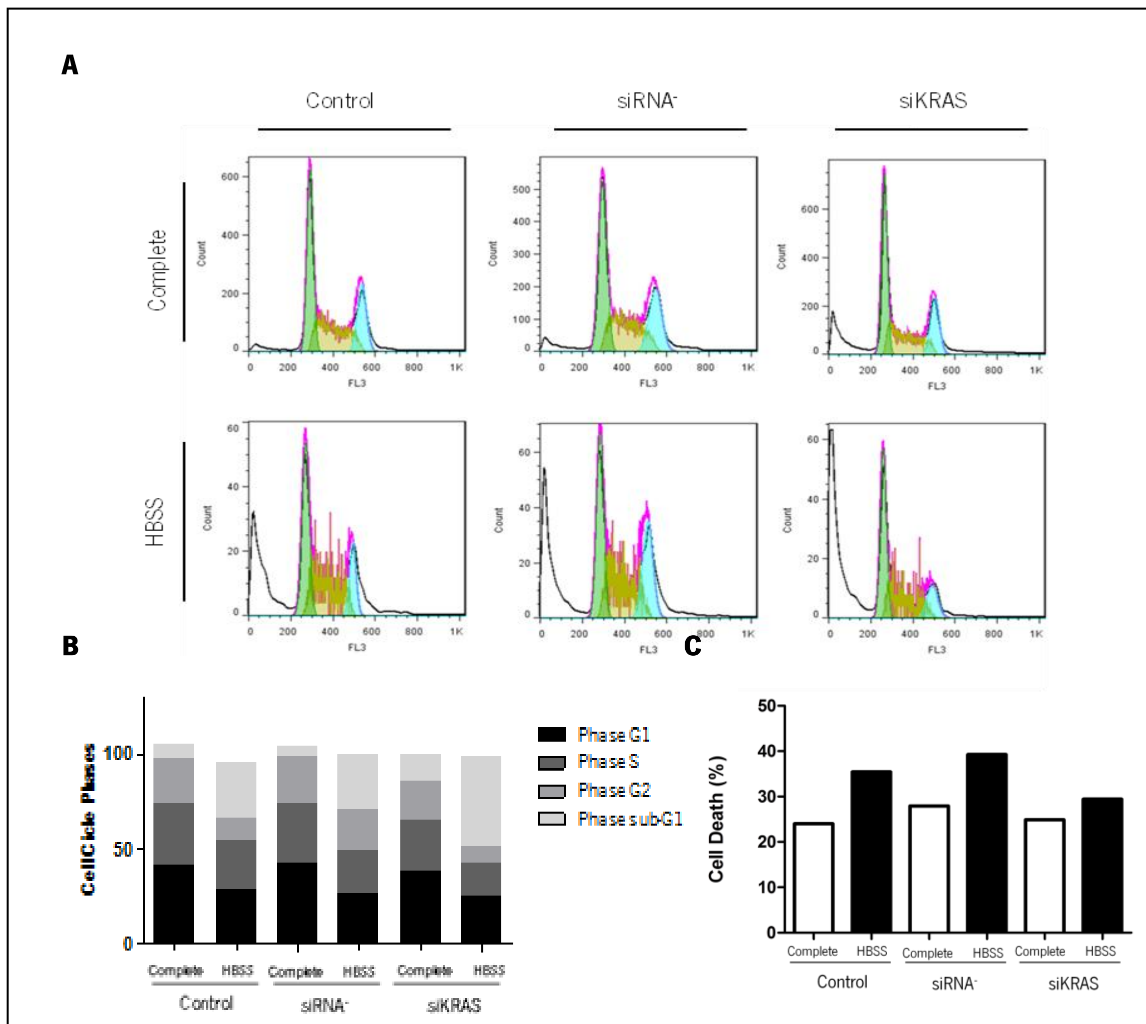


Figure 26: Role of KRAS protein on SW480 cell line survival. Cells were incubated 48 hours in RPMI complete medium and then switched to HBSS medium during 6 hours, or maintained in complete medium. siRNA negative conditions (siRNA-) correspond to an unspecific siRNA; siKRAS conditions correspond to the specific siRNA for KRAS. (A) Histograms obtained from cell cycle analyse that represent the number of cells (count) *vs* DNA content (FL3). Sub-G1, G1, S and G2 phases are represented in blank, green, yellow and blue colours, respectively. Results were treated with FlowJo 7.6 program; (B) Percentages of cells distribution on different cell cycle phases; (C) Cell death percentage obtained through the trypan blue exclusion assay.

After 6 hours of starvation induction with HBSS medium, the levels of cellular death (sub-G1 phase) increased in all the conditions, compared to cells maintained in complete medium (Figure 26). We could observe a decrease on S-phase and an increase of cellular death namely

after KRAS inhibition. The values of cellular death reached 46.96% compared with 28.94% in negative control siRNA and the values of S-phase cells decreased to 16.92% comparing with 23.61% in siRNA$^-$ condition. These results suggest that under conditions of nutrients deprivation, the ability of SW480 cells to survive is dependent on KRAS since the survival decreased upon KRAS inhibition. Altogether these results suggest that KRAS might provide survival of CRC cells under starvation conditions.

Analyzing the results obtained by the trypan blue exclusion assay (Figure 26C) was possible to observe that the levels of cellular death increased after incubation in HBSS, although in siRNA KRAS conditions there was a minor increase of death contrary to the results obtained by flow cytometry analysis (Figure 26A and 26B). We have to take into account that these results are only from a single experience and thus we need to repeat them to better clarify the results obtained.

3.2.4 Effect of autophagy inhibition in cell survival of SW480 cells

The role of autophagy in cellular survival of SW480 cell line harbouring *KRAS*^{G12V} mutation was study by flow cytometry, with cell cycle analysis, and by the trypan blue exclusion assay (Figure 27). For that propose, we analysed the effect of inhibition of autophagy by Bafilomycin A1, which is able to stop the autophagic process once it blocks the fusion of autophagosomes with lysosomes through vacuolar ATPase inhibition. Inhibition of autophagy in SW480 cells was performed by Bafilomycin A1 treatment (6h, 24h and 48h), before or after induction of autophagy by HBSS treatment.

Through the analysis of cell cycle results we saw that inhibition of autophagy in SW480 cells had a great impact in their ability to survive. The results showed that inhibition of autophagy by Bafilomycin A1 during 48 hours led to an increase in sub-G1 phase (75.17%) contrarily to the condition without autophagy inhibition (1.06%) where most of the cells were proliferating. Similar results were obtained upon an HBSS pre-treatment during 6 hours (autophagy inducer), in which autophagy inhibition by Bafilomycin A1 also led to cell death, being the percentage of cells in sub-G1 phase lower (67.51%), that suggests a possible protective role of starvation induced by HBSS in SW480 CRC cells harbouring *KRAS*^{G12V} mutation (Figures 27A and 27B).

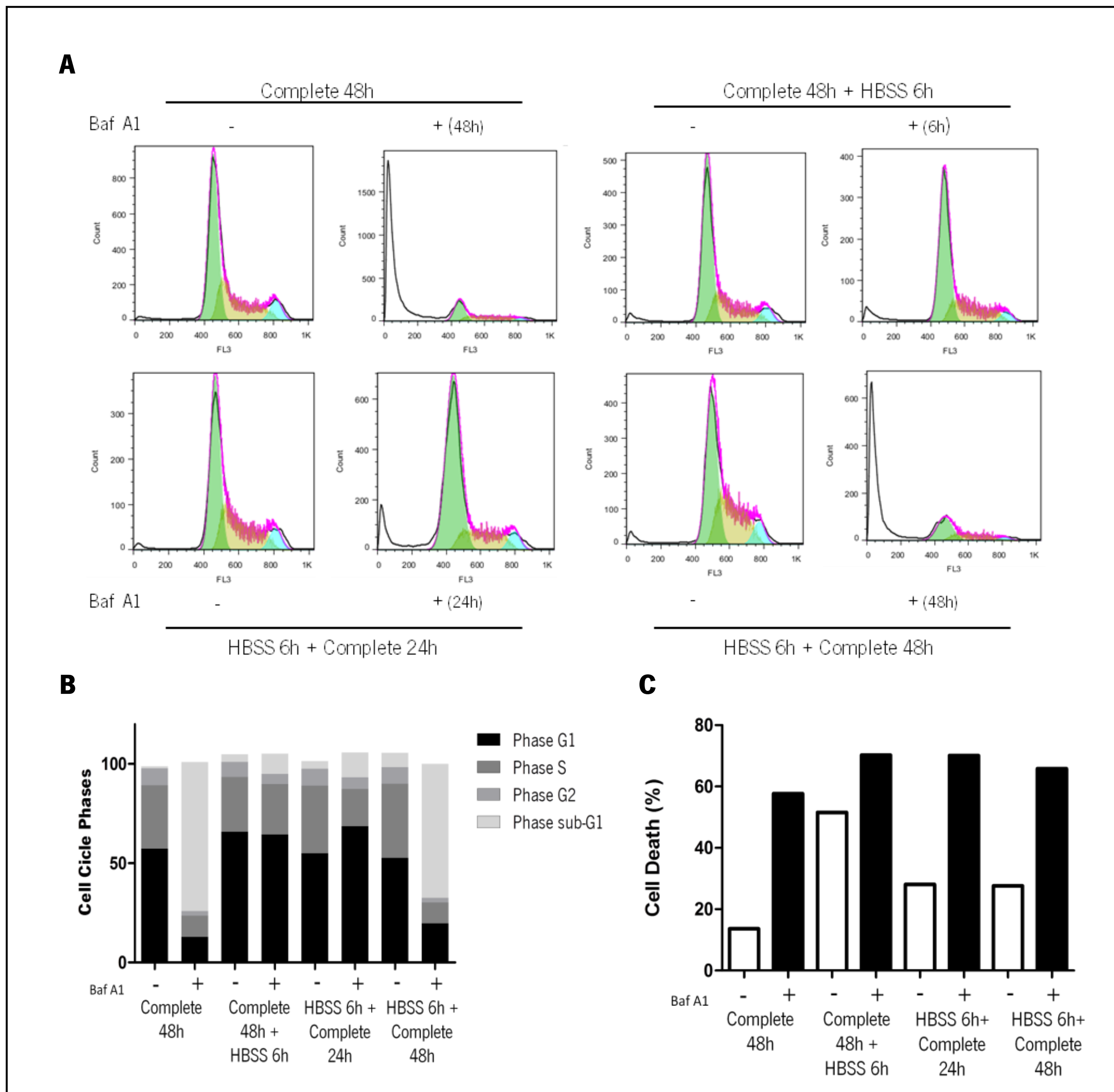


Figure 27: Role of autophagy in SW480 cell line survival. Cells were subjected to autophagy induction (6 hours of HBSS) and treated with an autophagic inhibitor, Bafilomycin A1 (Baf A1) during 6h, 24h and 48h. Complete 48h condition correspond to the control situation; In complete 48h + HBSS 6h condition SW480 cells grown in complete medium and after 48 hours, were submitted to an HBSS treatment during 6 hours with or without Baf A1. In HBSS 6h + Complete 24h and in HBSS 6h + Complete 48h conditions cells were submitted to a pre-treatment with HBSS for 6 hours and re-incubated with complete RPMI medium for 24h or 48h, respectively with or without Baf A1. (A) Histograms obtained from cell cycle analyse that represent the number of cells (count) *vs* DNA content (FL3). Sub-G1, G1, S and G2 phases are represented in blank, green, yellow and blue colours, respectively. Results were treated with FlowJo 7.6 program. (B) Percentages of cells distribution on different cell cycle phases. (C) Cell death percentage obtained through the trypan blue exclusion assay: [(blue cells/total of cells)*100]).

Short times of autophagy inhibition (Bafilomycin A1), such as 6 hours and 24 hours, did not have a high effect on SW480 cell survival, although the sub-G1 values increased comparing with control cells (10.27% and 12.56% for 6h and 24h respectively) (Figures 27A and 27B).

Cell death analysis was also performed by trypan blue exclusion assay (Figure 27C). The results obtained with this technique demonstrated that inhibition of the autophagic process by Bafilomycin A1 (black bars on Figure 27C) led to an increase in cellular death.

Altogether these results seem to demonstrate the importance of autophagy in SW480 ability to survive, although more studies are needed.

3.3 The cellular effect of KRAS mutations in HEK 293 cell line

3.3.1 Effect of KRAS mutations in autophagy induction in HEK 293 cells

To study the effect of *KRAS* mutations on autophagic induction, we used stable cell lines derived from HEK 293 cells, expressing the Flag-KRAS^{m} (Flag-KRAS^{G13D}, Flag-KRAS^{G12D}, and Flag-KRAS^{G12V}) as well as wild-type Flag-KRAS (Flag-KRAS^{WT}). The phenotype of transformed (*Flag-KRAS*^{G13D}*, Flag-KRAS*^{G12D}, *Flag-KRAS*^{G12V} and *Flag-KRAS*^{WT}) and parental HEK 293 cell line (at 18 passage) is represented in Figure 28.

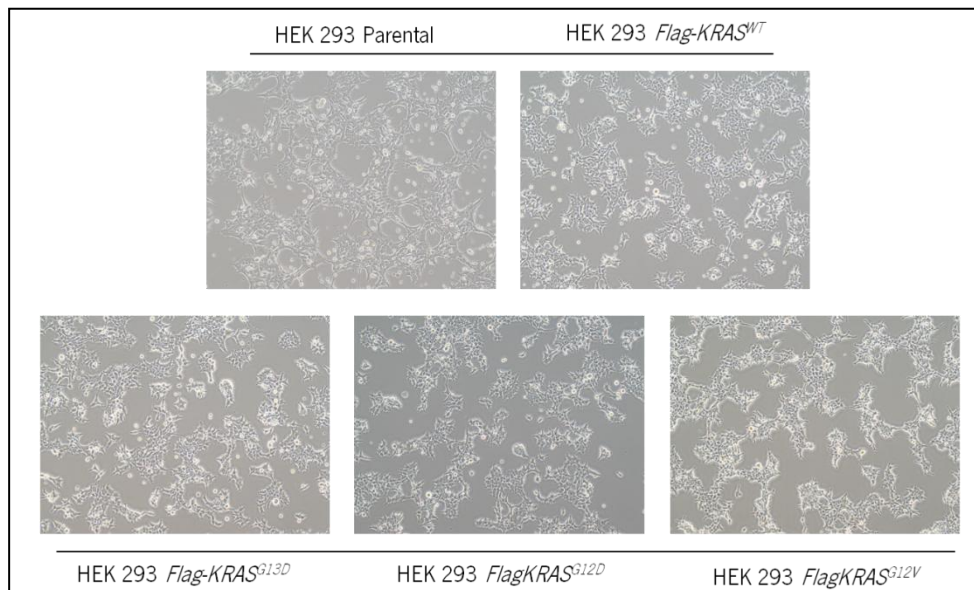


Figure 28: HEK 293 cell lines phenotype. HEK 293 Parental and transformed HEK 293 cell lines: (Flag-KRAS^{WT}, Flag-KRAS^{G13D}, Flag-KRAS^{G12D}, and Flag-KRAS^{G12V}). Images were obtained in a phase contrast microscope (10x).

After performing the experiments using HEK 293 cell lines, we first confirmed the KRAS^{m} expression in HEK 293 cells by Western-blot analysis using an antibody against Flag-KRAS (Figure 29).

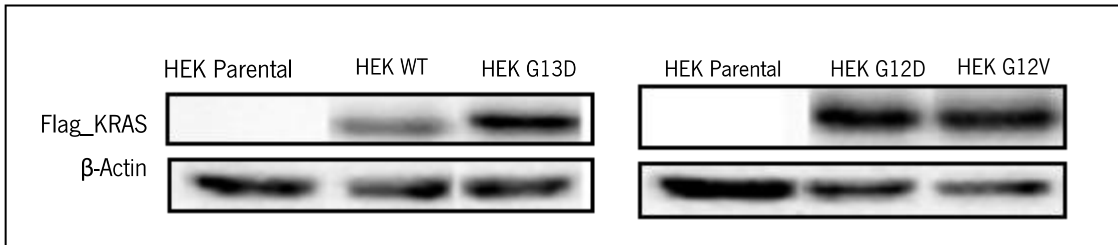


Figure 29: Western-blot analysis of Flag–KRAS protein in HEK 293 cell lines at 14 passage: (Parental, *KRAS*^{WT}, *KRAS*^{G13D}, *KRAS*^{G12D} and *KRAS*^{G12V}). β-Actin levels were used as an internal control to show equal protein loading.

Then, we assessed the effect of *KRAS* mutations in autophagy induction analyzing two proteins involved in autophagy process, Beclin 1 and LC3 (I/II). The results of Beclin 1 expression are represented in Figure 30.

Our results showed that the basal levels of Beclin 1 in HEK 293 cell lines are high, except in HEK 293 cell line with *KRAS*^{G12V} mutation. In response to starvation (HBSS), the expression of Beclin 1 in parental HEK 293 decreased, contrarily to the HEK 293 cell line with *KRAS*^{WT} that presents a small increase of this autophagic protein compared to the complete medium condition. HEK 293 cell lines harbouring the different *KRAS* mutations (*KRAS*^{G13D}, *KRAS*^{G12D}, and *KRAS*^{G12V}) also decreased Beclin 1 expression upon HBSS treatment compared with complete medium. Only in HEK 293 cell lines expressing KRAS^{WT} and KRAS^{G13D} the Beclin 1 expression was increased, upon autophagy induction comparing to parental HEK 293 cell line.

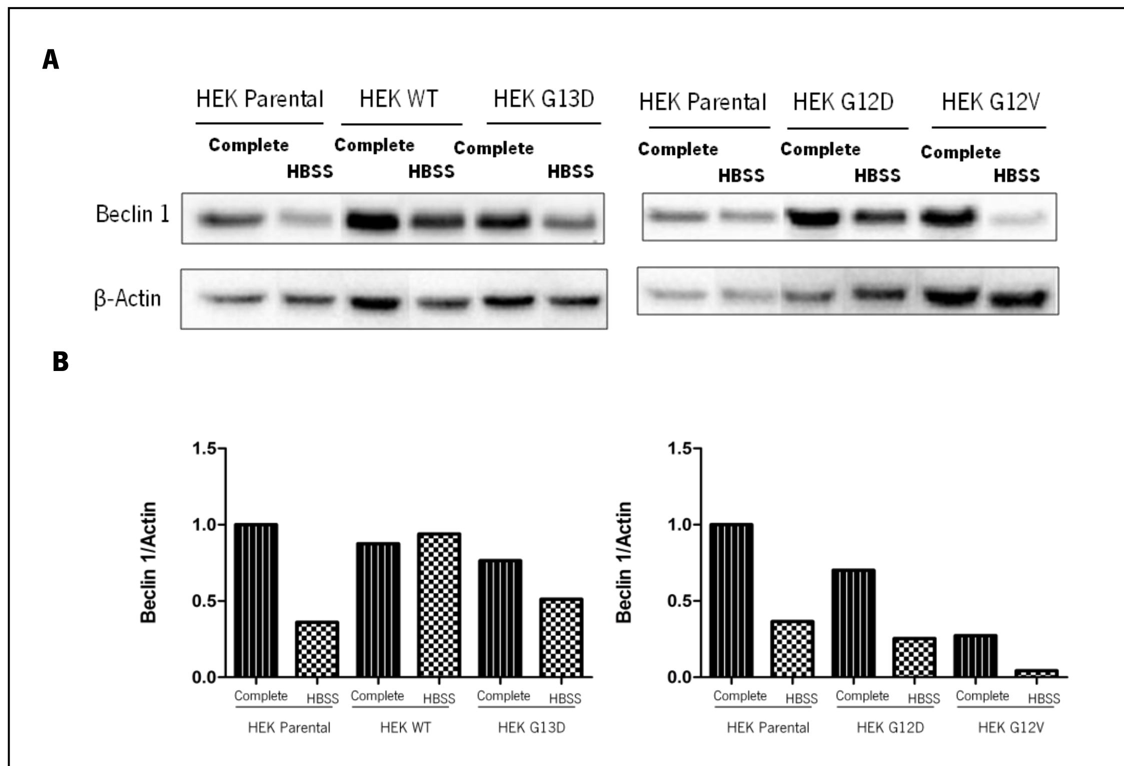


Figure 30: Expression of Beclin 1 protein by HEK 293 cell lines (Parental, *KRAS*^{WT}, *KRAS*^{G13D}, *KRAS*^{G12D}, and *KRAS*^{G12V}) in response to autophagy induction with 6 hours of HBSS. (A) Western-blot analysis of Beclin 1 protein in HEK 293 cell lines. β-Actin levels were used as an internal loading control; (B) Quantification of Beclin 1 protein was performed using the *Quantity One* software.

We also assessed autophagy induction by monitoring the amount of LC3 I/II. The expression of LC3 II protein by HEK 293 cell lines (Parental, *KRAS*^{WT}, *KRAS*^{G13D}, *KRAS*^{G12D}, and *KRAS*^{G12V}) is demonstrated in Figure 31. For all HEK 293 cell lines, the use of Bafilomycin A1 (Baf A1) in complete medium or in HBSS (autophagy inducer medium) allows the LC3 II accumulation, compared to conditions without Bafilomycin A1. In basal condition all HEK 293 cell lines expressing exogenous KRAS (KRAS^{WT}, KRAS^{G13D}, KRAS^{G12D}, and KRAS^{G12V}) led to an increase on the levels of LC3 II expression consistent with an increase in basal levels of autophagy by KRAS.

With the exception of parental HEK 293 cell line, in all HEK 293 cell lines expressing exogenous KRAS (KRAS^{WT}, KRAS^{G13D}, KRAS^{G12D}, and KRAS^{G12V}), the accumulation of LC3 II in HBSS + Baf A1 conditions was slightly lower than in complete medium + Baf A1 conditions. HEK 293

parental cell line seems to respond positively to the autophagy induction, since induction of starvation led to a major accumulation of LC3 II in HBSS + Baf A1 condition.

Only in HEK 293 cells expressing KRAS^{WT} the accumulation of the LC3 II protein increased after HBSS + Baf A1 treatment, comparing to the same treatment in parental HEK 293 cell line.

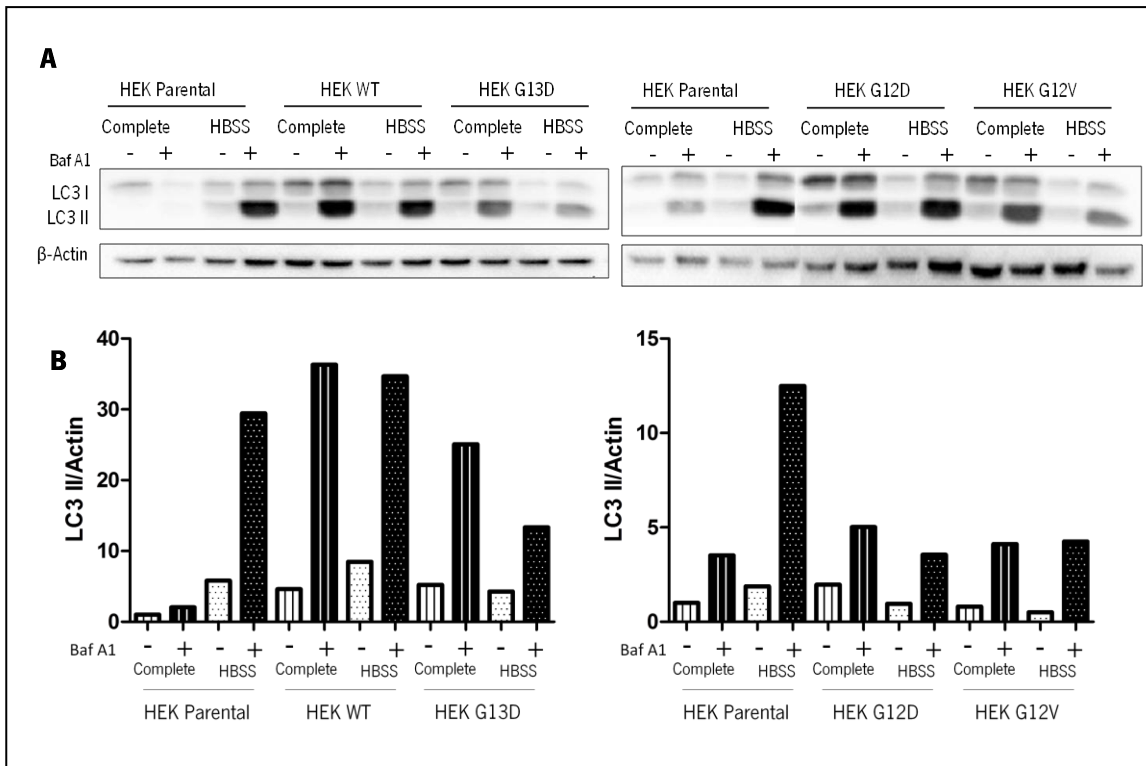


Figure 31: Expression of LC3 protein by HEK 293 cell lines (Parental, *KRAS*^{WT}, *KRAS*^{G13D}, *KRAS*^{G12D}, and *KRAS*^{G12V}) in response to starvation induction with 6 hours of HBSS. Baf A1 represents Bafilomycin A1. (A) Western-blot analysis of LC3 protein in HEK 293 cell lines. β-Actin levels were used as an internal loading control; (B) Quantification of LC3 II protein was performed using the *Quantity One* software.

3.3.2 Effect of expression of KRAS mutations on Colony Formation Ability of HEK 293 cells

In order to evaluate the effect of KRAS in the ability of cells to form colonies we performed the colony formation assay (Figure 32).

In parental HEK 293, the ability to form colonies was lower when compared with the HEK 293 cell line expressing $KRAS^{WT}$. In addition, HEK 293 cell lines expressing the *KRAS* mutations ($KRAS^{G13D}$, $KRAS^{G12D}$ and $KRAS^{G12V}$) showed a decrease in their ability to form colonies (Figure 32).

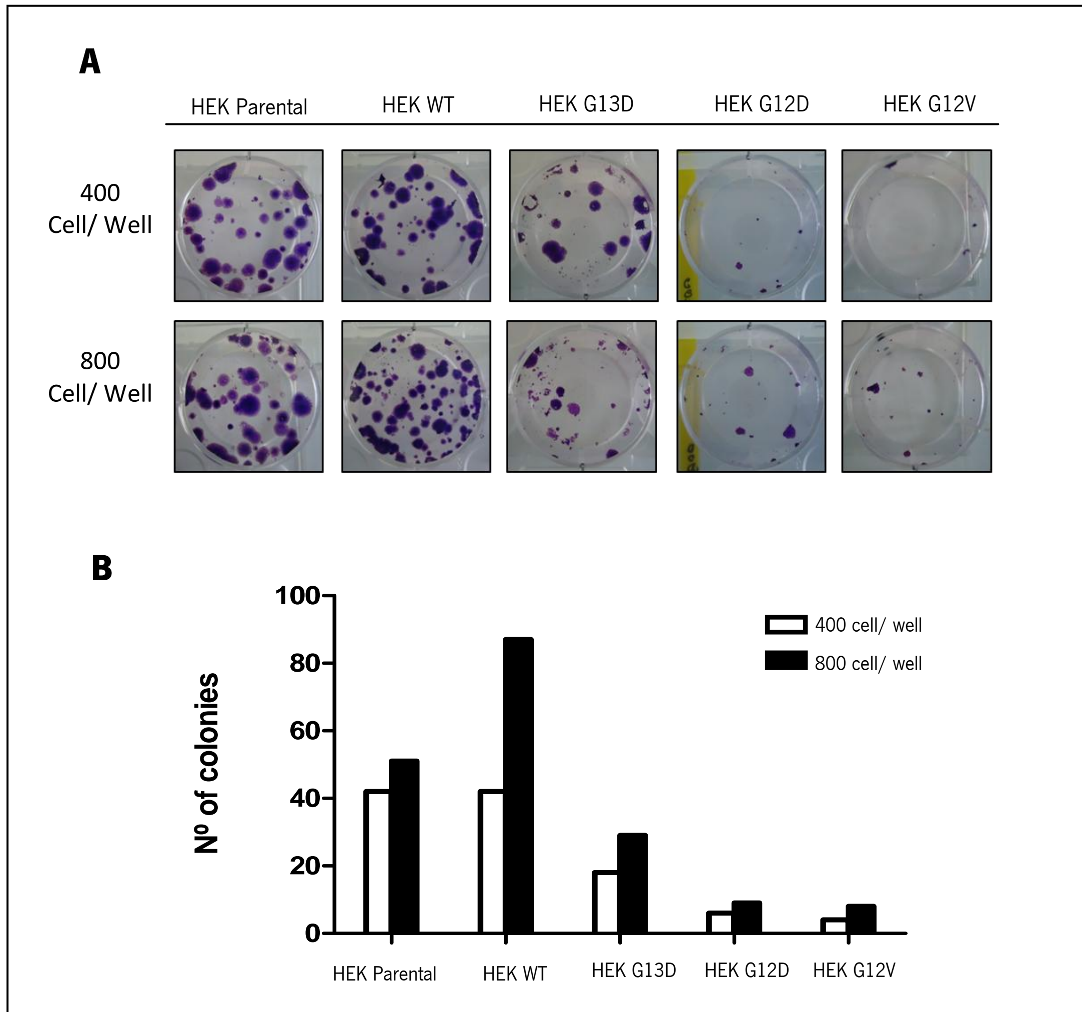


Figure 32: Effect of *KRAS* mutations on colony formation ability of HEK 293 cells. (A) Colony formation assay images of five HEK cell lines: (Parental, $KRAS^{WT}$, $KRAS^{G13D}$, $KRAS^{G12D}$ and $KRAS^{G12V}$ HEK 293) at passage 17; (B) Number of colonies formed by the 5 HEK cell lines, after 3 weeks in DMEM medium.

4. DISCUSSION

Colorectal carcinoma (CRC) is one of the most common types of cancer, leading to a high rate of mortality in Europe. As current anti-CRC therapies present poor outcomes for patients it becomes crucial to find new therapeutic approaches for CRC therapy.

Autophagy is a catabolic process that regulates normal turnover of organelles and removes those with compromised function [49]. This self-degradation process can be activated during various types of stress, resulting in cell protection. Many studies have shed light on the importance of autophagy in cancer but its precise role remains unclear. Indeed, autophagy has been suggested to function as a tumour suppressor pathway that holds cancer development [74, 119]. In the other hand this process has been implicated as a rescue mechanism of cancer cells, under unfavourable conditions like oxygen and nutrient depravation and as a fundamental process in cancer chemoresistance [104, 120, 121]. In fact, anticancer therapies, such as hormonal agents, chemotherapy and irradiation, frequently induce autophagy, in most cases as a pro-survival response, potentially contributing to treatment resistance [122].

Cancer cells, such as CRC cells, present higher levels of autophagy induction compared to normal cells, manly as a result of the unfavourable conditions common in tumour microenvironment and as a survival response to treatment-associated genotoxic and metabolic stress [104]. Chemotherapy combined with autophagy inhibition is expected to be a good approach for cancer therapies; in this way it is crucial to understand the precise role of autophagy in CRC.

Colorectal carcinogenesis is a result of transformations in apparently normal mucosa leading to benign adenomatous polyp that can, ultimately, progress to an invasive tumour. This progression requires several alterations in genes controlling cell proliferation, survival and invasion [10]. One of the most frequent events found in CRC is the presence of activating *KRAS* (Kirsten rat sarcoma viral oncogene homolog) mutations such as $KRAS^{G13D}$, $KRAS^{G12D}$ and $KRAS^{G12V}$ which have a role in the genesis and progression of these tumours [29, 123]. However, the precise role of *KRAS* mutations in the regulation of cellular survival through autophagy process is not well understood in CRC. Several studies have already demonstrated the importance of $KRAS^{G12V}$ expression in autophagy induction either in fibroblasts and epithelial cells from kidney, breast and ovarian [112, 113, 116, 124]. The major aim of this study was to understand the precise contribution of $KRAS^{G12V}$ in autophagy regulation in CRC using SW480 cell line as a study model.

For that purpose we inhibited *KRAS* in those cells and study the autophagy phenotype. After optimization of SW480 cell line transfection conditions for downregulation of KRAS protein through RNA interference (RNAi) technique, we assessed the expression of several autophagic proteins with function in distinct steps of this process: Beclin 1, Atg5, LC3 (I/II) and p62. We used as an autophagic stimulus, nutrient deprivation by HBSS, in order to study the respective autophagic proteins involved.

As mentioned before, Beclin 1 is a very important autophagic regulator that participates either in induction phase of autophagy and in autophagosomal maturation into autolysosome [66, 68-71]. Thus, there is a link between the induction of autophagy and increased expression of Beclin 1 [74, 75]. Our results showed that there was a decrease in the levels of Beclin 1 upon inhibition of KRAS and that decrease is higher after nutrient starvation (Figure 21). Despite preliminary, because the results are only from one experience, our results suggested that KRAS might regulate the levels of Beclin 1. On the other hand the decreased of Beclin 1 protein expression verified in all conditions, upon autophagy induction by HBSS, may suggests that SW480 cells are responding to autophagy induction through a Beclin 1- independent pathway. Beclin 1 is probably linked to Bcl-2 anti-apoptotic protein and is not interfering with autophagy, indicating that autophagy is activated through the mTOR/UIK pathway [64]. In future work, it will be important to perform at least 3 independent experiments to be sure of the obtained results.

Atg5 and LC3 (I/II), among others proteins, are essential for phagophore nucleation and elongation which leads to autophagosome formation, being LC3 a major constituent of this structure [76-79]. Our data showed an increase expression of these two proteins after autophagy induction and treatment with Bafilomycin A1, an inhibitor of autophagy, in LC3 study (Figure 22 and Figure 23). The greater accumulation of these autophagic proteins when compared with complete medium demonstrated that SW480 cells respond to the induction of autophagy by HBSS. Moreover, expression levels of LC3 II and Atg5 after KRAS inhibition were lower compared with control, which suggests that KRAS might be important in autophagy regulation by Atg5 and LC3 proteins. In fact, our results demonstrated that *KRAS*^{G12V} mutation increases autophagy in SW480 cells. The results regarding p62 protein expression were also in concordance with a role ok KRAS in autophagy regulation. p62 binds directly to LC3 II being transported into the autophagosome and degraded in autolysosome [81]. Basal autophagy is responsible for the degradation of p62 protein, and its inhibition by Bafilomycin A1, leads to the observed p62 accumulation [125]. Our results showed a decrease in p62 protein expression, upon KRAS

silencing, and also demonstrated that this decrease was even higher under starvation conditions (HBSS treatment). These facts suggest that KRAS might have an important role on p62 expression and consequently in the autophagic process.

Altogether, our results indicated that SW480 cell line appears to respond to autophagy activation and that KRAS protein might have a crucial role in autophagic process regulation in CRC cells, contributing for autophagy increase.

The extracellular-signal-regulated kinases (RAS – RAF – MEK – ERK) subfamily of MAPK signalling pathway have a large involvement in the pathogenesis, progression and oncogenic behaviour of CRC [23]. This subfamily of MAPK signalling pathway, when activated, may leads to the autophagy induction. In fact, the MAPK pathway has been implicated in autophagy induction in HT-29 colon cancer [98]. After an appropriated stimulus, the RAS – ERK cascade is activated and the signal is transferred from the cell surface to the nucleus. The final member of RAS - ERK signalling pathway (ERK protein) is phosphorylated and consequently activated by MEK protein leading to an increase of phosphorylated ERK protein (pERK) [21]. Thus, a down-regulation of the upstream protein of this pathway (KRAS protein) can prevent the activation of the downstream proteins, through this signalling pathway, and might prevents autophagy induction. Our preliminary results demonstrated that upon KRAS inhibition there was a decrease on pERK expression comparing to the control and that this decrease was even higher under starvation conditions (HBSS treatment) (Figure 25). These results suggest that pERK might be implicated in RAS-induced autophagy, although more studies need to be performed in order to prove it. For that, using ERK inhibitors, like PD98059, we can study the influence of ERK protein on autophagic proteins expression. KRAS inhibition also led to a decrease in total ERK expression upon an HBSS treatment so the analysis of pERK expression was also normalized by β-Actin protein expression.

KRAS mutation occurs in about 30% of CRCs cases [24, 29, 30] and this event has been recently suggested to be associated with increased proliferation [126]. In agreement, our results showed that inhibition of KRAS led to a decrease in the percentage of cells in G1, S and G2 cell cycle phases; and to a cellular death increase, shown by the raise of cells in sub-G1 phase (Figure 26). An activation of RAS protein leads to the activation of a phosphorylation cascade, RAS – RAF – MEK – ERK signalling pathway, culminating in the initiation of cell proliferation [22]. Moreover, this activated KRAS can interact with phosphatidylinositol-3 kinase class I which activates AKT. AKT activation leads to the activation of mTOR and to the inhibition of the RAS

protein, leading to an autophagy inhibition [98]. Thus, the regulation of cell survival by KRAS protein seems to occur due the MAPK signalling cascade and the mTOR pathway. Altogether these facts demonstrate that KRAS protein is important both in cell survival and proliferation. Our results showed that under nutrient deprivation condition, such as HBSS treatment, the ability for cells survival was further compromised, especially when KRAS was down-regulated. This might occur since the autophagic pathways seem to be inhibited however further studies are needed in order to confirm the obtained results.

As mentioned above the autophagic process plays a critical role in cellular survival by eliminating excessive, damaged and/or long-lived proteins and organelles, thus preserving the quality of essential cellular components [43]. Under stress condition, such as nutrient limitation, oxidative stress, hypoxia and heat, this process is commonly induced as a temporary cell survival mechanism to maintain cellular homeostasis [49]. Indeed this process is normally increased in cancer cells which may contribute to the survival of the cancer cells in their microenvironment [127, 128]. Our results showed that inhibition of autophagy in SW480 cells had a great impact in their ability to survive, since the inhibition of autophagic process by Bafilomycin A1 during 48 hours led to an enormous increase of cells in sub-G1 phase, correspondent to death cells, comparing with the control (Figure 27). These results are in concordance with several studies that demonstrated an increase of apoptotic cell death in various cancer cells as glioma cells, breast, colon and prostate carcinomas, upon an autophagy inhibition by Bafilomycin A1 combined with irradiation or chemotherapy treatments [104, 129, 130]. Short times of autophagy inhibition, such as 6 hours and 24 hours, do not have a high effect on survival of SW480 cells, although the sub-G1 values also increase comparing with control cells, suggesting once again the importance of the autophagic process to SW480 cell survival. However, these results were obtained from a single experience and need to be confirmed.

In order to understand the role of the different *KRAS* mutations (*KRAS*^{G13D}, *KRAS*^{G12D} and *KRAS*^{G12V}) in autophagy regulation we also performed a parallel study using HEK 293 cell lines transformed with all *KRAS* mutations. This study aimed to better understand the role of these mutations in autophagic process as well as their importance for cell survival. We used stable cell lines derived from HEK 293 cells, expressing the Flag-KRAS^{m} (Flag-KRAS^{G13D}, Flag-KRAS^{G12D}, and Flag-KRAS^{G12V}), the wild-type Flag-KRAS (Flag-KRAS^{WT}) and also the parental HEK 293, as a control. We first assessed the effect of *KRAS* mutations in autophagy induction condition through the analysis of two important autophagic markers expression (Beclin 1 and LC3 I/II) (Figure 30 and

Figure 31). According to our results, the basal levels of Beclin 1 in HEK 293 cell lines expressing KRAS^{m} (KRAS^{G13D}, KRAS^{G12D} and KRAS^{G12V}) were lower than those expressed in parental HEK 293 cell line. This suggests that the expression of Beclin 1 protein in complete medium conditions, in HEK 293 cell lines is not activated by *KRAS* mutations. As previously referred, the induction of autophagy by HBSS treatment leads to Beclin 1 expression increase [74, 75]. However, our results demonstrated that only HEK 293 cell lines expressing KRAS^{WT} and KRAS^{G13D} had an increase in Beclin 1 expression, upon autophagy induction comparing to parental HEK 293 cell line, pointing an important role for these KRAS protein forms to autophagic response in nutrient deprivation conditions. Relatively to LC3 II protein, we saw that in basal condition all HEK 293 cell lines expressing exogenous KRAS (KRAS^{WT}, KRAS^{G13D}, KRAS^{G12D}, and KRAS^{G12V}) had an increase on the levels of LC3 II expression consistent with an increase in basal levels of autophagy by KRAS. However, upon HBSS treatment only in HEK 293 cells expressing KRAS^{WT} the accumulation of the LC3 II protein increased after HBSS + Baf A1 treatment, comparing to the same treatment in parental HEK 293 cell line.

The expression of Beclin 1 and LC3 II proteins in the five HEK 293 cell lines demonstrated different responses to autophagy induction by HBSS, that were not consistent with the role of KRAS in autophagy regulation in CRC cells and namely with the results obtained in SW480 cells. The results were only from a single experiment and further experiences with these HEK 293 cell lines are needed. These contradictory results suggest that HEK 293 cells do not constitute a good model to study the role of KRAS in autophagy induction in CRC since these cells are not colon derived cell lines and possibly the introduction of exogenous *KRAS* might modify the cells behaviour. In future work the use of a different cell line model, as the normal colon cell line NCM460, might be a better and correct approach, which is already on the way in our laboratory [131].

In order to study the influence of the different *KRAS* mutations in the proliferative ability of cells we performed the colony formation assay. The ability of cells to form colonies corresponds to the competence of a cell to divide itself giving rise to a colony through successive divisions. Thus, this method reflects the proliferative ability of cells. As stated above, the MAPK signalling pathway is involved in very important cellular processes, being the key signalling pathway for cell proliferation from the cell surface to the nucleus [22]. The results obtained from colony formation assay demonstrated a higher proliferative ability of HEK 293 cell line transformed with *KRAS^{wt}* compared with parental HEK 293 (Figure 32). We might hypothesise

that this increased proliferation was due to a higher activation of MAPK signalling pathway (RAS – RAF – MEK – ERK), since the overexpression of KRAS was performed in the HEK 293 cell line that already have an endogenous $KRAS^{WT}$ expression. Contrary to previous studies describing an increased proliferation in *KRAS* mutation situations in CRC [126], our results using HEK 293 cell line showed that HEK 293 harbouring *KRAS* mutations ($KRAS^{G13D}$, $KRAS^{G12D}$ and $KRAS^{G12V}$) present less proliferative ability. It is known that, depending on the cellular context, activating *RAS* mutations can induce either proliferation or senescence. So, our results can be a consequence of activated senescence/cell cycle arrest promoted by *KRAS* mutations. In fact, recent studies demonstrated that activated *KRAS* induce senescence in several mouse tissues, including lung adenomas, pancreatic intraductal neoplasia, and colonic epithelium [132, 133]. Other studies show that oncogenic *RAS* promotes cellular arrest and senescence in vitro (fibroblasts) and in vivo (mouse tissue) [134, 135]. The entrance on senescent state can be a result of constitutive activation of RAS which is associated with the accumulation of p14 and p16 proteins, responsible for the activation of tumour suppressor proteins, such as p53 and Rb (Retinoblastoma tumour suppressor) proteins [134, 136, 137]. An analyse of senescence-related proteins and the cell cycle of the five HEK 293 cell lines could be helpful to better understand these results.

The results obtained with HEK 293 cell line were contradictory and therefore further experiments are required, particularly with the use of a different and more appropriated cell model, such as NCM460 cell line. These cells derive from normal colon cells and would be the “normal” clean model to study the role of KRAS in autophagy/survival regulation.

5. Conclusions and Future perspectives

5.1 Conclusions

The main goal of this work was to clarify the importance of *KRAS* mutations in autophagy regulation in colorectal carcinoma (CRC). Therefore SW480 a human CRC-derived cell line, harbouring *KRAS*^{G12V}, and stable cell lines derived from HEK 293 cells, infected with *Flag-KRAS*^{WT}, *Flag-KRAS*^{G13D}, *Flag-KRAS*^{G12D}, and *Flag-KRAS*^{G12V} mutations were used.

Taking into account all the results obtained during this work, it was possible to conclude that, KRAS is a crucial protein in autophagy regulation in SW480 cell line. In SW480 cells expressing KRAS protein the induction of autophagy, through HBSS (nutrient deprivation) led to an increase of autophagic markers such as LC3 II and Atg5 proteins. In conditions of KRAS inhibition and starvation induction, in SW480 cell line, there was a decreased of autophagy regulated proteins. Moreover upon KRAS inhibition we observed a decrease on pERK, what led us to hypothesise that RAS-MAPK signalling pathway might be implicated in KRAS induced autophagy. Altogether these results indicated that KRAS^{G12V} protein induces autophagy and that it might implicate a particular subfamily of MAPK signalling pathway, the RAS – RAF –MEK – ERK cascade.

This work also allowed us to conclude that basal levels of autophagy in SW480 cell line are essential for cellular survival. Indeed inhibition of autophagy in SW480 cells treated with Bafilomycin A1 led to increased levels of cellular death comparing with those obtained by SW480 cells under normal autophagy conditions.

KRAS inhibition in SW480 cells reveals an important role of this protein in cell survival and proliferation. The results obtained from SW480 cell cycle showed a decrease of cells in the initiating phases of cell proliferation (G1, S and G2) and an increased cellular death upon KRAS inhibition, contrary to cells expressing KRAS protein which mostly present a proliferative behaviour.

In summary, our preliminary results led us to conclude that KRAS protein, play a crucial role in several important processes like cell proliferation, survival and autophagy, and that this might be regulated at least through RAS – RAF – MEK – ERK signalling pathway.

The results obtain in this work for HEK 293 cell lines were controversial in relation to autophagy and proliferative studies, demonstrating that HEK 293 cell line is not a good model for study the role of *KRAS* mutations in autophagic regulation.

Autophagy is a very complex cellular process whose regulation involves numerous signalling pathways that often have crossing points. Thus, the total understanding of the mechanisms and proteins involved in this process and its regulation is very complicated and difficult to obtain. However, understanding the influence of *KRAS* mutations in the regulation of autophagy in colorectal carcinoma can now provide important answers which are essential for a better comprehension of CRC and hereafter help identify new targets to improve treatment for these tumours. The data from this work support the relevance of *KRAS* mutations in CRC cell survival through autophagic process suggesting that manipulation of KRAS protein expression and autophagy may be used as a therapy approach for CRC.

5.2 Future Perspectives

With the aim of further advance the present work, several studies still need to be performed in the future. For instance, in order to confirm if the autophagic process in SW480 cells is manly regulated by KRAS protein through RAS – RAF –MEK – ERK signalling pathway, it might be useful to study other RAS-induced signalling pathways, namely PI3K- AKT-mTOR, JNK1 and p38 pathways, known to be implicated in autophagy process. The inhibition of KRAS downstream pathways could be performed by the use of specific siRNAs or chemical inhibitors (LY294002 for PI3K; SP600125 for JNK1; SB203580 for p38 and PD98059 for ERK).

Another important study for a deeper understanding of the precise role of *KRAS* mutations in autophagy regulation in colorectal carcinomas may involves the use of other CRC cell lines harbouring different genetic background of *KRAS* mutations: HCT116 (*KRAS*^{G13D}) and Colo320 (*KRAS*^{G12D}). Indeed, our group team as already started these studies on HCT116 cell line. In order to overcame the genetic variability inherent to CRC cell lines harbouring different *KRAS* mutations, would be important to use a clea "normal" model like NCM460 cell line. NCM460 cells are normal colonic epithelial cells with a "clean" genetic background not showing transformational characteristics. Therefore we intent to study the autophagic phenotype after overexpressing of different *KRAS* mutations (*KRAS*^{G13D}, *KRAS*^{G12D} and *KRAS*^{G12V}) or *KRAS*^{WT} and identify genes that might be differentially expressed in NCM460 cells and specifically related to the different *KRAS* mutations. This approach could be an important tool in order to identify new genes implicated in *KRAS* mutations – induced autophagy regulation.

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