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Escola de Ciências

Rita Alexandra Batista Francisco

Understanding the role of KRAS-regulated autophagy in colorectal cancer: therapeutic implications

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Supervisors
Professora Doutora Ana Preto
Professora Doutora Maria João
Doutora Sara Alves

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DECLARAÇÃO

Nome: Rita Alexandra Batista Francisco

Endereço electrónico: rfrancisco@bio.uminho.pt

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Orientador:

Prof.ª Doutora Ana Preto

Co-Orientador(es):

Prof.ª Doutora Maria João Sousa

Doutora Sara Alves

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Understanding the role of KRAS-regulated autophagy in colorectal cancer: therapeutic implications

ABSTRACT

KRAS mutations (*KRAS*^{MUT}) are one of the most frequent genetic alterations present in colorectal cancer (CRC). Recently, KRAS has been described to be involved in autophagy, which is a cellular process whose role in cancer development still remains controversial, being designated as a double-edged sword. Recent data showed that CRC cells display high basal autophagic levels (a phenomenon denominated "autophagy addition"), which seem to be a metabolic advantage. In fact, our group has demonstrated that autophagy is important for CRC cells survival and that this catabolic process is regulated by KRAS^{MUT} in the colon model. Besides being an autophagy modulator, KRAS^{MUT} is also a molecular biomarker of resistance anti-EGFR inhibitors therapy.

This project general aim was to better understand how KRAS regulates autophagy in the colon model and how this impacts CRC therapeutic approaches. Specifically, we determined if upon autophagy induction by starvation the levels of KRAS changed and whether KRAS altered its localization in response to this stimulus. Furthermore, we explored the potential clinical impact of KRAS or autophagy inhibition in CRC. For these purposes, we used different cell lines: two CRC cell lines harbouring different KRAS^{MUT} and a non-cancer colon model stably expressing KRAS^{WT} and three hotspot mutations (KRAS^{G12D}, KRAS^{G13D} and KRAS^{G12V}). Additionally, in order to achieve our goals several molecular biology techniques were performed, including western blot analysis, SRB and immunofluorescence assays and RNA interference technique.

Our data reinforced our understanding of the crucial role of KRAS and autophagy in CRC carcinogenesis and suggested the use of KRAS and autophagy as effective targets for the development of more efficient CRC therapeutic strategies.

Caracterização do papel da autofagia regulada por KRAS no cancro colorretal: implicações terapêuticas

RESUMO

Mutações que afetam o *KRAS* (*KRAS*^{MUT}) são uma das mais frequentes alterações genéticas presentes no cancro colorretal (CCR). Recentemente, o KRAS foi descrito como estando envolvido na autofagia, um processo celular cujo papel no desenvolvimento no cancro permanece ainda controverso, sendo considerado uma "faca de dois gumes". Contudo, dados bastante recentes mostram que linhas celulares derivadas de CCR exibem elevados níveis basais de autofagia (um fenómeno denominado "adição autofágica"), o que parece constituir vantagem metabólica. Na verdade, o nosso grupo demonstrou que a autofagia é importante para a sobrevivência celular e que este processo catabólico é regulado pelo KRAS^{MUT} no modelo do cólon. Além de ser um modulador de autofagia, o KRAS^{MUT} é também um biomarcador molecular de resistência à terapia de inibidores anti-EGFR.

O objetivo geral do projeto foi compreender melhor como o KRAS regula a autofagia no modelo de cólon e qual poderá ser o impacto dessa regulação nas abordagens terapêuticas do CCR. Especificamente, determinámos se após a indução de autofagia por restrição de nutrientes os níveis de KRAS se alteravam e se esta proteína modificava a sua localização em resposta a este estímulo. Além disso, explorámos o potencial impacto clínico da inibição do *KRAS* ou da autofagia na terapia CCR. Para tal, utilizámos três modelos diferentes: duas linhas celulares de CCR com duas *KRAS*^{MUT} diferentes e um modelo de cólon não cancerígeno que expressa estavelmente *KRAS*^{WT} e três mutações "hotspot" (*KRAS*^{G12D}, *KRAS*^{G13D} e *KRAS*^{G12V}) deste oncogene. Adicionalmente, de modo a atingirmos os nossos objectivos, várias técnicas de biologia molecular foram desenvolvidas, incluindo análise de níveis de expressão proteica por western blot, ensaios de SRB e imunofluorescência e a técnica de RNAi, entre outras.

Os nossos resultados reforçam o papel crucial do KRAS e da autofagia na carcinogénese do CCR e, consequentemente identifica-os como alvos importantes e eficazes para o desenvolvimento de estratégias terapêuticas mais eficientes.

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LIST OF ABBREVIATIONS

3-MA 3 -methyladenine

5-FU 5-fluorouracil

AKT V-AKT murine thymoma viral oncogene

APC Adenomatous polyposis coli

Atg5 Autophagy related gene 5

ATP Adenosine triphosphate

BAX Bcl-2-associated X protein

Bcl-2 B-cell lymphoma 2

Bcl-xL B-cell lymphoma-extra large

Beclin-1 Bcl-2 interacting coiled-coil protein 1

BRAF V-RAF murine sarcoma viral oncogene homolog B

BSA Bovine serum albumin

c-MYC Myelocytomatosis viral oncogene

CASP5 Caspase 5, apoptosis-related cysteine peptidase

CIMP CpG island methylator phenotype

CIN Chromosomal instability

CHX Cycloheximide

COX-2 Cyclooxygenase 2

CQ Chloroquine

CRC Colorectal carcinoma

CRCSCs Colorectal cancer stem cells

EGF Epidermal growth factor

EGFR Epidermal growth factor receptor

ER Endoplasmatic reticulum

ERK Extracellular regulated MAP kinase

FAP Familial adenomatous polyposis

FBS Fetal bovine serum

GAPs Gtpase activating proteins

GDP Guanosine diphosphate

GTP Guanosine triphosphate

GTPase Guanosine triphosphatase

HBSS Hank´s balanced salt solution

HRAS Harvey rat sarcoma virus oncogene

JNK c-Jun N-terminal kinase

KRAS Kirsten rat sarcoma viral oncogene

LAMP2 Lysosomal-associated membrane protein 2

LC3 Microtubule-associated protein 1 light chain 3

MAPK Mitogen-activated protein kinase

mAb Monoclonal antibody

mCRC Metastatic colorectal carcinoma

Mcl-1 Myeloid cell leukemia 1

MEK Mitogen-associated extracellular signal-regulated kinase

MMR Mismatch repair

mRNA Messenger ribonucleic acid

MSI Microsatellite unstable

MSS Microsatellite stable

mTOR Mammalian target of rapamycin

NRAS Neuroblastome rat sarcoma virus oncogene

OHT 4-dehydroxy-tamoxifen

p38 p38 MAP kinase

PBS Phosphate buffered saline

PI3K Phosphoinositide 3-kinase

PFA Paraformaldehyde

PKC Protein kinase c

PM Plasma membrane

PTMs Post-translational modifications

RAB RAS-related GTP-binding protein, alternative splice

RAC1 RAS-related C3 botulinum toxin substrate 1

RALB RAS-like protein B

RAS Rat sarcoma

RIPA Radio imunoprecipitation assay

RNA Ribonucleic acid

RNAI RNA interference

ROS Reactive oxygen species

RPM Revolution *per* minute

RPMI Roswell Park Memorial Institute medium

RT Room temperature

SDS Sodium Dodecyl-Sulphate

siRNA Small interference RNA

SMURF2 Small ubiquitination regulatory factor 2

TKIs Tyrosine kinase inhibitors

TOM20 Translocase of outer mitochondrial membrane 20 homolog

TOR Target of rapamycin

TP53 Tumor protein p53

VEGF-A Vascular endodermal growth factor A

VMP1 Vacuole membrane protein 1

WNT Wingless and INT1

I – INTRODUCTION

I.1 **Cancer: hallmarks of a heterogeneous genetic disease**

Cancer is a highly heterogeneous disease characterized by deregulated genetic and cellular behaviour. Acquirement of oncogenic features, loss of tumour suppressive functions and of physiological tissue architecture, interactions with and alterations in the cellular microenvironment are processes and features that enable malignant cells to evade the mechanisms of cellular homeostasis in an organism (Kristensen et al., 2012).

The acquired functional capabilities that allow cancer cells to survive, proliferate, and disseminate have been defined as hallmarks of cancer in 2000 by Hanahan and Weinberg (Hanahan & Weinberg, 2000). These hallmarks, which have been recently revised and complemented by the same authors, range from resistance to apoptosis, sustained proliferative signalling and angiogenesis, replicative immortality and capability to invade and metastasise to deregulated cellular metabolism, genome instability and mutation and tumour promoting inflammation (Hanahan & Weinberg, 2011) (Fig. I.1). Combinations of these biological features, differently expressed in space and time, account for most aspects of the disease (Floor et al., 2012).

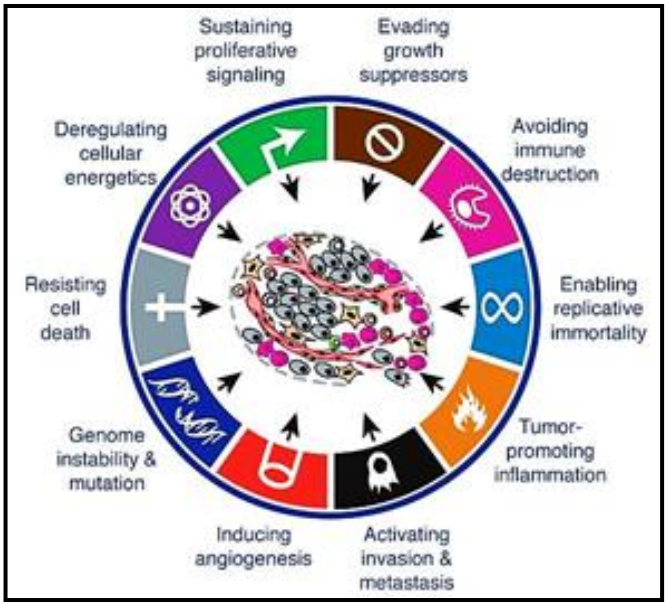


Fig. I.1 - **The Hallmarks of Cancer**. Schematic representation of the acquired capabilities of tumour cells necessary for tumour growth and progression. Adapted from (Hanahan & Weinberg, 2011).

Taking into account the molecular features and capabilities of cancerous cells, it is not surprising that cancer is a leading cause of death worldwide having accounted for 8.2 million deaths in 2012 (WHO, 2014).

I.1.1 Colorectal cancer

Colorectal cancer (CRC) is one of the most common cancers in developed countries; in fact nearly one million people develop CRC each year (Newton et al., 2011). CRC accounts for over 10% of all cancer incidence, being the third most common cancer worldwide and the fourth most common cause of death (Marisa et al., 2013;Stefano & Carlomagno, 2014). CRC is traditionally divided into sporadic and familial (hereditary) cases, being the wide majority (75% - 80%) of the tumours of a sporadic origin (Morán et al., 2010). Indeed, cases associated with well-defined hereditary syndromes like Lynch Syndrome or Familial Adenomatous Polyposis (FAP) account for less than 5% of all CRCs (Grande-Pulido et al., 2011).

At the molecular level, CRC is a heterogeneous disease and this can be attributed to three carcinogenesis pathways currently accepted to be responsible for the initiation, promotion and progression of this cancer: microsatellite instability (MSI), chromosomal instability (CIN) and the CpG-island methylator phenotype (CIMP) (Fearon, 2011). It is important to highlight that these pathways are not mutually exclusive and, therefore more than one may be involved in both sporadic and hereditary forms of CRC (Jass, 2007).

As far as the MSI pathway is concerned, this so called “mutator” pathway is present in approximately 12-15% of sporadic CRCs, besides being the underlying mechanism of Lynch syndrome (Boland & Goel, 2011; Imai & Yamamoto, 2008). This pathway is triggered by mutations in the mismatch repair (MMR) system that, consequently result in instability in the number of nucleotide repeats found within the microsatellite regions. Microsatellites are scattered throughout the genome, inclusively in the promoters of some genes implicated in CRC carcinogenesis, among which are *BAX*, *CASP5* and *E2F4*, thus leading to abnormal and uncontrolled cell growth (Blanes & Diaz-Cano, 2006).

As for the pathway that accounts for 65-70% of sporadic CRC (Pino & Chung, 2010) it is called the "canonical" (adenoma-carcinoma sequence) or "suppressor" pathway (Fig.I.2), being microsatellite stability (MSS) retained and chromosomal instability (CIN) involved (Walther et al., 2009). This pathway is based on the model proposed by Fearon and Vogelstein in 1988, which correlates specific genetic events with evolving tissue morphology. CIN occurs due to defects in different phases of the cell cycle at a genomic level, which leads to the formation of cancer cells characterised by a high frequency of allelic imbalance, chromosomal amplifications, and translocations (Kanthan et al., 2012). The CIN pathway is associated with mutations in the tumour suppressor gene *APC* and/or loss of chromosome 5q that includes this gene, mutations of the *KRAS* oncogene and loss (chr 18p) or deletion (chr 17p) of the tumour suppressor gene *TP53* (Walther et al., 2009).

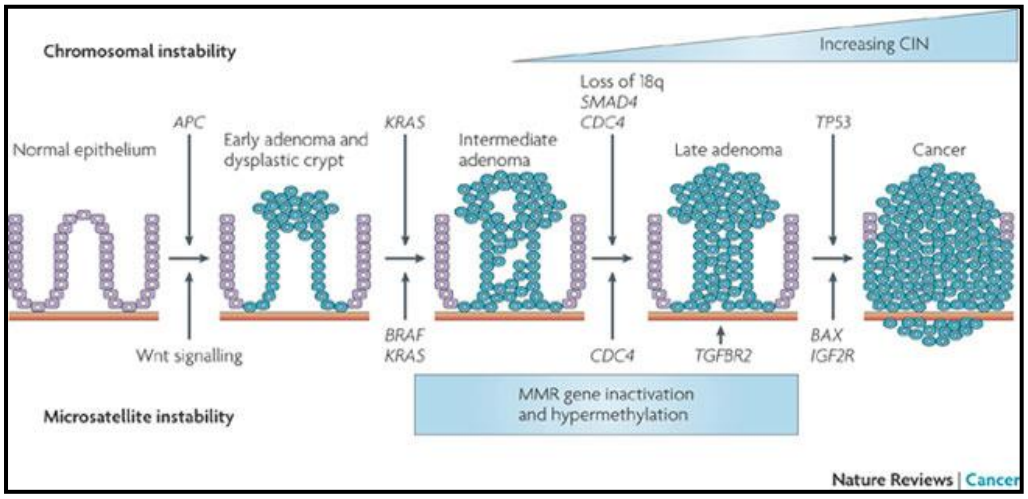


Fig. I.2 **- The adenoma-carcinoma sequence or suppressor pathway**. A stepwise accumulation of molecular alterations accompanied by increasing CIN, which results in the formation of 60-75% of sporadic CRCs. Adapted from (Walther et al., 2009).

Lastly, the importance of epigenetic instability has been clearly implicated in colorectal carcinogenesis through the identification of the CpG-island methylator phenotype (CIMP) (Jass, 2007; Wong et al., 2010). In CIMP the promoters of some genes, namely tumour suppressor genes such as *MLH1* and *P16*, are hypermethylated, what results in gene silencing (Imai & Yamamoto, 2008). In contrast with the two previously referred carcinogenesis models, CIMP tumours do not display the same genetic instability and tend to have *BRAF* – in highly methylated tumours- and *KRAS*- in

intermediate or low methylated tumours- mutations and few *APC* and *TP53* mutations (Hughes et al., 2012; Issa, 2008).

Summing up, CRCs are multistep disorders generally characterised by a number of epigenetic and genetic mutations, which mainly affect tumour suppressor genes, namely *APC* and P53 and proto-oncogenes, such as *BRAF* and *KRAS* (Samowitz et al., 2007; Arrington et al., 2012).

Importantly, the mechanism through which any of the CRC carcinogenesis pathways develops is still debatable. Recently, defying the well-accepted stochastic model – which states that every cell within the tumour has tumourigenic potential - another carcinogenic model has been proposed to explain CRC development – the Colorectal Cancer Stem Cells (CRCSCs) theory. CRCSCs are a restricted subpopulation of cells within the tumour that contain self-renewal capacity and the ability to differentiate, being tumour-initiating cells. These cells can be identified at a molecular level by the presence of several stem cell surface markers, such as CD133 and CD44, pluripotency transcription factors, namely Oct-4 and c-myc, and by the overexpression of signalling pathways related to cell differentiation and proliferation, such as the Wnt/β- catenin pathway (Kemper et al., 2010; Todaro et al., 2007; Vaiopoulos et al., 2012). Interestingly, these cells display an increased resistance to apoptosis induction that has been, among other factors, correlated to an up-regulation of the levels of anti-apoptotic B cell lymphoma 2 (Bcl-2) proteins (Abdullah & Chow, 2013). Moreover, a RAS-RAF-MEK-ERK pathway hyperactivation has been implicated in the regulation of the expression of the stem cells marker CD133 in CRCSCs, which in turn was related to poor disease prognosis (Kemper et al., 2012a), nonetheless the role of KRAS in CRCSCs still remains widely unexplored. In conclusion, CRCSCs are thought to be the driving force behind tumour growth and metastasis and responsible for tumour recurrence after treatment (Kemper et al., 2012b).

I.2 **The RAS family: KRAS a very special case**

The *RAS* family GTPases is included in a large superfamily of small GTP-binding proteins (Arozarena et al., 2011). This superfamily is composed of more than 150 small GTPases, which are divided into at least 5 distinct families (Ras, Rho/Rac, Rab, Arf, and Ran) on the basis of primary sequence relationships. The RAS family encompasses the proto-oncogenes Harvey-RAS (*HRAS*), Neuroblastome-RAS (*NRAS*) and Kirsten-RAS (*KRAS*), the most commonly mutated oncogenes in human cancer (Castellano & Santos, 2011).

The 21kDa GTP-binding proteins encoded by the three canonical members of the RAS gene family are HRAS, NRAS, KRAS4A and KRAS4B. The two KRAS isoforms originated from an alternative splicing event in exon 4, being the latter the form that is ubiquitously expressed, thus hereafter it will be designated KRAS (Hancock, 2003; Karnoub & Weinberg, 2008). These proteins act as central control elements in signal transduction pathways that touch on multiple aspects of cell biology, including proliferation, differentiation, apoptosis, survival, motility and adhesion, among many others (Fernández-Medarde & Santos, 2011). The multitude of functions performed by these proteins stems from the fact that they are crucial nodes in signalling networks, which connect a great variety of upstream signals (e.g. mitogens, cytokines and hormones) to an even wider set of downstream effector pathways (e.g. Mitogen Activated Protein Kinase (MAPK) cascade , PI3K/AKT) (Stites & Ravichandran, 2009).

The importance of RAS proteins in cell physiology is clearly highlighted by the dramatic results of their deregulation in some pathological conditions, namely in cancer. Activating mutations in *RAS* genes are detected in about 30% of human cancers (Prior et al., 2012). Among the three *RAS* isoforms *KRAS* is the most frequently mutated (20%-30% of human cancers) (Prior et al., 2012), being mutated in a large number of human epithelial cancers, particularly in pancreatic cancer (90%) (Collins et al., 2012), and CRCs (45-50%) (Vaughn et al., 2011).

In comparison to the other two isoforms of the *RAS* gene, *KRAS* has many other particularities besides its confined prevalence to a subset of tumours. It also possesses a unique pattern of expression in a restricted number of cell lineages and/or tissues both during development and in other physiological settings (Bar-sagi, 2001).

Moreover, KRAS activates RAF-1 with much more efficiency than any other of the RAS proteins (Yan et al. 1998; Plowman et al. 2008), this increased affinity translates into a more potent activation of the MAPK cascade, which regulates cell proliferation, growth and survival that ultimately promotes tumour development (Tanoue & Nishida, 2003).

As far as its roles in tumour promotion and development are concerned, it has been reported that oncogenic *KRAS*^{G12D} stimulates colonic epithelium hyperproliferation (Haigis et al., 2008) and that only this isoform (more specifically *KRAS*^{G12V}) can trigger stem cell expansion to initiate endodermal tumours (Quinlan et al., 2008). Moreover, KRAS stimulates cell motility by preferentially activating RAC1, which is a protein involved in the control of cell polarity and cytoskeleton (Walsh & Bar-sagi, 2001). Additionally, RAC1 activation by *KRAS*^{G12D} promotes cell survival and hyperproliferation in oral epithelium (Samuel et al., 2011). Interestingly, this KRAS downstream effector has been found to interact with Bcl-2 at the mitochondrial membrane, enhancing Bcl-2 anti-apoptotic activities (Kang & Pervaiz, 2013). In addition to this, *KRAS*^{G12V} has been reported to alter the expression of intercellular proteins, such as β1-integrin and N-cadherin in colon epithelial cells, thus inducing loss of cell-cell and cell-substrate adherence (Yan et al., 1997). Also, *KRAS* has been stated to be solely responsible for the steady-state production of (metalloproteinase-2) MMP-2 in fibroblasts (Liao et al., 2003). Altogether these properties can account for the highly invasive and metastatic phenotype of *KRAS* derived tumour cells.

In conclusion, it is very likely that the intrinsically different biological potency of the different *RAS* isoforms, combined with the different cellular contexts in which these isoforms are expressed, are among the contributing factors responsible for the functional specificity and subsequent associated phenotypes observed in each particular case (Castellano & Santos, 2011).

I.2.1 KRAS post-translational modifications towards activation, stability and protein turnover

RAS isoforms share a high degree of sequence identity, however they do not display redundant functionality. This is particularly surprising since the regions of the proteins that interact with downstream effectors as well as the controlling and activating sites where GDP/GTP and regulatory proteins bind, thus mediating RAS activation state, are identical among the three RAS isoforms (Prior & Hancock, 2012). RAS proteins specific roles may be explained by various factors: cellular context, differential interaction with effectors, compartmentalized signalling and post-translational modifications (PTMs) (Arozarena et al., 2011).

H, N and KRAS proteins are composed by 188 aa and are synthetized in the cytosol, nonetheless they require subsequent PTMs to enable them to stably associate with membranes where they can function (Henis, Hancock, & Prior, 2009). These PTMs take place in the C-terminal hypervariable region (HVR), which differs significantly among the isoforms and has been shown to be essential for membrane interaction and cellular trafficking (Krens et al., 2010).

In the HVR these proteins have a carboxyl-terminal “CAAX” motif that undergoes three sequential post-translational processing events. Firstly, the cysteine (i.e., the C of the CAAX sequence) is isoprenylated by either farnesyltransferase (FTase) or geranylgeranyltransferase type I (GGTase I) proteins in the case of KRAS, what facilitates the association of RAS proteins with the ER where the second PTM occurs. In this organelle, the last three amino acids of the protein (i.e., the –AAX sequence) are cleaved off by Rce1. Thirdly, the newly exposed isoprenylcysteine is methylated by an endoplasmatic reticulum (ER) membrane–bound methyltransferase, isoprenylcysteine carboxylmethyltransferase (Icmt). All of these modifications confer more hydrophobicity to these proteins, increasing their membrane binding aptitude (Fig.I.3) (Bergo et al., 2004).

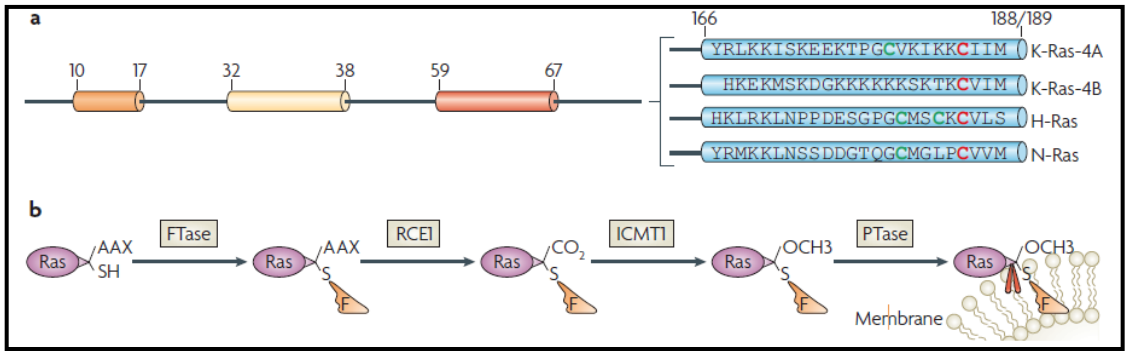


Fig. I.3 - **C-terminal (HVR) processing of RAS proteins. (a)** The HVR present in the C-terminal is highly divergent among the RAS family members and contains the membrane-targeting sequences. **(b)** Schematic representation of the PTMs in the C-terminal region common to all RAS isoforms. Adapted from (Karnoub & Weinberg, 2008).

Nevertheless, these modifications result in weak membrane binding ability, which is enhanced by a second motif in the HVR that strengthens membrane interactions. This motif varies amongst RAS isoforms, being comprised of a string of six positively-charged lysine residues upstream of the C terminal in KRAS case (Karnoub & Weinberg, 2008). As a consequence, KRAS is located within nanoclusters in the plasma membrane (PM) which are cholesterol depleted and enriched in negatively charged lipids, like phosphatidylinositol- 4,5-bisphosphate that is a substrate for PI3K, a key RAS effector (Hancock, 2003). Moreover, these PTMs also increase KRAS stability and, as a result the protein half-life (Bergo et al., 2004). Reports by various groups state that the half-life of KRAS, more specifically of $KRAS^{G12V}$ mutation, varies from 12 to 13.9 hours (Elad et al., 1999; Rabien et al., 2012; Shukla et al., 2014). Despite of this, as the polybasic domain results in a lower PM anchorage efficiency, KRAS has a membrane residency half-life of only a few minutes, what results in this isoform having a greater cytosolic pool than the other two RAS proteins, consequently one might speculate this affords KRAS an increased time window opportunity to bind to endomembranes (Silvius et al., 2006).

However, the signalling pathways activated by this protein from the membranes of intracellular organelles are just starting to be uncovered, so a lot remains to be elucidated and fully understood. In spite of this fact, a PTM has been described to be the underlying mechanism for steering KRAS towards the mitochondria – phosphorylation of this protein in Ser181 results in its binding to the mitochondria

where it triggers apoptosis by forming a complex with pro-survival protein Bcl-xL (Bivona et al., 2006).

I.2.2 KRAS localization towards signalling activation – from the plasma membrane to endomembranes

Signalling pathways allow cells to respond to their environment by activating intracellular signal transduction pathways that make decisions about cell identity, behaviour and survival (Pryciak, 2009). Despite the fact that cancer cells share the same regulatory circuits that govern normal cell proliferation and homeostasis in the former these signalling pathways are deregulated (Hejmadi, 2009). As the RAS oncogenes, namely KRAS, can affect multiple pathways as referred above, its effects on cell physiology are immense.

The most predominant types of genetic altering events that culminate in a mutated $KRAS^{MUT}$ isoform are single-point mutations in codons 12, 13 and 61. These mutations result in reduced RAS GAP (GTPase Activating Protein) GTPase activity, therefore locking the protein in the active RAS-GTP conformation and rendering it constitutively active. Due to their high frequency these genetic alterations have been named "hotspot" mutations (Ellis & Clark, 2000). Although less common, additional missense mutations at codons 19, 22 and 146 have been described in single colorectal tumour case reports (Akagi et al., 2007) as well as other types of mutations, such as gene amplification (Fig.I.4) (Smith et al., 2010).

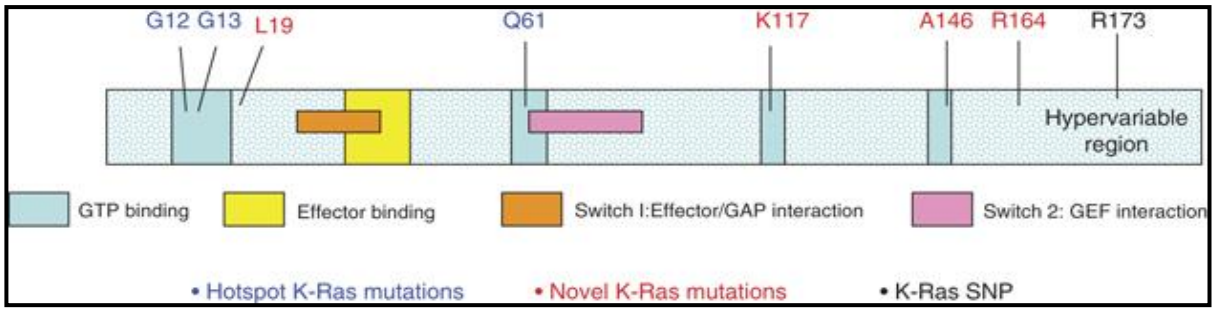


Fig.I.4 - **Schematic representation of novel and hotspot *KRAS* mutations**. Codons 12, 13 and 61 are the most commonly mutated in KRAS, rendering it constitutively active. Adapted from (Smith et al., 2010).

The presence of $KRAS^{MUT}$ has been widely associated with worse prognosis in CRC, thus the disclosure of specific molecular characteristics of individual KRAS mutations may be important determinants of both tumour progression and response to targeted therapy (e.g. *EFGR* therapy) (Heinemann et al., 2009).

Mutations in the codons 12 and 13, which will be the ones under study in this work and that correspond to 25%-40% of KRAS mutations in CRC (Chaiyapan et al., 2013), lead most commonly to the incorporation of aspartate or valine at codon 12 and of aspartate at codon 13, whereas in wild-type (WT) KRAS both codons encode glycine residues (Naguib et al., 2011). Some studies have identified different transformation potencies among these point mutations, relating codon 12 mutations with more aggressive tumours. The tumours display higher growth rates, lower apoptosis levels as well as higher levels of Bcl-2 proteins, E-cadherin and β-catenin when compared to tumours containing codon 13 mutated (Guerrero et al., 2000; Smith et al., 2010).

Studies concerning oncogenic $KRAS^{MUT}$ point towards its early activation during tumour development (Liu et al., 2011) and also suggest that $KRAS^{MUT}$ may coordinate different biological effects resulting from the differential activation of its downstream effectors as specified by KRAS activation kinetics (Romano et al., 2013).

In part the selective activation of downstream KRAS targets might be unravelled by the several cellular membranes it was found to signal from, besides the PM, which are endosomes/lysosomes (Lu et al., 2009), Golgi complex, ER (Hancock, 2003) and mitochondria (Bivona et al., 2006; Rebollo, Perez-Sala, & Martínez-A, 1999). This brings into light the important role of space in the transmission of KRAS signals, since it orchestrates the interactions with specific regulatory and effector proteins, thus creating another degree of complexity and diversity to this isoform signalling (Kholodenko et al., 2011).

Evidencing the significance of endomembrane signalling with concrete examples, for instance it has been reported that $KRAS^{WT}$ present in the mitochondria activates *STAT3* in order to achieve malignant cellular transformation (Gough et al., 2010). Additionally, $KRAS^{MUT}$ has been found to participate in the activation of signalling pathways from mitochondria, such as apoptosis in kidney cells (Bivona et al.,

2006). Conversely, in T cells KRAS was only located in mitochondria in non-apoptosis inducing conditions (Rebollo et al., 1999). Moreover, *KRAS*^{12V181E} has been reported to travel from the PM to the mitochondria through the phosphorylation of its Ser181 residue by PKC, a process designated farnesyl-electrostatic switch, which is a mechanism analogous to the myristoyl-electrostatic switch that regulates the subcellular localization MACKS (myristoylated alanine-rich protein kinase C substrate) (Bivona et al., 2006). Furthermore, Thissen and collegues described an interaction between isoprenylated KRAS and microtubules, which could play a role in the cellular movement of this protein (Thissen et al., 1997). Given that mitochondria are associated with the cytoskeleton and use the microtubules to move along the cell (Karbowski et al., 2000), it might be possible that this common platform may provide a physical connection between mitochondria and *KRAS*. Finally, another possible trafficking mechanism that has been put forward entails the involvement of chaperone proteins that may direct KRAS towards the mitochondria (Bivona et al., 2006).

As far as KRAS location in mitochondria is concerned, studies have suggested it to localize at the outer membrane, where *KRAS* was found to coimmunoprecipitate with the Bcl-2 family protein– Bcl-xL (Bivona et al., 2006), while others state it is mostly present at the inner membrane (Hu et al., 2011).

KRAS^{WT} has also been described to localize to early and late endosomes and, ultimately to lysosomes, where this protein suffers degradation. This de-localization occurs upon Epidermal Growth Factor (EGF) stimulation and in a clathrin-dependent manner. In these experimental settings, KRAS has been found to build a signalling platform at late endosomes from where it can activate the MAPK cascade (Lu et al., 2009).

A variable that has been constantly emphasized in KRAS signalling (both WT and MUT) is time, therefore the definition of a KRAS signalling threshold has been proposed (Prior et al., 2012). Several studies using inducible systems have concluded that time of expression plays a critical role in the selective activation of signalling effectors or pathways, being mitochondrial metabolism a very important example. In fact, KRAS^{G12V} tumourigenesis has been associated with a metabolic shift from oxidative phosphorylation to glycolysis as well as to increased ROS production and to the inducement of mitochondrial dysfunction (Hu et al., 2011). Additionally, KRAS^{G12D}

tumour growth and proliferation have been associated with dependence on glutamine degradation and increased ROS production, respectively (Weinberg et al., 2010).

Finally, a ground-breaking study has put forward the hypothesis that oncogenic *KRAS*^{G12D} is not constitutively active as has been widely believed, rather they propose that KRAS^{MUT} can be more readily and strongly activated by upstream stimulants, like EGF (Huang et al., 2013).

In conclusion, KRAS^{MUT} signalling network is intricate and widely diverse, thus the enlightenment of its mechanisms is essential to understand in depth its role in physio-pathological diseases such as cancer.

I.2.3 KRAS and autophagy: a tight bond

Autophagy is a self-catabolic process that provides macromolecular precursors through nutrient recycling, cellular energy homeostasis maintenance and degradation of damaged or toxic cytoplasmic organelles, such as the ER and mitochondria. In the autophagic process the degradation of cellular components occurs through the formation of autophagosomal structures, which engulf the cellular constituents that will undergo destruction. The autophagosome then fuses with lysosomes forming the autophagolysosomes where the breakdown of cellular components is achieved (Weidberg et al., 2011). Under stress, this genetically regulated and evolutionary conserved program is commonly induced as a temporary cell survival mechanism that is exceedingly present and important in nutrient or growth factors depletion as well as in hypoxic environments, among other stressful conditions. Autophagy accumulates various functions in cell metabolism, protein and organelle turnover, cell survival, proposing a dynamic and extremely complex role of this process in cancer promotion, evolution and settlement (Apel et al., 2009; Mancias & Kimmelman, 2011).

Actually, the role of autophagy in cancer seems to be a double-edged sword, and, in spite of the contradictory evidences and conclusions, the accumulating knowledge over the years has been suggesting that the aberrant control of autophagy may be among the key hallmarks of cancer. Whereas in “normal” cells autophagy is under tight control, in cancer many of its pathways/intermediates/effectors are

deregulated, namely PI3K/AKT/mTOR, Bcl-2, Beclin-1 and RAS (Bach & Ramm, 2011; Kimmelman, 2011).

The attribution of tumour suppressor or tumour promoting properties to autophagy has to take into consideration cell type, tumour type, tumour characteristics (e.g. aggressiveness, invasiveness), stage of cancer development, genetic mutations, microenvironment conditions (e.g. hypoxia, glucose deprivation), intensity and duration of this program (Bellot et al., 2013; Selvakumaran et al., 2013). At an early phase in the tumourigenic process autophagy seems to essentially suppress malignant cell transformation given that oncogenes like *PI3K*, *AKT* or *mTOR* inhibit autophagy when activated (Guertin & Sabatini, 2007). Also Bcl-2 family protein Noxa (a BH3-only protein), has been reported to bind to Beclin-1, consequently inhibiting autophagy in human ovarian surface epithilial cell line transfected with a doxycycline inducible $HRAS^{G12V}$ system (Elgendy et al., 2011). Furthermore, a number of studies have reported an increase in genomic instability and aneuploidy due to loss of autophagy, what may promote tumourigenesis (Karantza-wadsworth et al., 2007; Mathew et al., 2007)

Since autophagy is a pathway that promotes cell-autonomous survival under metabolic stress the finding of its up-regulation in a number of established/advanced tumours is not unexpected (Kimmelman, 2011). Particularly in tumours driven by oncogenic RAS, autophagy appears to be necessary for transformed cells metabolism (Mancias & Kimmelman, 2011). Although, Yoo and collegues have shown that oncogenic $KRAS^{G13D}$ blocks autophagy by down-regulating Beclin-1 in colon epithelial cells (HCT116, Hkh-2, and Hke-3) (Yoo et al., 2010) an increasing number of studies point towards a different direction.

Oncogenic *KRAS* malignant transformation has been correlated with autophagy induction through several downstream targets (Kim et al., 2011; Guo et al., 2011; Ré et al., 2012). $KRAS^{G12V}$ promotes anchorage-independent growth in breast epithelial cells (Brito & Scorrano, 2011), and also enables cell survival during starvation whilst its inhibition disturbs mitochondrial metabolism in mouse kidney epithelial cells (Guo et al., 2011). Additionally, *KRAS*-driven cell lines and tumours have been described to display high basal autophagy levels (Yang et al. 2011; Guo et al. 2011) a phenomenon termed “autophagy addiction”, which may prove to be metabolically advantageous.

Actually, Zhai and co-workers have disrupted the autophagic flux in two colorectal cancer cell lines (HCT116 and SW480) through the overexpression of microRNAs, what led to cell growth inhibition both *in vitro* and *in vivo*, confirming once more that in the CRC model autophagy has a pro-survival role (Zhai, Song, Xu, Zhu, & Ju, 2013). Another very recent study reached the same conclusion through a distinct approach, in this case using a combination of therapeutic drugs and autophagy inhibitors in several colon cancer cell lines (HT29, HCT116, HCT15, SW620, KM12, BE, WiDr, and LoVo). It concluded that autophagy had a cellular protective effect against therapeutic agents (Selvakumaran et al., 2013). Furthermore, Wang *et al.* using the *in vitro* model mouse fibroblasts transformed with $HRAS^{G12V}$ and as a confirming model HCT116 ($KRAS^{G13D}$) cell line have proposed that the levels of autophagy determine whether oncogenic RAS expression results in a senescence-induced phenotype or if it promotes tumour survival and growth (Wang et al., 2012), this evidence indirectly ascertains the pro-tumourigenic role of high basal autophagy levels.

I.2.4 The role of KRAS in the cross-talk between apoptosis and autophagy: Bcl-2 family proteins interaction

Apoptosis and autophagy are not mutually exclusive pathways, indeed they have been shown to cooperate synergistically as well as to antagonize each other. A molecular link between the intrinsic pathway of apoptosis and autophagy has been provided by the Bcl-2 family of proteins (Mariño, Niso-santano, Baehrecke, & Kroemer, 2014). This protein family is identified by the presence of at least one of four Bcl−2 homology (BH) domains (BH1-BH4), thus being divided into three groups: the proteins containing three to four BH domains, namely Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bcl-B and A1 all of which possess anti-apoptotic properties, hence functioning as oncogenes in the tumourigenic process; the other two groups subdivided according to their different number of BH domains and apoptotic mechanisms, encompass proteins with pro-apoptotic activities, therefore acting as tumour suppressors in cancer development. Among these pro-apoptotic members, there are the multidomain proteins, containing three BH domains (Bax, Bak and Bok), that directly initiate apoptosis and the BH3-only

proteins (Bad, Bid, Bim, Bmf, BIK, Hrk, Noxa and Puma), which have a conserved BH3 domain and that can bind to the anti-apoptotic Bcl-2 proteins to prevent their activity or directly to the pro-apoptotic proteins in order to promote apoptosis (Kirkin et al., 2004; Kelly & Strasser, 2011).

Recently, this growing family had a new addition – Beclin-1, which has been proven to have a BH3 domain (Sinha & Levine, 2008) and so the intertwine between apoptosis and autophagy began to be disclosed. Beclin-1 has been found to bind to anti-apoptotic proteins, such as Bcl-2, Bcl-xL, Mcl-1 and Bcl-w and this connection has been reported to inhibit autophagy (Fig. I.5) (Erlich et al., 2007; Kang et al., 2011; Pattingre et al., 2005), in the same way that the binding of these proteins to pro-apoptotic members of the family has been known to inhibit apoptosis (Kelly & Strasser, 2011). Defying the generally reported anti-apoptotic as well as anti-autophagic activities of Bcl-2 and Bcl-xL, Priault and colleagues have attributed a pro-autophagic role to both proteins in a CRC cell line (HCT116) with pro-survival consequences. Overexpression of both proteins led to an increase in the autophagic flux, although Bcl-xL promoted this augment more robustly than its counterpart and in a Beclin-1 independent manner, whilst Bcl-2 fully relied on Beclin-1 binding to stimulate autophagy (Priault et al., 2010).

In a lymphoid/myeloid precursor cell line, growth factor deprivation induced autophagy that resulted in increased levels of BH3-only protein Bim, which at early starvation stages supported autophagy in Bcl-2 and Bcl-xL expressing cells, probably through the disruption of the complex(es) Beclin-1/Bcl-2/Bcl-xL (Altman et al., 2009). Luo *et al.* reached a completely contradictory conclusion as far as Bim action on autophagy is concerned. According to this group, Bim inhibits autophagy by recruiting Beclin-1 to microtubules and in response to stress Bim dissociates from the microtubules and moves to the mitochondria becoming pro-apoptotic (Luo et al., 2012), therefore another co-regulatory mechanism of both autophagy and apoptosis has been proposed.

Conversely, the BH3-only protein Bad has been reported to stimulate autophagy by actively competing for Beclin-1 BH3 binding domain (Maiuri et al., 2007a) resembling its activity in apoptosis. However, pro-apoptotic Bax has been shown to disrupt the complex Beclin-1/Bcl-2 and enhancing caspase mediated

cleavage of Beclin-1, thus causing the reduction of autophagy (Luo & Rubinsztein, 2010). Interestingly, Beclin-1 whose primary function entails autophagy mediation, when cleaved (e.g. by caspases) its C-terminal fragment acquires a new function and becomes involved in the amplification of mitochondrion-mediated apoptosis (Rikiishi, 2012). Keeping in mind the central role of Bax in apoptosis, as one of the proteins responsible for Mitochondrial Outer Membrane Permeabilization (MOMP) (Tait et al., 2010), the interaction with Beclin-1 suggests that this protein may have a direct or indirect role in regulating autophagy that is somehow connected to its role in apoptosis modulation (Maiuri et al., 2007b). Also very intriguing is the pro-apoptotic role displayed by Bcl-2 and Bcl-xL as a result of PTMs and specific protein interactions (Zhou & Yang, 2011). Bcl-2 and Bcl-xL are believed to carry their anti-autophagic and anti-apoptotic effects from within different organelles, the ER and the mitochondria, respectively, thus adding yet another regulatory mechanism to their actions within the cell (Pattingre et al., 2005; Maiuri et al., 2007a). A summary overview of the dual role of these proteins is presented in Figure I.5.

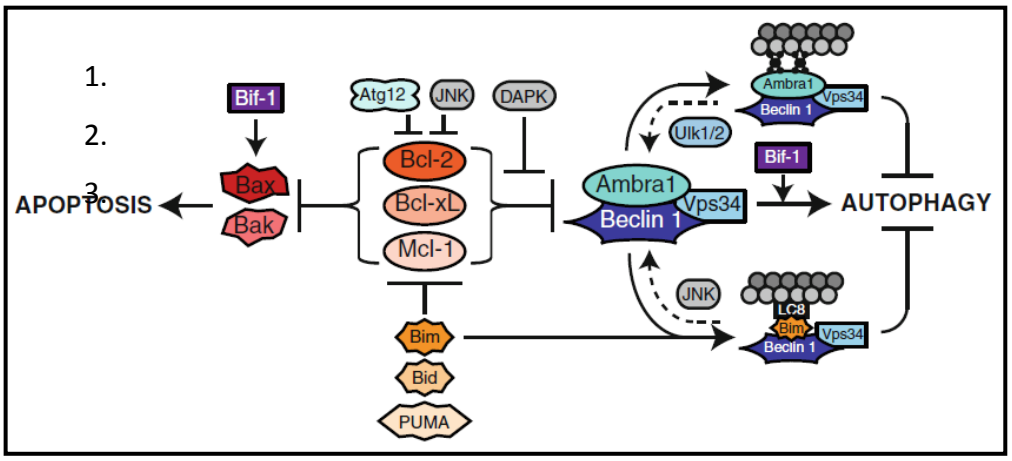


Fig. I.5 - **Cross-talk between apoptosis and autophagy.** The interaction between Beclin-1 and other Bcl-2 family proteins involved in the regulation of both apoptosis and autophagy. Adapted from (Young & Wang, 2013).

The instigating stimulus and the activated pathways might determine whether autophagy or apoptosis dominates (Maiuri et al., 2007b). KRAS is the upstream regulator of many signalling pathways involved in apoptosis and in autophagy (Young and Wang, 2013). $KRAS^{MUT}$ constitutively activates the PI3K/AKT pathway, which has opposing actions in apoptosis and autophagy. This pathways has been demonstrated

to have an inhibitory effect on apoptosis, for instance through AKT mediated phosphorylation of Bad, which elicits its dissociation from Bcl-2, thus freeing Bcl-2 to inhibit Bax/Bak-mediated apoptosis, but also through AKT mediated phosphorylation of Bax in neuroblastoma cell lines upon H_20_2 stimulus (Sadidi et al., 2009). Conversely, a promoting effect on autophagy has been reported through the inhibition of mTORC1 under starvation (Eisenberg-Lerner et al., 2009).

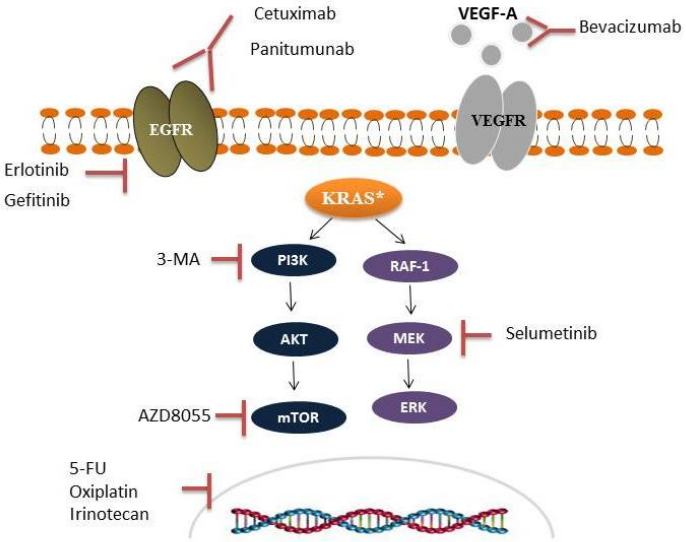
Moreover, the best described signalling circuit activated by KRAS is the MAPK cascade, which ensures continuous cell proliferation, therefore apoptosis evasion as well. It is also responsible for the activation of autophagy through the c-Jun Kinase (JNK) phosphorylation of Bcl-2 leading to its dissociation from Beclin-1 (Wei et al., 2008). Also, in a mouse model, KRAS mediated activation of ERK1/2 resulted in a decrease in the levels of Bim and consequently in apoptosis inhibition (Wiener et al., 2014).

As for a direct interaction of KRAS protein product and Bcl-2 family proteins resulting in either apoptosis or autophagy, the reports remain scarce and intangible. The few exceptions involve mitochondrial *KRAS* having been found to co-immunoprecipitate with Bcl-xL (Bivona et al., 2006). Additionally, Luca Scorrano's group postulated the importance of RAS binding site present on MFN2 (Brito & Scorrano, 2009), which physically bounds mitochondria and ER. Given that the ER pool of Bcl-2/ Bcl-xL seems to be highly responsible for regulating autophagy one might conjecture that MFN2 may act as a signalling platform from which KRAS can regulate autophagy and perhaps apoptosis as well. Actually, Hamasaki *et al.* has shown that the ER-mitochondria contact site is essential for the precipitation of the autophagic process, given that under starvation conditions several autophagic proteins localize at this site. Moreover, this group reiterated the relevance of the protein MFN2 by silencing its expression using a siRNA approach, which led to a great attenuation of autophagy (Hamasaki et al., 2013).

I.3 **Colorectal cancer therapeutic approaches**

CRC remains one of the main causes of death worldwide mainly due to the resistance to therapy inherent to this disease. Currently, there are four key approaches

to CRC management, ranging from conventional cancer treatments such as surgery, radiation and systemic cytotoxic chemotherapy, to more selective and targeted therapies based on increased understanding of tumour biology and on specific tumour subtypes (Abdullah & Chow, 2013). In early disease stages, surgical removal is the most effective course of treatment, in spite of this in late CRC stages alternative and adjuvant measures need to be taken (Hagan et al., 2013). A schematic overview of some of those adjuvant treatments are summarized in Figure I.6.



Inhibitor	Target(s)	Mechanism(s) of action
ABT-263 (BH3 minetics)	Pro-survival Bcl-2 and Bcl-xLproteins	Binding to the BH3 groove in Bcl-2 proteins
Cloroquine (CQ)	Lysosome	↑pH
Celecoxib	Cox-2	inhibition of prostaglandin synthesis
Quercetin	Free radicals (e.g$O2^-$ and OH^-)	Causes the overexpression of superoxide dimustase 2(SOD)
Lentinin	T-cells	T-cell- dependent immunopotentiation, balancing the Th1 Th2 tumour-associated populations

Fig. I.6 - **Schematic overview of some of the current adjuvant therapies available for CRC**. Illustration and enumeration of some of the existing therapeutic agents, their known targets and mechanisms of action.

A great majority of CRC chemotherapy regimens include a confined number of drugs, such as 5- Fluorouracil (5-FU), oxaliplatin and irinotecan, used either alone or in combination. All these cytotoxic agents mechanisms of action interfere with DNA

synthesis, thus promoting cell death (Bracht et al., 2010; Nallapareddy & Eckhardt, 2008).

However, a major challenge for conventional chemotherapy agents relies on their lack of specificity, which results in ineffective treatments with substantial toxicity side-effects (Walther et al., 2009). As a result, several tailored therapeutic agents have been designed. The rationale for this approach has been the identification of pathways that might be disrupted with these new biological targeted therapies, thus constituting a more proficient line of treatment (Tabernero et al., 2004).

One of the first selected targets was the Vascular Endothelial Growth Factor (VEGF), given that it has been demonstrated that the inhibition of the angiogenic process hinders both tumour growth and metastasis formation. The most successful anti-VEGF clinical agent is Bevacizumab - a recombinant humanised monoclonal antibody that specifically targets VEGF-A, which is synthesised during tumour growth. This antibody has the ability to prevent VEGF from interacting with appropriate receptors in vascular endothelial cells, consequently imparing the neovascularisation process of growing tumours (Zouhairi et al., 2011). Besides pinpointing cellular growth factors, cell surface receptors themselves have been chose as potential therapeutic targets, being the Epidermal Growth Factor Receptor (EGFR), due to its generalized overexpression in CRCs a main subbject of interest. Also, EGFR is the upstream effector of two extremely relevant signalling pathways in CRC - RAS-MEK-ERK and PI3K/AKT pathways (Heinemann et al., 2009). Indeed, two classes of anti-EGFR therapies exist: the monoclonal antibodies to EGFR (cetuximab and panitumumab) and tyrosine kinase inhibitors (TKIs), such as erlotinib and gefitinib (Hagan et al., 2013).

In addition to this, a cyclooxygenase-2 (COX-2) inhibitor – celecoxib – has also proved to be efficient, namely in the treatment of the hereditary form of CRC FAP (Roelofs et al., 2014; Tabernero et al., 2004). This enzyme is involved in the synthesis of prostaglandins and thromboxanes, which are regulators of biologic processes such as cell proliferation and angiogenesis, and has been found to be overexpressed in a considerable percentage of CRCs.

Moreover, BH3 minetics are a new class of anticancer drugs that mimic the function of endogenous BH3-only proteins (Bad, Bid, Bim, Puma, Noxa) and that serve to neutralize pro-survival Bcl-2 proteins (Adams and Cory 2007). Some reports in CRC

cell lines have shown that the use of BH3-only mimetics in combination with other inhibitors primes cells to undergo apoptosis. For instance, Corcoran and co-works showed that the co-inhibition of MEK by selumetinib and of Bcl-2 and Bcl-xL by the BH3-mimetic ABT-263 resulted in higher levels of apoptosis in HCT116 and SW620 cell lines (Corcoran et al., 2013). Another example of the beneficial achievement of a synthetic lethal drug cocktail was published this year by Faber *et al.* whose group undisclosed that CRC cell lines harbouring either mutated KRAS ($KRAS^{MUT}$) or BRAF ($BRAF^{MUT}$) exhibited greater sensitivity to the combined inhibition of Bcl-2, Bcl-xL and Mcl-1 than wild-type (WT) cell lines. This inhibition was attained through the employment of ABT-263 and of the TORC1/2 inhibitor AZD8055, having the latter a deleterious effect on Mcl-1 protein levels, in this fashion surpassing the inability of ABT-263 to inhibit this anti-apoptotic protein (Faber et al., 2014).

Nutraceuticals, also termed “functional food” or phytochemicals, are natural compounds (e.g essential oils and antioxidants) present in diverse alimentary sources that act by controlling DNA damaging factors and/or by regulating DNA transcription in cancer cells. Several coumpounds, namely quercetin (a natural antioxidant) or lentinan (a β-1,3-glucan) have proved their efficiency in CRC therapeutics, inclusively lentinan is used clinically in CRC treatment whereas quercetin has proved its efficiency *in vitro* and *in vivo* (Kuppusamy et al., 2014).

Furthermore, autophagy inhibition, in a number of CRC cell lines, has exhibited promising results as a therapeutic approach. Li *et al*. have shown that the combinatorial application of 5-FU and 3 – methyladenine (3-MA), a autophagy inhibitor, significantly enhanced cell death of HT29 and colon26 cell lines, being this increase in apoptosis associated with the decrease in the levels of expression of Bcl-xL protein (Li et al., 2009). Yet another group have determined that in HCT116 and HT29 cell lines the autophagy inhibitor chloroquine (CQ) in addition to chemoradiotherapy (5-FU + radiotherapy) leads to a decrease in cell viability in both cell lines, although a diminution in cellular proliferation was only observed in HT29 cells (Schonewolf et al., 2014). Additionally, Huang and Sinicrope demonstrated that the tripartite approach of the chemotherapeutic agent celecoxib + the BH3 mimetic ABT – 737 + autophagy inhibitor 3-MA resulted in a significant increase in apoptosis cell death in a diverse panel of CRC cell lines (Huang & Sinicrope, 2010), thus reinforcing that not only

autophagy targeting is an important therapeutic strategy, but also that a multiple-targeted approach may be the most reliable and successful course of action.

I.3.1 KRAS: a marker of therapy resistance in colorectal cancer

Nowadays, the extensive and accumulated knowledge of the pathologic and molecular mechanisms underlying cancer in general and CRC in particular have promoted a paradigm shift in terms of therapeutics. Indeed, the recognition of CRC as a highly heterogeneous disease combined with the massive advances in diagnosis tools have motivated the need of identifying clinical and/or molecular predictive markers to ensure appropriate use of targeted therapies (Hagan et al., 2013; Heinemann et al., 2013). Although, there is still a long way to go to systematically and clearly correlate the genetic background of a tumour to its responsiveness to a certain therapy and a longer way still to implement it routinely in clinic, presently, one of the most well-studied cases entail KRAS mutation status and EGFR therapy response (Lièvre et al., 2006; Mello, Marques, & Araújo, 2013; Misale et al., 2012).

EGFR is a transmembrane glycoprotein composed of an extracellular ligand binding domain and an intracellular tyrosine kinase activator domain. The growth factors which bind to this receptor include the transforming growth factor-alpha (TGF-α) and EGFs. Upon ligands binding, EGFR dimerizes and stimulates an intracellular phosphorylation cascade, mediated by the RAS-MAPK and/or the PI3K/AKT pathways, that stimulates migration, angiogenesis, cell cycle progression proliferation, survival of cancer cells and drug sensitivity (Herreros-villanueva et al., 2014) The main mechanism of EGFR activation, so far characterised in CRC, corresponds to the somatic mutations of KRAS oncogene (Fiore et al., 2010).

The prominence of KRAS mutations was truly recognized with the introduction of anti-EGFR monoclonal antibodies as a therapeutic regimen for metastatic CRC (mCRC). These were implemented in clinical use around 2004 and presented response rates of 10%–15% in mCRC (Hagan et al., 2013). Actually, in 2006, Lièvre and collegues conducted a study in which they correlated KRAS mutation status with decrease response to cetuximab therapy, being $KRAS^{MUT}$ significantly less responsive to this

antibody than $KRAS^{WT}$ tumours (Lièvre et al., 2006). Since then many other studies have supported this premise (Fiore et al., 2007; Amado et al., 2008; Lièvre et al., 2008). However, the broad and extensive study of this matter revealed that the subtype of $KRAS^{MUT}$ may also influence the efficacy of anti-EGFR treatment. Indeed, among the different mutations, $KRAS^{G13D}$ tumours have been associated with a better response to this therapy (Messner et al., 2013). Moreover, a more recent study resorted to the use of two CRC cell lines with different genetic backgrounds (DiFi: overexpress EGFR/ Lim1215: display "normal" EGFR levels), but with similar sensitivities to cetuximab (sensitive). Nevertheless, after continuous treatment with this agent both cell lines became resistant to treatment and that resistance was attributed to alterations in *KRAS* oncogene. In DiFi cells *KRAS* suffered a gene amplification and in Lim1215 this oncogene acquired *G13D* and *G12V* mutations. Both genetic alterations led to increased KRAS signalling output, therefore providing evidence that anti-EGFR therapy resistance is connected to KRAS augmented activity (Misale et al., 2012).

This year a groundbreaking study has unveiled yet another new mechanism though which KRAS mediates EGFR-inhibitors resistance. In a set of epithelial cancers, this group described that the expression of the integrin αvβ3 was correlated with resistance to erlotinib and that this resistance was mediated by an association of this adhesion molecule to KRAS, consequently forming a complex that could account for the drug resistance and stemness-like phenotypes manifested by these tumours due to the downstream signalling targets of these complex: RalB and its effectors, TBK1 and NFκB (Seguin et al., 2014).

All these studies reveal that, despite this being a thoroughly explored subject, there are still many debatable and controversial subtleties in need of further clarification. Nevertheless, $KRAS^{MUT}$ status is considered by the Food and Drug Administration (FDA) as a biomarker of reduced or absent efficiency of anti- EGFR therapies and since last year, both FDA and European Medicines Agency (EMA) recommend KRAS genetic testing before the administration of these drugs (Misale et al., 2014).

I.4 *Rationale* and aims

- ***Rationale***

KRAS mutations play an important role in the regulation of crucial cellular programs, such as autophagy. Our group has already established the pro-survival role of KRAS-mediated autophagy in the colon model (Alves et al., submitted). Nevertheless, many aspects of how KRAS regulates autophagy in the colon model and its implications in terms of CRC therapy need further investigation. Namely, it remains to be determined if upon autophagy induction by starvation the levels of KRAS change and whether KRAS alters its localization in response to this stimulus. Moreover, given that the Bcl-2 family members are important regulators of apoptosis-autophagy loop and that KRAS has been described to interact with Bcl-2 (Rebollo et al., 1999) and Bcl-xL (Bivona et al., 2006), in the latter case regulating apoptosis, we wondered whether KRAS acts as an upstream regulator of Bcl-2 family members in our model. Furthermore, we intended to explore the potential clinical impact of KRAS and/or autophagy inhibition on CRC therapy.

- **General aim**

Our **general aim** is to better understand how KRAS regulates autophagy in the colon model, towards the identification of new therapeutic approaches for CRC therapy.

- **Specific aims**

Our **specific aims** are in the frame of the following questions:

- Does KRAS levels change upon autophagy induction by nutrient starvation and can it be correlated with protein stability?

- Does KRAS re-localize to either the mitochondria or the lysosome upon starvation induction in the NCM460 cell model harbouring different KRAS mutations?
- Does starvation induced-autophagy affect the Bcl-2 family proteins?
- Do *KRAS* mutations regulate the levels of Bcl-2 family members?
- Can a combinatorial approach of KRAS/autophagy inhibition and anti-EGFR therapies overcome the resistance in CRC cells thus being a more efficient treatment strategy?

In order to answer these questions we used three cell lines:

- CRC derived cell lines: SW480 and HCT116, harbouring a $KRAS^{G12V}$ and $KRAS^{G13D}$, respectively.
- NCM460 cell line: Parental cells and cells infected with $KRAS^{WT}$, $KRAS^{G12D}$, $KRAS^{G13D}$ and $KRAS^{G12V}$.

II. MATERIAL AND METHODS

II.1 **Cell lines and culture conditions**

In order to achieve the aims proposed in the present study, several cell lines derived from human colon and bearing different genetic backgrounds were used, namely: SW480^{KRASG12V}, HCT116^{KRASG13D} and NCM460 cell lines.

SW480^{KRASG12V} is a human colon derived cell line (ATCC) that was established from a caucasian male and characterized as a Duke's class B colon adenocarcimona (Lebovitz et al., 1976). This cell line, which harbours a *KRAS*^{G12V} mutation, (Ahmed et al., 2013) was obtained from Cell Lines Bank from IPATIMUP/INEB and kindly provided by Maria Oliveira (IPATIMUP/INEB). HCT116^{KRASG13D}, a human CRC-derived cell line (ATCC) harbouring *KRAS*^{G13D} and *PIK3CA*^{H1047R} mutations (Ahmed et al., 2013; Shigeta et al., 2013) was provided by Muriel Priault (IBGC, CNRS). As for NCM460, a normal, non-transformed epithelial cell line derived from the human transverse colonic mucosa (Moyer et al., 1996) was received by a material transfer agreement with INCELL Corporation, San Antonio, TX. This cell line stably expresses FLAG-tagged wild-type KRAS (KRAS^{WT}) and three FLAG-tagged KRAS hotspot mutations (KRAS^{G12V}, KRAS^{G12D} and KRAS^{G13D}) previously constructed by our group through lentiviral infection (Alves et al., submitted).

SW480^{KRASG12V} and NCM460 cells were grown in RPMI 1640 medium with stable glutamine (Biowest) supplemented with 1% penicillin–streptomycin (Biowest) and 10% fetal bovine serum (FBS; Gibco, Invitrogen); HCT116^{KRASG13D} cells were propagated in McCoy's 5A (Biowest) 1% penicillin–streptomycin (Biowest) and 10% FBS (Gibco, Invitrogen). All cell lines were plated onto 25 cm^2 tissue culture flasks and maintained in a humidified incubator with 5% CO_2 at 37 ^oC, fed every 3 to 4 days and subcultured when 80% confluence was reached, by treatment with a 0.05% trypsin/EDTA solution (Sigma).

For the experiments cells were seeded onto six (1.5 mL) and twenty-four (500 µL) well plates at optimized densities (cell/well) for each cell line and protocol. To assess basal protein levels, cells were grown in complete medium until 80% confluence at the end of experience in the negative control. When protein levels were evaluated under starvation conditions, in order to carry out autophagy assays, cells growing in complete

medium were washed 3 times with PBS and incubated for varying time points (6,18 and 24 h) in Hank's Buffered Salt Solution (HBSS;PAA, Austria) buffered with 2.2 g/L $NaHCO_3$.

II.2 **Protein extraction and western blotting analysis**

II.2.1 Preparation of total protein extracts

Cells were treated for varying periods of time, depending on the drug used and experiment performed. After treatment, both floating and attached cells were collected and maintained on ice. Firstly, floating cells were directly collected to a 15 mL falcon tube, whilst attached cells were detached using a cell scraper and collected to the same falcon. All wells were washed with PBS 1x to ensure the collection of the remaining cells. Cells were then centrifuged at 2000 rpm for 10 min at 4 ^{o}C and the pelleted cells resuspended in 500 µL PBS 1x and transferred to eppendorf tubes. After this, cells were centrifuged at 2000 rpm for 5 min at 4 ^{o}C and supernatant was discarded. Cells were then lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40), supplemented with 20 mM NaF, 20 mM Na3VO4, 1 mM PMSF and 50 µL/mL protease inhibitor cocktail (Roche) and incubated on ice for 20 min. After a final centrifugation at 14 000 rpm for 15 min, the protein extracts present in the supernatants were transferred (5 µL) to a new eppendorf tube and kept at -20 ^{o}C until protein quantification. The remaining supernatant was transferred to another eppendorf tube and kept at -80 ^{o}C until western blot analysis.

II.2.2 Protein quantification

Protein quantification was performed using a Bio-Rad DC Protein Assay Kit, according to manufacturer instructions. This is a colorimetric assay to determine protein concentration following detergent solubilisation. This method entails the preparation of protein standards (from 0.25 mg/mL to 5 mg/mL) in RIPA buffer from a 5% BSA (bovine serum albumin) stock solution. After being prepared, 5 µL of each

protein standard solution was transferred to a 96-well plate. Moreover, 5 µL of RIPA buffer (blank) or 1 µL of sample + 4 µL of RIPA buffer were added to each well. Afterwards, 25 µL of reagent A'(1000 µL reagent A + 20 µL reagent S) and 200 µL of reagent B were added to each well. Consecutively, the plate was incubated for 15 min at room temperature (RT) in the dark and absorbance was read at 720 nm (Spectra Max 340PC – Molecular Devices).

The calibration curve was attained using the values of the BSA standards from which the concentration of the samples was extrapolated.

II.3 **Western blotting**

Protein samples were prepared by dilution in dH_2O and by addition of Laemmli sample buffer 4× (0.25 M Tris-HCl, 9.2% SDS, 40% glycerol, 5% β-mercaptoethanol, 0.5% bromophenol blue) (final concentration 1×). To certify a more efficient separation during electrophoresis, samples were denatured by heat (at 95 ^{o}C for 5 min), being then ready to be loaded onto the polyacrylamide gel.

The polyacrylamide gel is composed by 2 gels of different polyacrylamide concentrations, pH and separating efficiencies: A resolving gel (at a concentration of 15%, 12.5% or 10%) (375 mM Tris-HCl pH 8.8 SDS 0.4%, 0.1% TEMED, 0.05% APS), which allows an efficient protein separation and a stacking gel of 5% polyacrylamide (125 mM Tris-HCl pH 6.8 SDS 0.4%, 0.1%TEMED, 0.05% APS), permitting that proteins reach the resolving gel at the same time. A final volume of 20-30 µL of protein (25-50 µg) and 2 µL of marker (PageRuler™ Prestained Protein Ladder) were loaded and electrophoresis ran in a Mini-Protean III electrophoresis system at 100 V for about 1.30 – 2hs. The buffer used during electrophoresis was the Running Buffer (10×), which contains 0.25 M Tris base, 1.92 M Glycine, 1% SDS, however it must be diluted 1:10 before usage. After electrophoretic separation, proteins were transferred to PVDF (polyvinylidene difluoride; Thermo Scientific) membranes, using the Mini Trans-Blot System, Bio-Rad, at 100V for 2 hs, using Transfer Buffer (10× - 0.25 M Tris base, 1.92 M Glycine; final concentration 1×) and a cooling coil. Following this, membranes were blocked, a process that eliminates nonspecific binding sites on membranes, for at least

1 h at room temperature or overnight at 4 ^{o}C, with moderate stirring, in PBST (PBS 1× and 0. 1% Tween-20) containing 2.5% soy milk. After blocking, membranes were briefly washed in PBST and then incubated overnight with the primary antibody, at 4 ^{o}C, or for 1h at RT, using a Roller Mixer. After, to ensure total unbound primary Ab removal, the membranes were washed 3 times in PBST, 5 min each with moderate stirring. Subsequently, membranes were incubated for 1 h at room temperature with the secondary antibody conjugated with IgG horseradish peroxidase and successively washed 3-5 times in PBST, 7-9 min per wash. Immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.). Band intensity was quantified using ImageJ.

The primary antibodies used were the following: anti-KRAS, anti-FLAG, anti–Atg5, anti-Beclin-1, anti-Bcl-2, anti-Bcl-xL, anti-Bax, anti-Bcl-2, anti-Mcl-1 and anti-β-actin. As for the secondary antibodies, peroxidase conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch) and horseradish peroxidase-labeled goat anti-mouse immunoglobulin IgG (Jackson ImmunoResearch) were used (**Table II.1**).

Table II.1: List of the primary and secondary antibodies applied in Western Blot analysis

Primary antibody	Size	Temp (°C)	Dilution	Incubation time	Blocking solution dilution	Secondary antibody (1:2000)
Anti-KRAS (Santa Cruz)	21KDa	4°C	1:100	ON	2,5%Soy milk	Anti-mouse
Anti-FLAG (Sigma)	25KDa	4°C	1:1000	ON	2,5%Soy milk	Anti-mouse
Anti-Atg5 (Sigma)	60kDa	4°C	1:500	ON	2,5%Soy milk	Anti-rabbit
Anti- Beclin-1 (Sigma)	60KDa	4°C	1:2000	ON	2,5%Soy milk	Anti-rabbit
Anti-Bcl-2 (Leica)	26KDa	4°C	1:500	ON	5% non-fat milk	Anti-mouse
Anti-Bcl-xL (Santa Cruz)	30/34KDa	4°C	1:1000	ON	2,5%Soy milk	Anti-mouse
Anti – Bax (Sigma)	20KDa	4°C	1:5000	ON	2,5%Soy milk	Anti-rabbit
Anti- Mcl-1 (Leica)	41KDa	4°C	1:1000	ON	5% non-fat milk	Anti-rabbit
Anti-β-actin (Sigma)	42 KDa	RT	1:2000	1h	5% BSA	Anti-mouse

In order to re-probe the same membrane for immunodetection of different proteins, membranes were either stripped with β-mercaptoetanol stripping buffer (Tris-Hcl 1 M pH 6.8, β-mercaptoetanol and SDS 10%) for 30 min at 50 °C, and then washed 5 times with PBST for 5 min or with glycine stripping buffer (1 M Glycine and SDS 20%) for 20 min, at RT and then washed 2 times with PBST for 10 min.

II.4 **Immunofluorescence assay**

NCM460 cells stably expressing the different FLAG-KRAS constructs were seeded on glass coverslips at 3 x 10^5 cells/well in 12-well plates. Following autophagy induction by nutrient starvation, cells were fixed for 40 min with 4% paraformaldehyde (PFA) (Sigma), after which remaining aldehyde radicals from PFA fixation were removed using an ammonia chloride (NH_4Cl) solution at 50 nM. Afterwards cells were permeabilized with 0.1% SDS for 10 min, blocked with 3% BSA for 20 min and incubated with primary antibodies: anti-FLAG (Sigma), anti-LAMP2 (Abcam) and Anti-TOM20 (Santa Cruz) for 1 h at RT. This incubation with the primary Ab was followed by the addition of the secondary antibodies, FITC-conjugated anti-mouse (DakoCytomation) and AlexaFluor647 anti-rabbit (Molecular Probes) for 30 min. This procedure was performed for single staining. As for double-staining experiments, both TOM20 and LAMP2 were incubated first and overnight. On the following, day the secondary antibody anti-rabbit AlexaFluor647 was incubated for 1 h at RT. Following this, the anti-FLAG antibody was incubated for 1 h at RT, after which the secondary FITC-conjugated anti-mouse was added for 30 min. **Table II.2** summarizes the antibodies used in this assay. For both single and double stainings, in the last protocol step coverslips were mounted with Vectashield anti-fading solution containing DAPI onto a microscope slide. Finally, images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

Table II.2: Summary of the primary and secondary antibodies used ((1) represents single staining and (2) double staining experiments).

Primary antibody	Temp (°C)	Dilution	Incubation time	Secondary antibody	Dilution	Incubation time
Anti-FLAG (Sigma)	4°C	1:100	1 h,RT	FITC-conjugated anti-mouse	1:100	30 min, RT
Anti-LAMP2 (Abcam)	4°C	1:25	1 h,RT (1) ON (2)	AlexaFluor647 anti-rabbit	1:50	30min,RT
Anti-TOM20 (Santa Cruz)	4°C	1:100	1 h,RT (1) ON (2)	AlexaFluor647 anti-rabbit	1:50	30min,RT

II.5 **SRB assay**

To assess the anti-proliferative and/or cytotoxic effects of cetuximab, erlotinib and cycloheximide we performed sulphorhodamine B (SRB) test. This assay was first implemented by Skehan and colleagues in 1990 with the purpose of measuring drug-induced cytotoxicity and cell proliferation for large-scale drug screening applications (Voigt 2005). This colorimetric technique relies on the uptake of the negatively charged pink aminoxanthine dye, SRB by basic amino acids in the cells. Consequently, it indirectly gives the measurement of the number of cells present since it gives an estimate of the existing protein mass. Thus, when cells are lysed the intensity of colour observed is translated into absorbance, enabling in this way the calculation of the cellular mass (Houghton et al., 2007; Keepers et al., 1991). This method has been repeatedly and importantly applied, including in anticancer drug research (Ashraf et al., 2012; Lemos et al., 2009) due to its advantages, namely sensitivity, simplicity, practicality, reproducibility and better linearity in comparison to other tests such as MTT (Houghton et al., 2007; Papazisis et al., 1997).

NCM460 Parental, HCT116^{KRASG13D} and SW480^{KRASG12V} cell lines were seeded in 24-well plates at densities of 1.6x10^5 and 8x10^4, 2.76 x10^4 and 1.35x10^4, 6.4x10^4 and 3.2x10^4 cells per well (final volume 500 µL), respectively for 48 and 72 h for cetuximab

and erlotinb (using a range of different concentrations – cetuximab: 5 µg/mL – 100 µg/mL; erlotinib: 5 µM – 20 µM) treatments. As for cycloheximide treatment, SW480^{KRASG12V} and HCT116^{KRASG13D} cell lines were plated onto 6-well plates and treated with the drug at 100 µg/mL for 6,12,18 and 24 h; for this compound the plating densities were as follows: 6.8x10^5 (HCT116^{KRASG13D}) and 9.5x10^5 (SW480^{KRASG12V}) cells per well (final volume 1.5 mL). After treatment, culture medium was discarded; cells were then washed with PBS 1× and fixed with a solution of ice cold methanol containing 1% acetic acid for a minimum period of 1 h 30 min at – 20 ^{o}C. Following this step, the fixation solution was discarded; plates were dried at 37 ^{o}C, and then incubated with 250 or 500 µL of 0.5% (wt/vol) SRB dissolved in 1% acetic acid for 1 h 30 min at 37 ^{o}C. The dye that was not taken up by the cells was removed by repeated washings (2 to 3 times) with 1% acetic acid, whereas the bound dye was solubilized with 10 mM Tris base solution (pH=10). SRB dye was solubilized through mild plate agitation, and lastly, 200 µL from each well were transferred to a 96-well plate and absorbance was read at 540 nm (Spectra Max 340PC – Molecular Devices), using 10 mM Tris base solution as a blank. Results were normalized relatively to the negative control (untreated cells), which represents 100% of cell proliferation/cell viability.

II.6 **RNA interference assay**

RNA interference is a gene regulatory mechanism that limits the transcript level by a homology-dependent degradation process. In mammalian cells, small interfering RNAs (siRNA) are the product of an intricate machinery that carries out a process which entails: double strand RNA (dsRNA) cleavage by the RNaseIII endonuclease Dicer, involvement of the induced silencing complex (RISC), in which argonaute-2 (Ago-2), due to its active catalytic domain for cleavage activity cleaves and releases the sense strand, leading to an activated form of RISC (RISC-siRNA complex). Ultimately, this complex recognizes, binds to and degrades complementary mRNA, leading to the silencing of the target gene (Agrawal et al., 2003; Kim & Rossi, 2009)

II.6.1 *KRAS* and *BECN1* silencing in both complete and starvation conditions

siRNA sequences targeting human *KRAS* and *BECN1* were designed by Qiagen. The sense siRNA target sequences were: 5' –AAGGAGAATTTAATAAAGATA –3` (Hs_KRAS2_8) for *KRAS* silencing and 5' – CACTTGGTTCTTATACTGAAT – 3' (Hs_BECN1_5) for *BECN1* silencing. The control (non-silencing) siRNA target sequence was 5' – AATTCTCCGAACGTGTCACGT – 3' and was also designed by Qiagen. All siRNAs were resuspended in siRNA buffer as recommended by the manufacturer. Transfection was performed as follows: SW480^{KRASG12V} cells were plated at a density of 4x10^5 cells per well with a final volume 1 mL per well (250 µL OPTIMEM + Lipofectamine + siRNA mix + 750 µL cell suspension) in RPMI medium supplemented with 10% FBS. Firstly, an OPTIMEM (Gibco) and Lipofectamine 2000 (3 µL/well) (Invitrogen) mix was prepared and incubated 5 min at RT prior to the addition of the siRNA (at final concentration of 150 nM), in a total volume of 250 µL (per well). Secondly, after a-20 min incubation at RT this transfection mix (250 µL) as well as the cell suspension (750 µL) were added to the respective well followed by a gentle shake of the plate to allow the two to mix. Control cells (blank) were left untreated. All procedures were performed under RNAse free conditions. Thirdly, 24 hs later the medium was replaced by RPMI complete medium and 42 hs following transfection, in the assigned wells, HBSS medium was added after each well was washed 3 times with PBS 1X. Finally, 48 hs after transfection cells were harvested and protein extraction was performed.

II.6.2 *KRAS*, *ATG5* and *BECN1* silencing by siRNA in either the absence or presence of erlotinib

In addition to the aforementioned silencing sequences, the siRNA sequence targeting *ATG5* also designed by Qiagen 5' –AACCTTTGGCCTAAGAAGAAA – 3' (Hs_APG5L_6) was used in this assay. Twenty-four hs following the transfection the medium was replaced by RPMI complete medium and erlotinib at a concentration of 10 µM was added to the assigned wells. Finally, 24 hs after medium replacement, at

the consigned wells, HBSS medium with or without erlotinib (µM) was added. On the following day Annexin V/PI assay was performed.

II.7 **Annexin V/PI assay**

Studies of cellular apoptosis have been significantly impacted since the introduction of flow cytometry-based methods. Propidium iodide (PI) is widely used in combination with Annexin V to ascertain whether cells are viable, apoptotic, or necrotic relying on alterations in plasma membrane integrity and permeability. Annexin V binds to translocated phosphatidylserine, a phenomenon that happens in early apoptosis, whereas PI is a nucleic acid dye that only enters the cells when membrane integrity is lost, which occurs during late apoptosis/early necrosis. Thus, using Annexin V as a fluorescein isothiocyanate (FITC) conjugate together with propidium iodide (PI) as an exclusion dye for cell viability, this assay allows the detection of apoptotic cells and discriminates between apoptosis and necrosis (Rieger et al., 2011; Wilkins et al., 2002). Both floating and attached cells from each condition were collected to their respective falcon and maintained on ice. In order to collect attached cells, a cell scraper was used and each well was washed once with PBS 1X to ensure minimal cell loss. After this, cellular suspensions were centrifuged at 1000 rpm for 5 min and washed with PBS 1X followed by a second centrifugation identical to the previously described. Cells were resuspended and transferred to properly identified cytometer tubes. Following this, cells were incubated in 100 µL of ligation buffer, 5 µL of Annexin V and 10 µL of PI 50 µg/mL for 15 min. Finally, 200 µL of PBS 1X was added to each tube and acquisition was performed with an Epics XL-MCL (Beckman Coulter) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. At least ten thousand cells were counted per sample at low flow rate and data was analysed using *FlowJo* software.

II.8 **Statistical analysis**

Data are expressed as means ± SEM of at least two independent experiments. Statistical analysis was performed by One-way ANOVA test followed by Tukey's and Dunnet's Multiple comparison tests. Differences were considered significant when p-values ≤0.05 (* $p \leq 0.05$; ** $p \leq 0.01$;***$p \leq 0.001$). All statistical analyses were performed through the GraphPad Prism 5.0 software (San Diego, CA, USA).

III. RESULTS

III.1 **KRAS protein levels and stability upon starvation induction in non-cancer colon and colorectal cancer cell lines**

Previous results from our group, in the yeast *Saccharomyces cerevisiae ras2*Δ strain transformed with the pCM184 plasmid harbouring $KRAS^{WT}$ and the three *KRAS* hotspot mutations ($KRAS^{G13D}$, $KRAS^{G12D}$ and $KRAS^{G12V}$), under the control of a *tet-off* promoter (doxycycline-repressive promoter), showed that after nutrient starvation (autophagic stimulus) there was an increase in the level of KRAS protein correlated, in the case of $KRAS^{WT,}$ with increased autophagy levels (Alves et al., submitted)(SFig. VI.1). Taking into account these results, we set forward to understand whether in non-cancer colon (NCM460) and colorectal cancer (HCT116^{KRASG13D} and SW480^{KRASG12V}) cell lines the levels of KRAS protein also increased upon starvation induction.

III.1.1 KRAS protein levels upon starvation induction in normal colon cells

Similarly to what was performed by our group in the yeast model (Alves et al. submitted), we used NCM460 cells stably infected with a lentivirus vector harbouring *FLAG-*$KRAS^{WT}$ or the three different *FLAG-*$KRAS^{MUT}$ and analysed FLAG-KRAS as well as endogenous KRAS protein levels upon nutrient starvation. In NCM460 parental cells the levels of $KRAS^{WT}$ protein displayed a tendency to increase upon nutrient starvation induction, particularly after 6 h of incubation in starvation medium. In spite of this tendency being consistent, it was not statistically significant (Fig. III.1). Among NCM460 transformed cells, only in the cells infected with $KRAS^{WT}$ a similar tendency in endogenous KRAS levels was observed, being the endogenous levels of this protein, particularly in the case of cells infected with either $KRAS^{G12D}$ or $KRAS^{G12V}$, highly variable. Furthermore, contrary to the observed in the yeast model, we did not see an increase in FLAG-$KRAS^{WT}$ or FLAG-$KRAS^{MUT}$ protein levels in the same conditions. In fact, the level of FLAG-KRAS protein proved to be highly variable in this non-cancer colon cell model and did not reach statistical significance in starvation conditions (Fig. III.1).

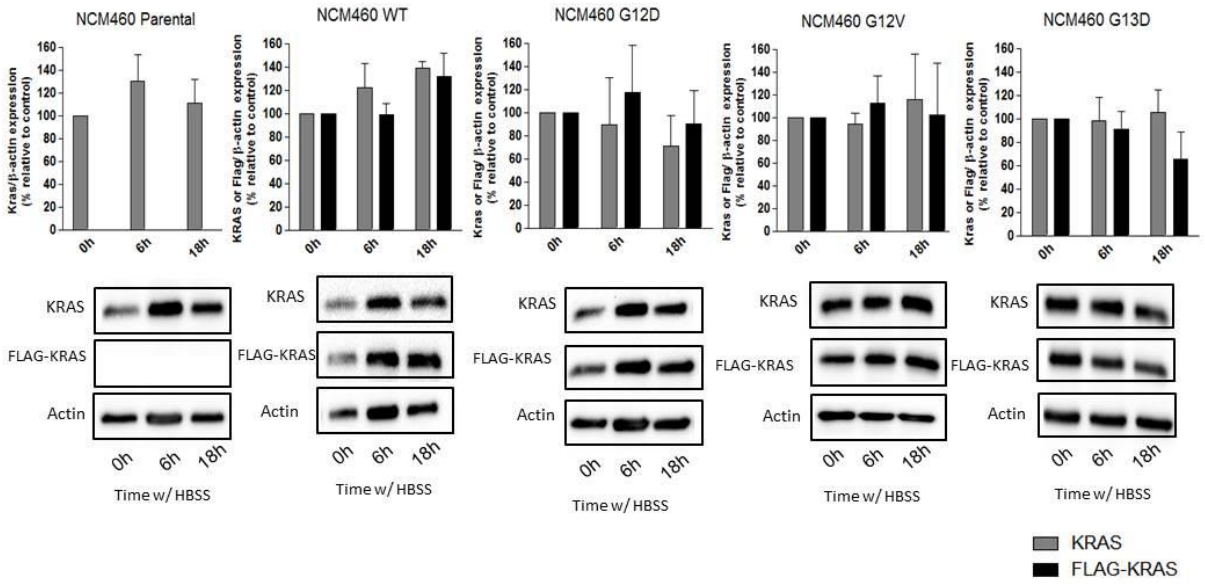


Fig. III.1 - **KRAS and FLAG-KRAS protein levels in NCM460 cells are not significantly altered upon starvation induction.** Endogenous KRAS and FLAG-KRAS levels were assessed by western blot in NCM460 parental cell line and in cells stably expressing *KRAS*^{WT}, *KRAS*^{G12D}, *KRAS*^{G12V} and *KRAS*^{G13D}. Cells were incubated in HBSS for 6 h and 18 h before protein extraction. FLAG antibody was used to detect exogenous KRAS. FLAG-KRAS and endogenous KRAS levels were firstly normalized relative to actin and then to the 0h, no significant alterations being observed. Representative western blots are shown for NCM460 cells. Quantification was performed using *ImageJ* software. The results presented are the mean ± SEM of at least two independent experiments. Statistical analysis was estimated by One-way ANOVA test followed by Tukey's test .

III.1.2 KRAS protein levels and stability in colorectal cancer cell lines

Taking into account the results obtained in the NCM460 model, we wanted to address the same question using two CRC cell lines – HCT116^{KRASG13D} and SW480^{KRASG12V}, harbouring *KRAS*^{G13D} and *KRAS*^{G12V} mutations, respectively (Ahmed et al., 2013). Moreover, we also wanted to unravel if the putative increase in KRAS levels was due to a higher stability of the protein. For that purpose cells were incubated with the protein synthesis inhibitor cycloheximide (CHX) at a concentration of 100 µg/mL. At the cellular level, CHX impairs the translation of mRNA on 60S ribosomes

(Schneider-poetsch et al., 2010). In nutrient-depleted medium (Fig.III.2a), the level of $KRAS^{MUT}$ protein fluctuates in the presence or absence of CHX, in both CRC cell lines. NCM460 parental cell line harbouring $KRAS^{WT}$ was used as a control in order to determine if KRAS mutation status had any influence in proteins stability. In spite of the variability observed, after 12 h in HBSS the level of protein decreased in the absence of CHX, while it increased in the presence of this inhibitor, being this true for all the cell lines. Moreover the only cell line harbouring $KRAS^{WT}$ showed a decrease in the levels of KRAS protein after 18 h of incubation in HBSS in the presence of CHX, suggesting that $KRAS^{WT}$ is less stable than $KRAS^{G13D}$ and $KRAS^{G12V}$ mutations. In addition to this, we performed the same experiment in complete medium. Also in these experimental settings, KRAS protein levels did not alter among time points and with respect to the presence or absence of the protein synthesis inhibitor CHX in both CRC cell lines (Fig. III.2b). In order to determine if CHX, at the concentration of 100 µg/mL, was affecting cell proliferation we performed a SRB assay (Fig. III.2c). We observed that in complete medium with CHX there was a constant decrease tendency in cell proliferation in both cell lines, whereas in HBSS medium in the presence of CHX there was an increase in cell proliferation, with exception of the 12 h time point. In spite of the observed increase in cell proliferation in HBSS medium, it is neither constant nor accentuated. Thus, even though further experiments need to be performed in order to confirm these results, they suggest that our CHX concentration in inhibiting protein synthesis and that cell number variation was not affecting our results.

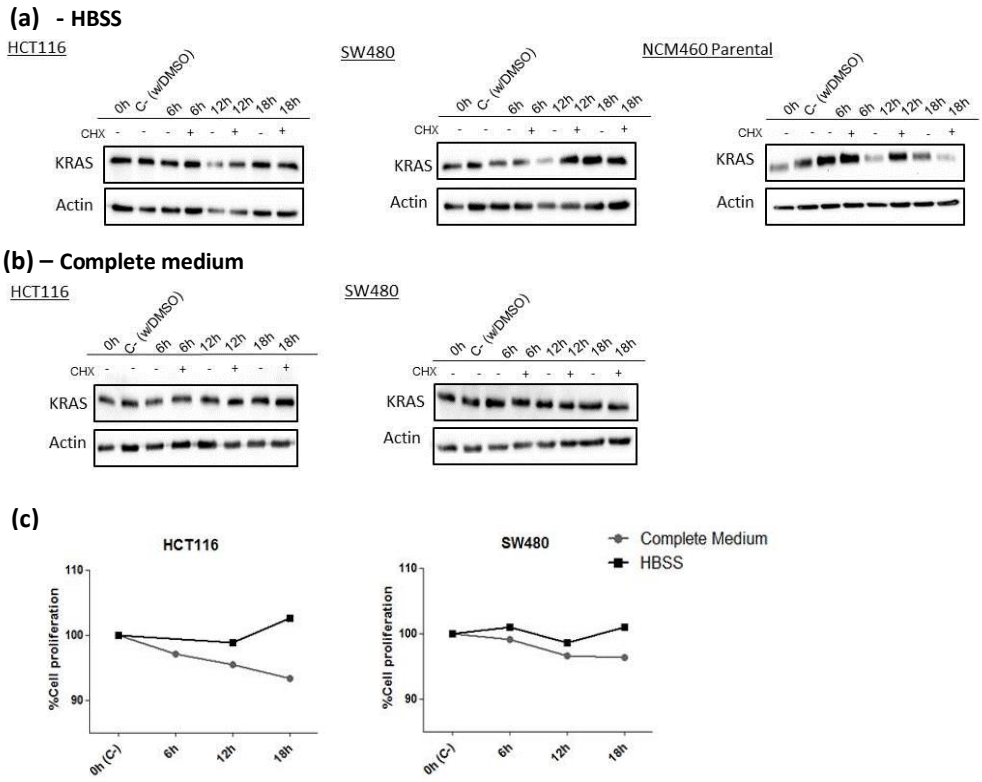


Fig.III.2 **– KRAS levels and stability are not changed in CRC cell lines either in basal conditions or upon starvation induction.** Cells were incubated with CHX for 6, 12 and 18 h before protein extraction and KRAS protein levels were analysed in the presence and absence of cycloheximide (CHX) at 100 µg/mL, both in HBSS **(a)** or complete medium **(b)**. In CRC cell lines, SW480^{KRASG12V} and HCT116^{KRASG13D} KRAS^{MUT} levels remained unaltered for a period of 18 h in HBSS **(a)** and in complete medium **(b)**, whereas in the non-cancer colon cell line NCM460, KRAS levels appear to decrease and stability is lost after 18 h **(a)**. **(c)** SRB assay in HCT116^{KRASG13D} and SW480^{KRASG12V} at 6, 12 and 18 h after CHX addition suggests that the CHX dose is affecting cell proliferation in both CRC cell lines.

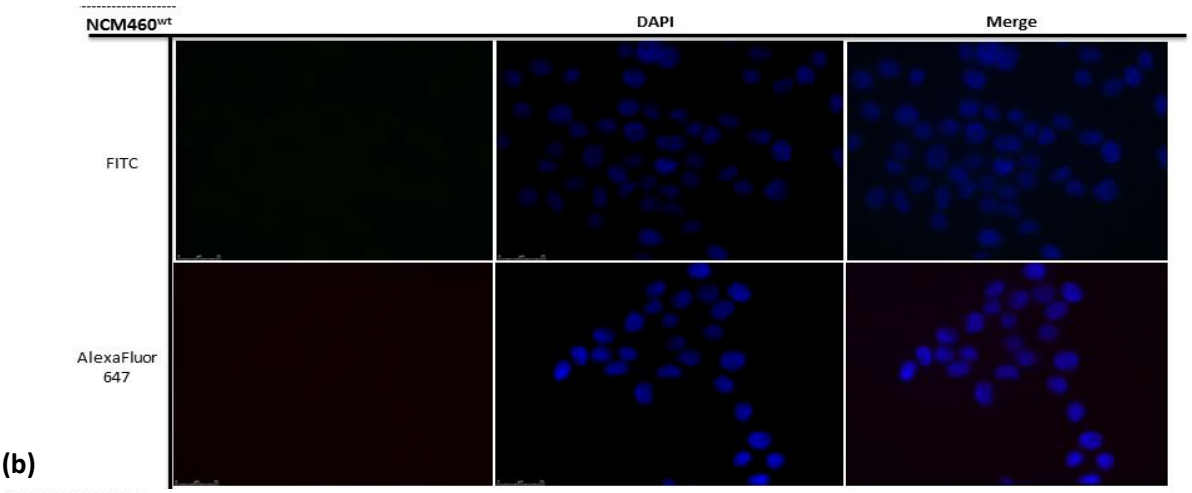
II.2 **Study of KRAS cellular localization in NCM460 cells upon starvation induction**

Several reports have described that KRAS has been found to localize at cellular membranes, other than the plasma membrane, namely at the mitochondria (Bivona et al., 2006) and at the lysosome (Lu et al., 2009) in response to additional PTMs and to growth factor deprivation, respectively. Even though that under nutrient starvation conditions KRAS^{MUT} has been shown to induce autophagy associated with

mitochondrial changes (e.g. metabolism, membrane polarization and ROS production) (Kim et al., 2011), whether this stimulus induces $KRAS^{MUT}$ re-localization to this or to another intracellular organelle has not been established yet.

In order to test this hypothesis, we used three antibodies: anti-FLAG antibody – which allowed us to assess the localization of the FLAG-$KRAS^{WT}$ and FLAG-$KRAS^{G13D}$ constructs in NCM460 cells; LAMP2 antibody – which marks lysosomes and TOM20 antibody – that served as a mitochondrial marker. We optimized the concentration of each antibody (Fig.III.3). Our group had already observed a FLAG-KRAS re-localization in NCM460 cells under starvation conditions (SFig. VI.2). Anti-FLAG antibody had already been optimized, and we were able to reproduced the results obtained (Fig. III.4a, 4b). Using TOM20 antibody, we evaluated if the mitochondrial network suffered alterations after incubation with HBSS medium and we observed that the staining pattern is more diffuse in cells incubated in complete medium in comparison to cells incubated in HBSS medium (Fig. III.4b).

(a)


NCM460wt
DAPI
Merge
FITC
AlexaFluor 647

(b)

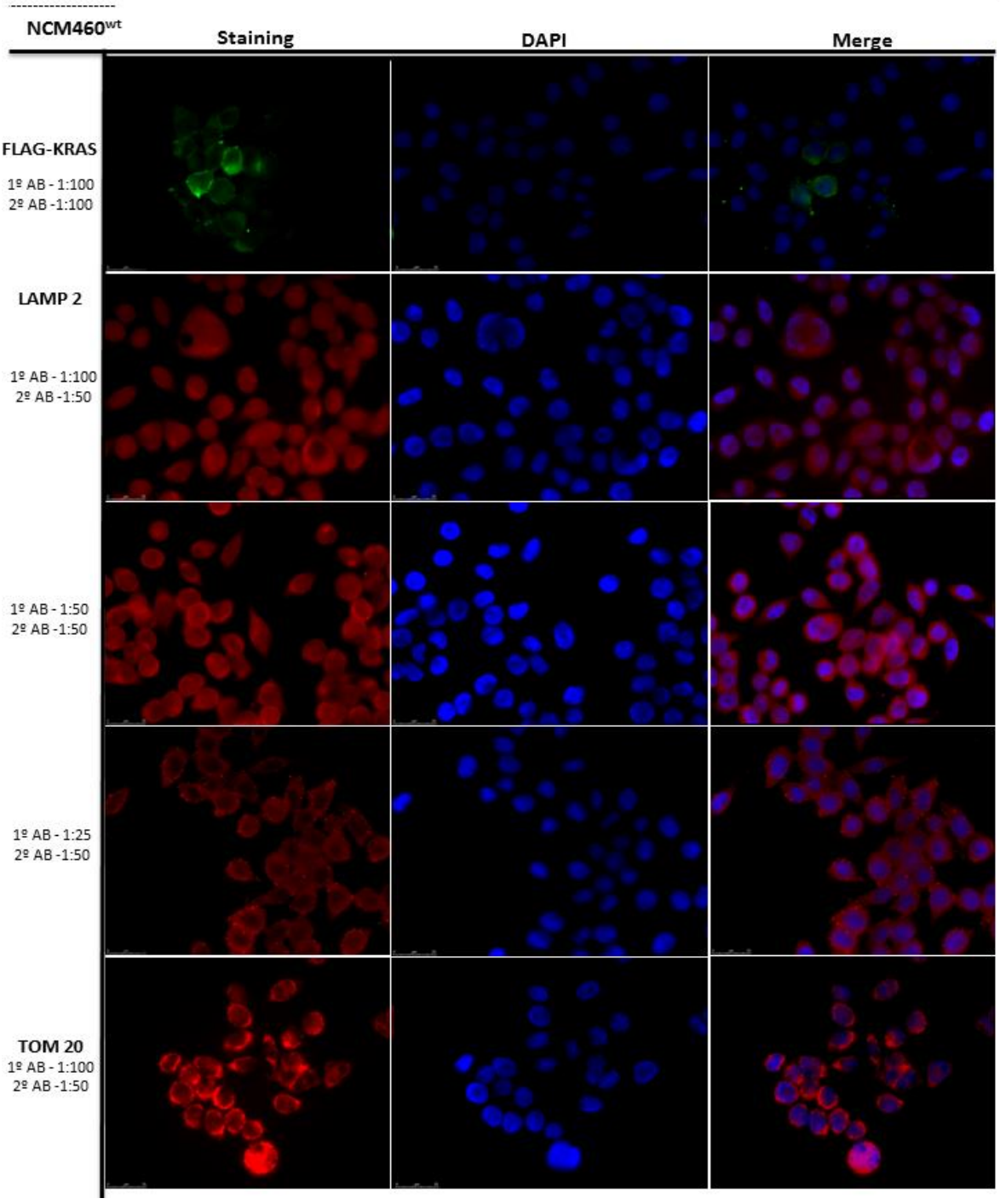

NCM460wt
Staining
DAPI
Merge
FLAG-KRAS
1º AB - 1:100
2º AB -1:100
LAMP 2
1º AB - 1:100
2º AB -1:50
1º AB - 1:50
2º AB -1:50
1º AB - 1:25
2º AB -1:50
TOM 20
1º AB - 1:100
2º AB -1:50

Fig. III.3 – **Optimization of LAMP2 and TOM20 antibodies concentrations in immunofluorescence. (a)** Secondary antibodies control in NCM460 FLAG-KRAS^{WT} cell lines, anti-mouse FITC (green) and anti-rabbit AlexaFluor647 (red). **(b)**Fluorescence microscopy images of Anti-FLAG, anti-LAMP2 and anti-TOM20 antibodies in NCM460 FLAG-KRAS^{WT} cell line in complete medium – 40x magnification.

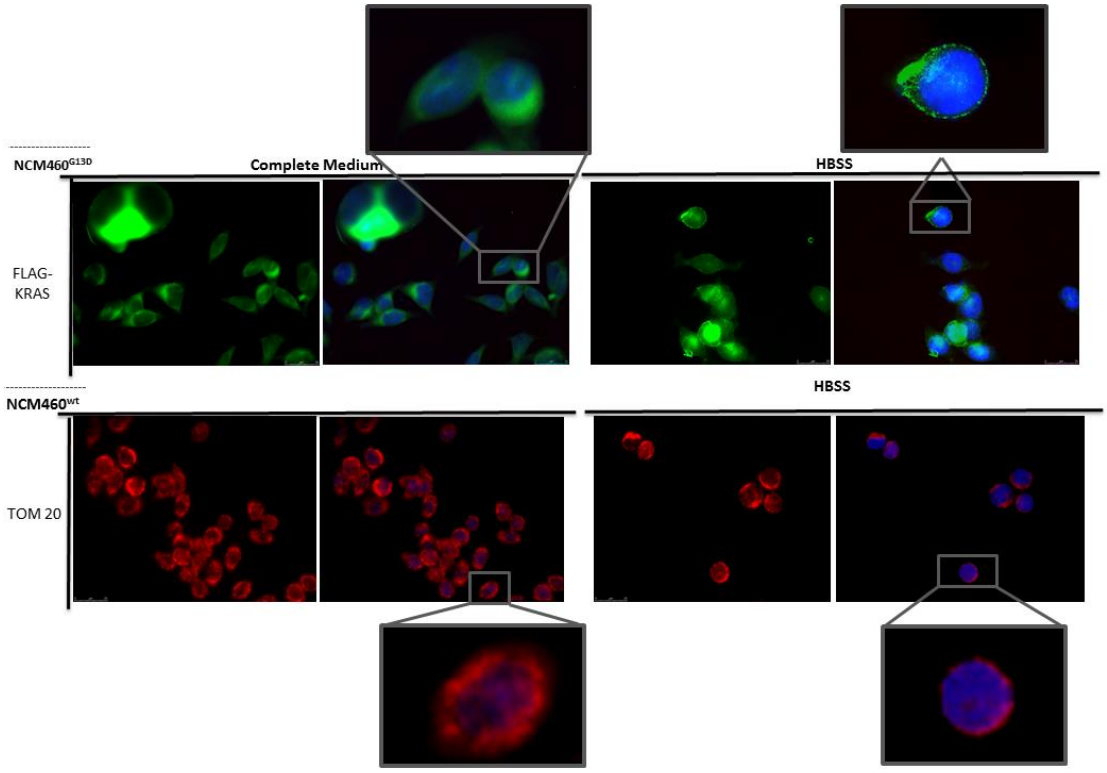


Fig. III.4 – Starvation induces FLAG- KRAS re-localization and causes morphological alterations in mitochondrial networks in NCM460 cells. (a) After a 6 h incubation in HBSS medium FLAG -KRAS changes its intracellular distribution from an uniform scattering along the plasma membrane to a more punctuate and sub-membrane localization in NCM460 FLAG-KRAS^{G13D} cells. **(b)** In NCM460 FLAG-KRAS^{WT} the mitochondrial network acquires a more perinuclear distribution after 6 h in starvation medium – 40x magnification.

We them performed co-localization of FLAG-KRAS^{WT} with either the mitochondria (TOM20) or lysosome (LAMP2) in cells maintained in complete medium (Fig. III.5).Both secondary antibodies, anti-mouse FITC (green) and anti-rabbit AlexaFluor647 (red) did not cross-react when co-incubated without primary antibodies (Fig. III.5a), but when we performed the complete co-immunofluorescence the signal of one of the primary antibodies was masked, thus not allowing to conclude about the localization of FLAG-KRAS upon starvation induction in NCM460 cells (Fig III.5b).

Consequently, further optimizations are needed to ascertain to which structures KRAS localizes (Fig. III.4a) under starvation conditions.

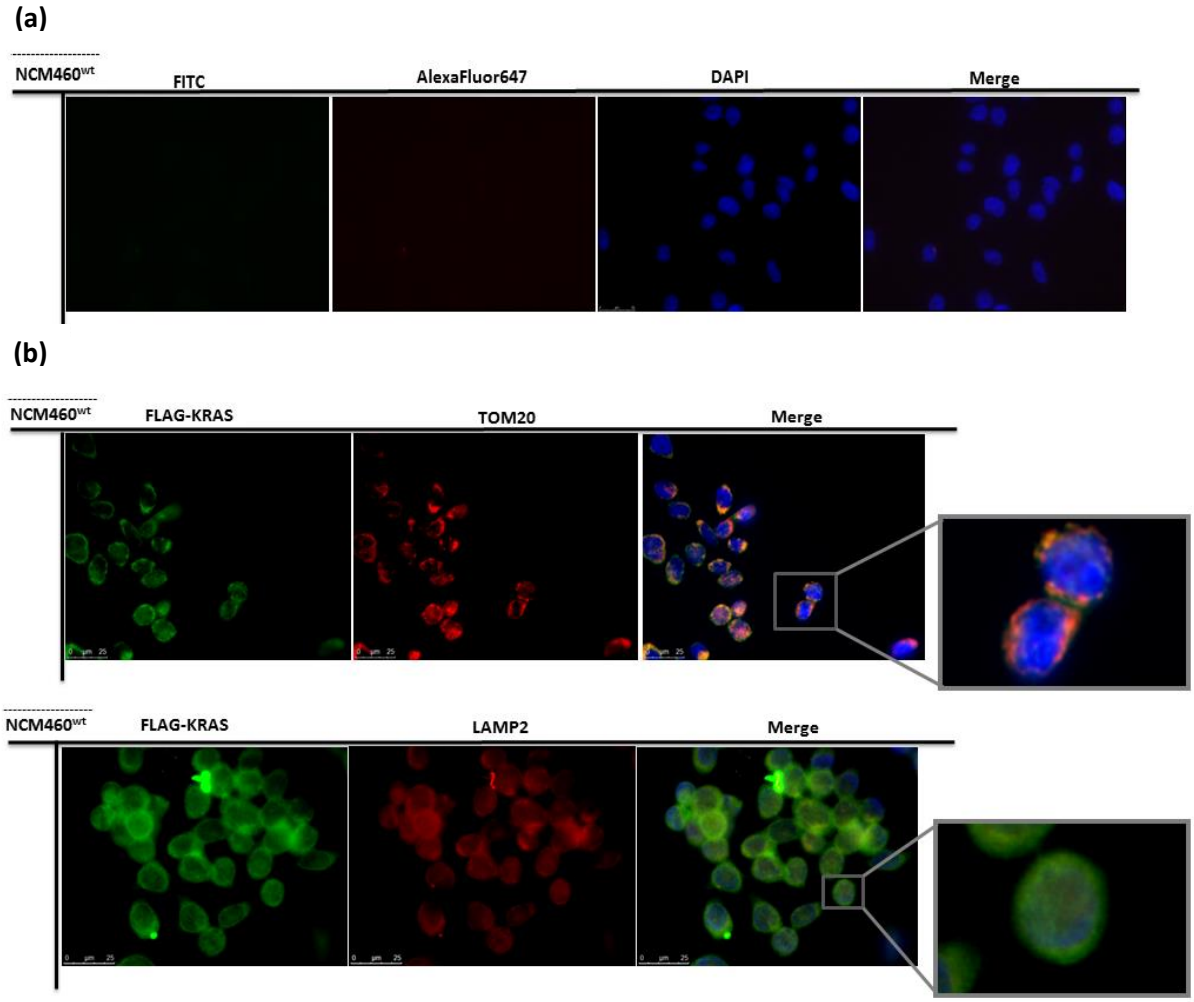


Fig. III.5 - Co-immunofluorescence of FLAG-KRAS with either mitochondria (TOM20) or lysosome (LAMP2) in NCM460 FLAG- KRAS^{WT} cells in complete medium. (a) Co-incubation of both secondary antibodies, anti-mouse FITC (green) and anti-rabbit AlexaFluor647 (red) without any of the primary antibodies. **(b)** Fluorescence microscopy micrographs of co-immunofluorescence assay of FLAG-KRAS (FLAG) + mitochondria (TOM20) and of FLAG-KRAS (FLAG) + lysosome (LAMP2) in NCM460 FLAG-KRAS^{WT} cells under nutrient-rich conditions – 40x magnification.

III.3 **Role of KRAS and autophagy in the levels of Bcl-2 protein family in colorectal cancer cell lines**

The Bcl-2 family of proteins is one of the molecular links between the mitochondrial-dependent apoptotic pathway and autophagy (Mariño et al., 2014),

having their role in autophagy been repeatedly documented (Wei et al., 2008; Altman et al., 2009; Priault et al., 2010). Taking that into account, as well as the fact that the mitochondria was hypothesized as one of the possible locations of KRAS in the NCM460 cell model upon autophagy induction and given that this organelle is also an important platform for many members of the Bcl-2 proteins family (Bivona et al., 2006; Tait et al., 2010) we set forward to explore if/how autophagy influences these proteins levels.

III.3.1 Protein levels of Bcl-2 family members under basal and starvation conditions

With the purpose of determining whether autophagy affects the levels of some members of the Bcl-2 family, including Beclin-1, Mcl-1, Bcl-2, Bcl-xL and Bax proteins in the CRC cell lines HCT116^{KRASG13D} and SW480^{KRASG12V}, we determined the optimal amount of protein that allowed the assessment of the levels of expression of the members of the Bcl-2 family and decided to proceed to further experiments with the concentration of 50 µg/µL (Fig. III.6). Starvation was induced by incubating cells 6 h in HBSS medium. This period of incubation was selected, as after analyzing the basal levels of these proteins under complete and starvation medium we intended to proceed to a siRNA assay, being the time of incubation in HBSS for this experiment of 6 h, as optimized by our group.

Atg5-Atg12 complex (using an anti-Atg5 antibody) was used as an autophagic marker, because this complex is involved in the formation of autophagosomes (Mariño et al., 2014), and it increases when autophagy is promoted. Although not reaching statistical significance, a tendency for Atg5-Atg12 increase was observed in both cell lines. In order to confirm these results, another autophagy marker, such as LC3-I/II, should be used. As for the Bcl-2 family proteins, none of the proteins belonging to this family varied its expression significantly in response to starvation induction, in both CRC cell lines. In HCT116^{KRASG13D} cells, with the exception of Bcl-2, all the other anti-apoptotic proteins, which are generally reported to be anti-autophagic, levels (Bcl-xL and Mcl-1) (Erlich et al., 2007; Kang et al., 2011) were diminished in starvation conditions, nonetheless this decrease was not accompanied by an increase in pro-

apoptotic Bax protein levels (Fig. III.6). Beclin-1 also maintained a constant level under starvation conditions. In SW480^{KRASG12V} cells, the levels of Beclin-1, Bcl-xL and Bax remained unchanged in HBSS medium. Curiously, KRAS proteins levels appeared to be significantly increased upon autophagy promotion in SW480^{KRASG12V} cell line (Fig. III.6), an effect that is not in accordance with results obtained in the same cell line and in similar experimental settings (Fig.III.2a). This indicates that KRAS levels are very changeable under autophagy promoting conditions, at least in SW480 cells.

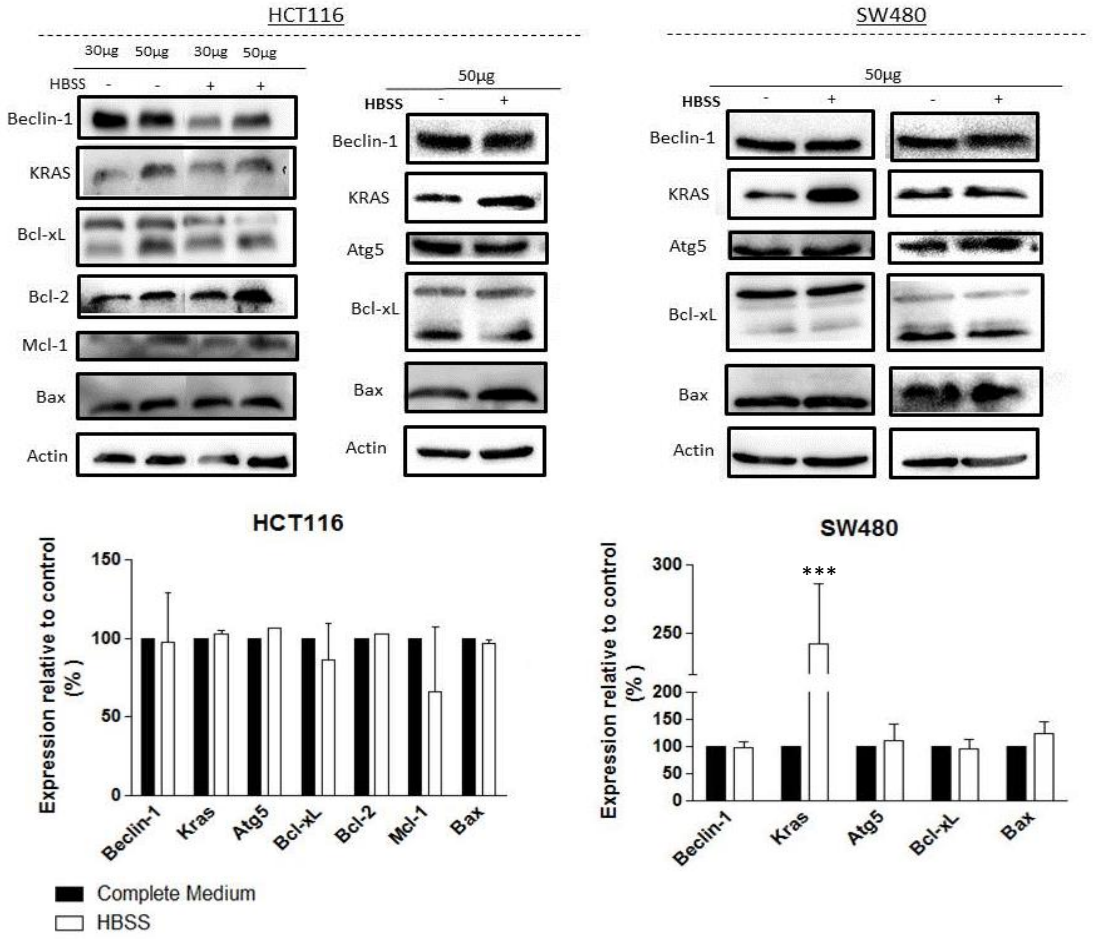


Fig. III.6 **- Bcl-2 family proteins levels do not change significantly in colorectal cancer cell lines upon starvation induction.** The levels of some members of the Bcl-2 protein family (Bcl-2, Bcl-xL, Mcl-1, Beclin-1 and Bax in HCT116^{KRASG13D} cell line; Bcl-xL, Beclin-1 and Bax in SW480^{KRASG12V} cell line) were analysed in both complete and HBSS medium. No significant changes were observed for any of these proteins either in HCT116^{KRASG13D} or SW480^{KRASG12V} cells. Western blott quantification was performed using *ImageJ* software and protein levels were normalized relative to actin and then to complete medium. Results are the mean ± SEM of at least two

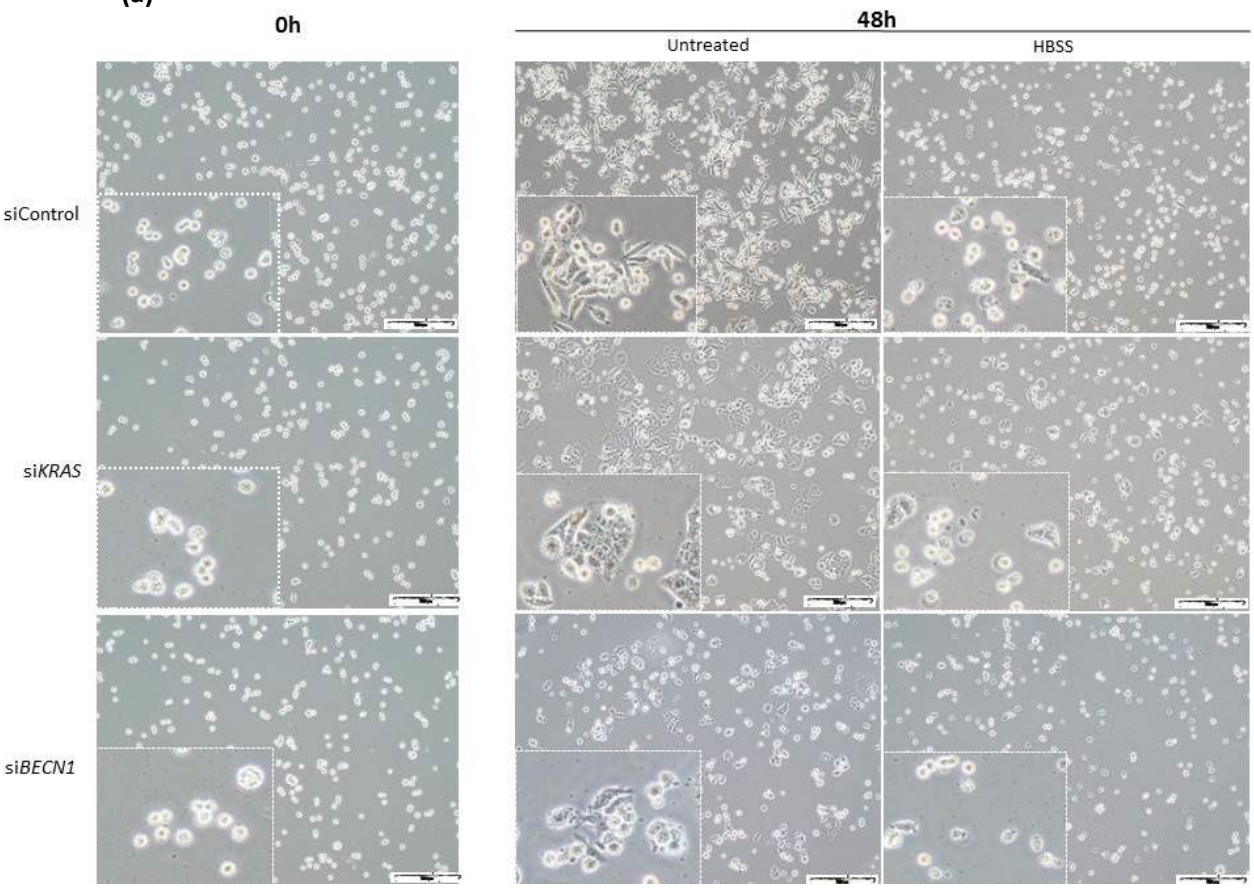
independent experiments. Statistical significance was estimated by One-way ANOVA test followed by Tukey's Multiple Comparison test ***p≤0.001.

III.3.2 Levels of Bcl-2 family members under basal and starvation-inducing conditions after *KRAS* or *BECN1* silencing in SW480^{KRASG12V} cells

Aiming at unveiling if KRAS plays a part in the regulation of the levels of some of the proteins of the Bcl-2 family (Beclin-1, Bcl-xL, Bcl-2, Mcl-1 and Bax) we inhibited *KRAS* by RNA interference (RNAi) in SW480^{KRASG12V} cell line, so that we could determine whether the silencing of this gene influenced the amount of any of the aforementioned proteins. In addition to *KRAS* silencing, we also silenced *BECN1* (Beclin-1 coding gene), since Beclin-1 is documented to physically interact with both Bcl-2 and Bcl-xL, and in that way controlling autophagy (Young & Wang, 2013). Our results revealed that both *KRAS* and *BECN1*, although to apparently different extents, exert a regulatory mechanism on some members of the Bcl-2 family protein. In complete medium, *KRAS* silencing led to a decrease of Bcl-xL and to an almost complete abrogation of Mcl-1 and Bcl-2 proteins. In nutrient-replete conditions, *KRAS* silencing caused a slight decrease in Bax levels. In nutrient-depleted conditions, *KRAS* silencing originated a minor increase in Bax levels as well as a major augment in Mcl-1 expression. Both Bcl-xL and Bcl-2 expression levels were similar to those registered in complete medium. *BECN1* silencing resulted in an augment in the levels of Bcl-2 family proteins, with the exception of Bcl-2, whose expression was not affected by *BECN1* depletion. Moreover, the effect of *BECN1* silencing on Bax and Bcl-xL levels of expression was less evident in comparison to Mcl-1 (Fig III.7b). In nutrient starvation medium, *BECN1* depletion was not correlated with Bcl-xL overexpression, whilst both Bax and Mcl-1 levels were increased. Bcl-2 levels did not suffer any alteration, similarly to what was observed in complete medium after *BECN1* silencing. Atg5 levels did not change much both in complete and HBSS medium as well as after *KRAS* and *BECN1* knockdown, although an increasing tendency was observed either in *KRAS* or *BECN1* silencing in starvation-medium. In response to each other silencing, both KRAS and Beclin-1 marginally diminished their expression levels in nutrient-replete and depleted medium (Fig. III.7b).

Morphologic alterations after *KRAS* and *BECN1* silencing as well as after and in combination with starvation induction were visible in cells in culture as shown in Fig. III.7a. Apart from the obvious differences in confluence, demonstrating control siRNA condition to have more cells than either *KRAS siRNA* or *BECN1 siRNA* after 48h. *KRAS* silencing appeared to impair cells to properly stretch and acquire their normal morphology while KRAS-depleted cells exhibited more aggregates. As for *BECN1* silencing, it seemed to affect cell elongation to a greater extent than *KRAS* silencing, being these cells more round-like. Nutrient starvation clearly made cells adopt a more round morphology and reduced cell confluence in all conditions (control siRNA, *KRAS* siRNA and *BECN1* siRNA).

(a)



(b)

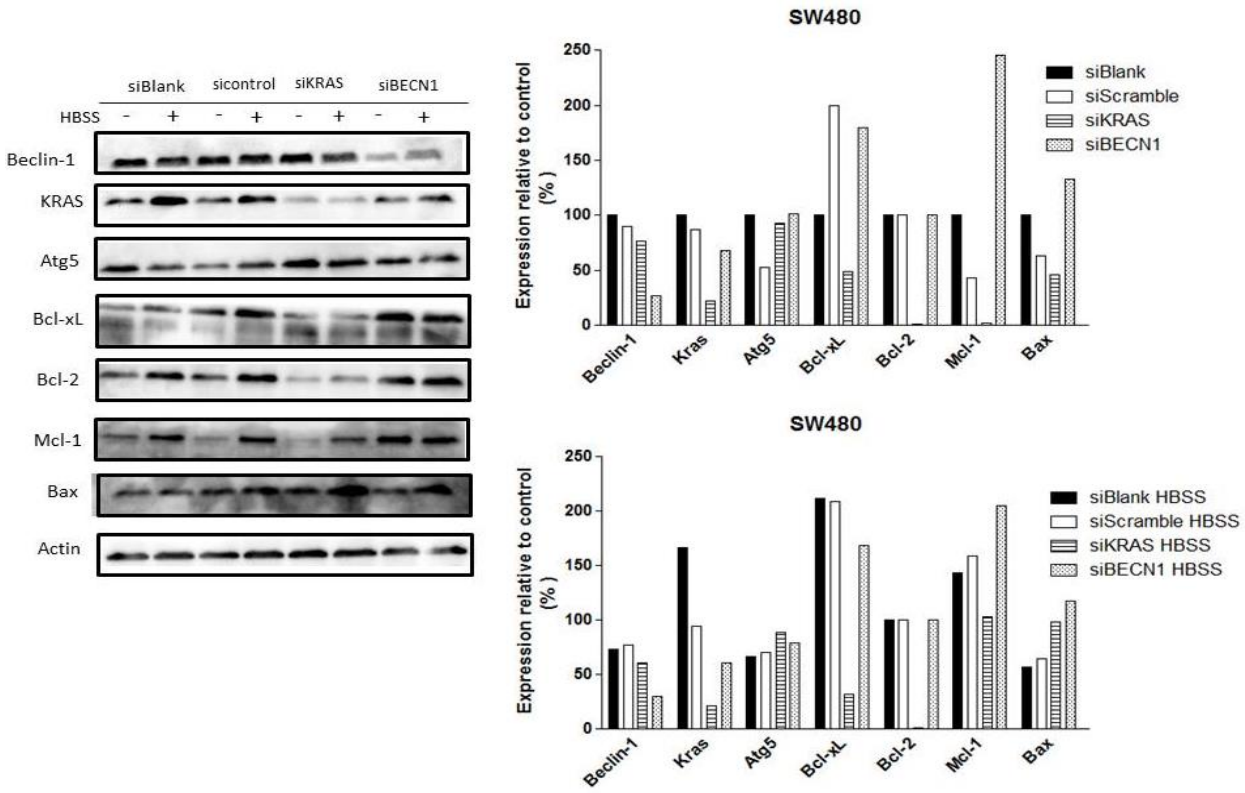


Fig. III.7 **- KRAS and Beclin-1 regulate Bcl-2 family proteins**. **(a)** Representative phase contrast photos of the modifications in cell morphology in SW480^{KRASG12V} cells following the silencing of *KRAS* and *BECN1* both in complete medium and after 6h incubation in HBSS – phase contrast 10x magnification. **(b)** In SW480^{KRASG12V} cell line, KRAS and Beclin-1 both modulate Bcl-2 family proteins in complete and HBSS medium. Western blott quantification was performed using *ImageJ* software and protein levels were normalized in relation to actin.

III.4 **Effect of EGFR inhibitors in non-cancer colon and cancer cell lines with KRAS mutations**

KRAS^{MUT} is a recognized biomarker of anti-EGFR therapies, since KRAS mutation status has been repeatedly associated with resistance to these therapies (F. D. Fiore et al., 2007; Lièvre et al., 2006, 2008). To test our CRC cell lines response/resistance to anti-EGFR targeted therapies we used: the monoclonal antibody cetuximab (Fig.III.8) and the tyrosine kinase inhibitor erlotinib (Fig. III.9/10).

III.4.1 Effect of cetuximab on cellular proliferation in non-cancer colon and colorectal cancer cell lines

We initiated by testing cetuximab, since this therapeutic agent has been applied in clinic and unsuccessfully in KRAS^{MUT} genetic backgrounds (Amado et al., 2008; Fiore et al., 2007; Lièvre et al., 2006, 2008). From an extensive search in literature concentration range was chosen (Ashraf et al., 2012; Dahan et al., 2009; Jhawer et al., 2008). Also, our search revealed that in the majority of literary publications both SW480^{KRASG12V} and HCT116^{KRASG13D} are both deemed resistant to anti-EGFR therapy (Dunn et al., 2011; Troiani et al., 2014; Wild et al., 2006), nonetheless, a few reports have found SW480^{KRASG12V} cells to be more sensitive to cetuximab than HCT116^{KRASG13D} cell line (Shigeta et al., 2013; Seo et al., 2014). In spite of this, both cell lines appeared to be equally resistant to this drug (Fig. III.8). However, to certify that our results stemmed solely from the fact that our CRC cell lines were resistant to cetuximab we used NCM460 parental cell line, which does not harbour a KRAS^{MUT} or other genetic mutations (Moyer et al., 1996) that are viewed as predictive biomarkers of cetuximab therapy resistance (Jhawer et al., 2008). Even in this cell line we could not observe any effects on cell proliferation (Fig. III.8). Moreover, we tested two much higher doses of this mAb – 200 and 400 µg/mL- in SW480^{KRASG12V} cell line (data not shown) and once again a decrease in cell proliferation of more than 20% could not be attained, as a consequence we considered that this drug was not active and selected another anti-EGFR inhibitor erlotinib.

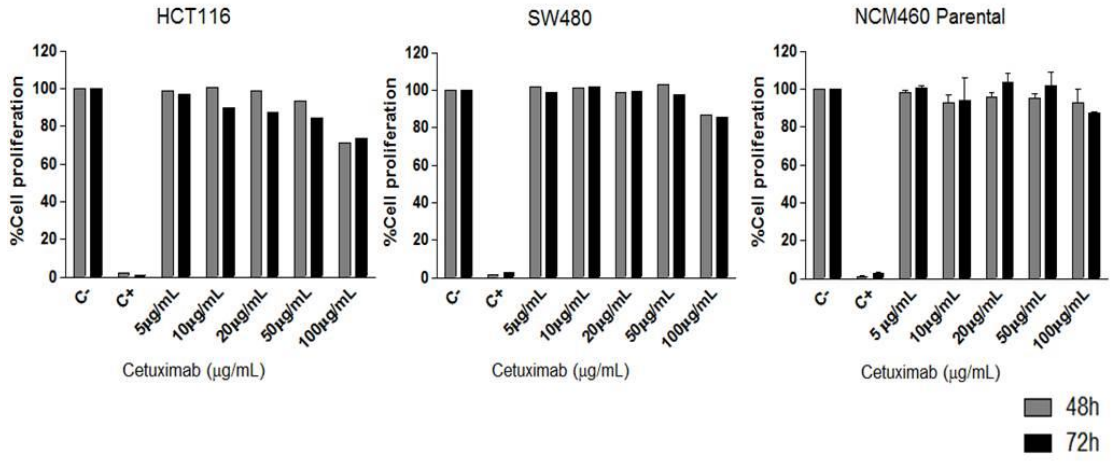


Fig. III.8 - **Cetuximab does not present an anti-proliferative effect in non-cancer colon and CRC cell lines.** Cetuximab does not affect cell proliferation in HCT116^{KRASG13D}, SW480^{KRASG12V} and NCM460 parental cell lines after either 48 or 72h of incubation even at the highest dose employed (100 µg/mL). Results are the mean ± SEM of at least two independent experiments. Statistical analysis was performed using One-way ANOVA test followed by Dunnet's test, no significant results were obtained.

III.4.2 Effect of erlotinib on cellular proliferation in colorectal cancer cell lines

Since our results indicated that our batch of the monoclonal antibody cetuximab was not active, we decided to test another EGFR inhibitor – erlotinib. We determined its effect on cell proliferation by SRB assay using an increasing set of concentrations ranging from 5 µM to 20 µM. We observed a decrease in cell proliferation in both SW480^{KRASG12V} and HCT116^{KRASG13D} cell lines, being that decrease statistically significant for SW480^{KRASG12V} cells from the concentration of 10 µM and for HCT116^{KRASG13D} from the concentration of 5 µM.

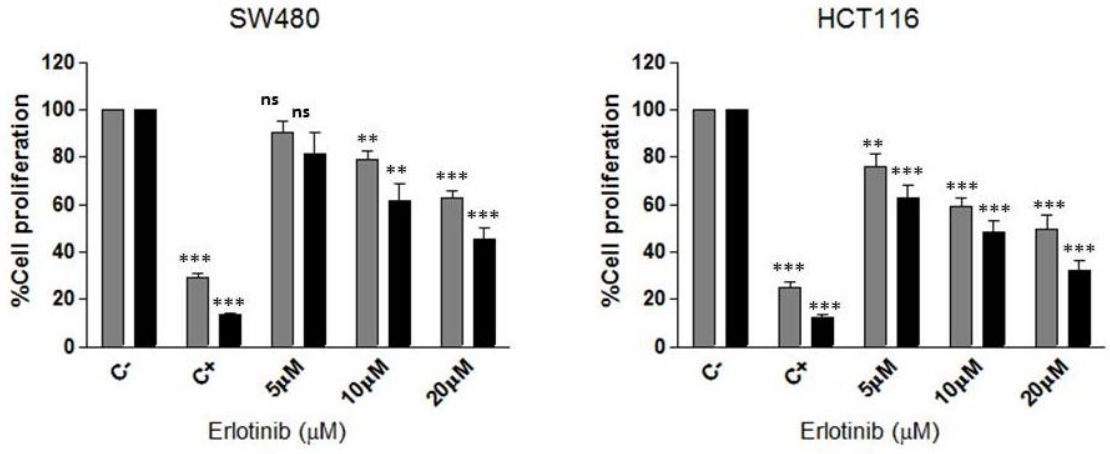


Fig. III.9 – Erlotinib exhibits an anti-proliferative action in colorectal carcinoma cell lines. Cell proliferation is negatively affected by erlotinib in HCT116^{KRASG13D} and SW480^{KRASG12V} cell lines determined by SRB assay following both 48 h and 72 h incubation and using a set of concentrations from 5 µM to 20 µM. Results are the mean ± SEM of at least three replicates of

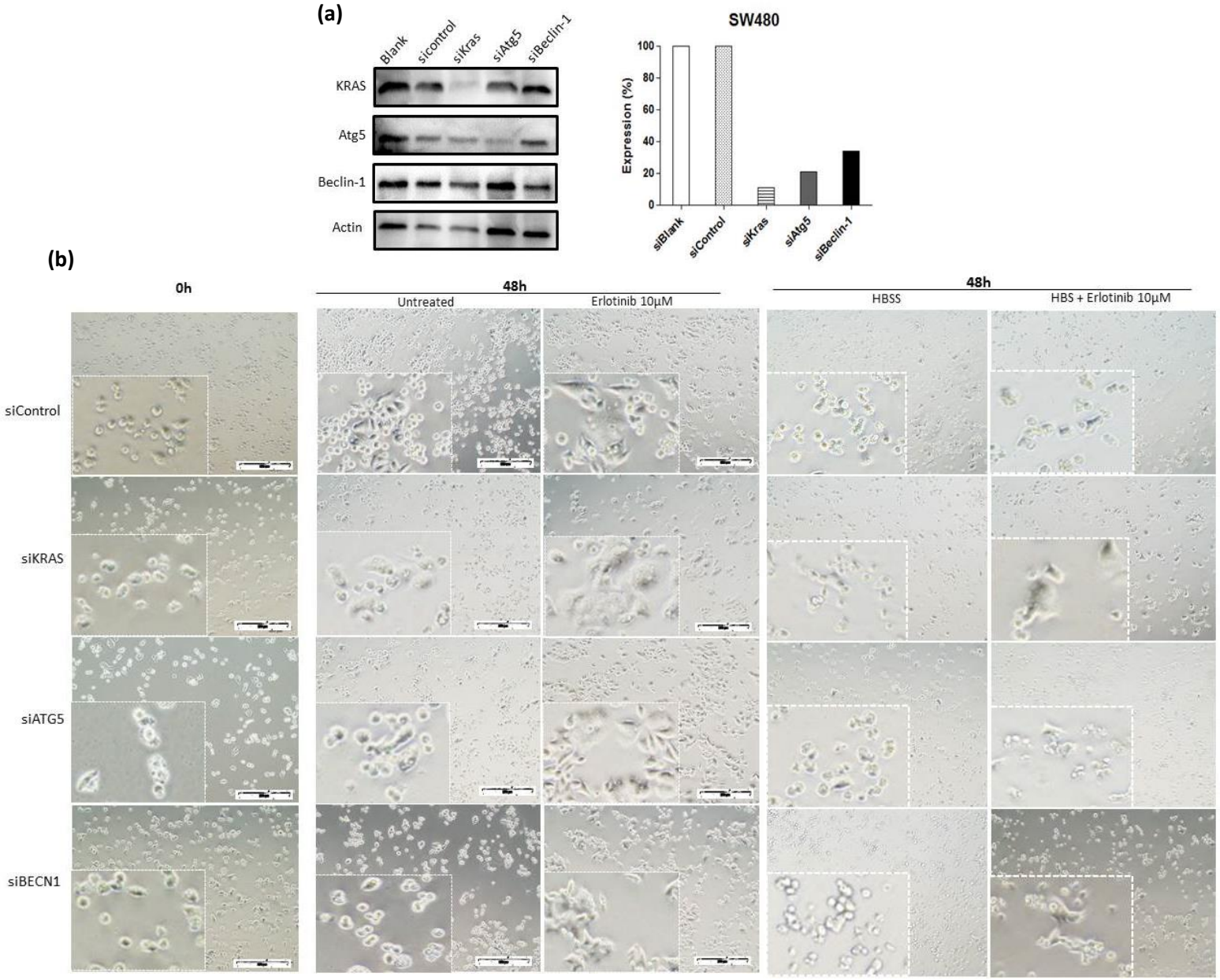
three independent experiments. Statistical significance was estimated by One-way ANOVA test followed by Dunnet's test ***p ≤ 0. 001, **p<0.01, *p<0.05, ns – not significant.

III.4.3 Effect of KRAS/autophagy inhibition with EGFR- inhibition by erlotinib on cell viability in SW480 cell line

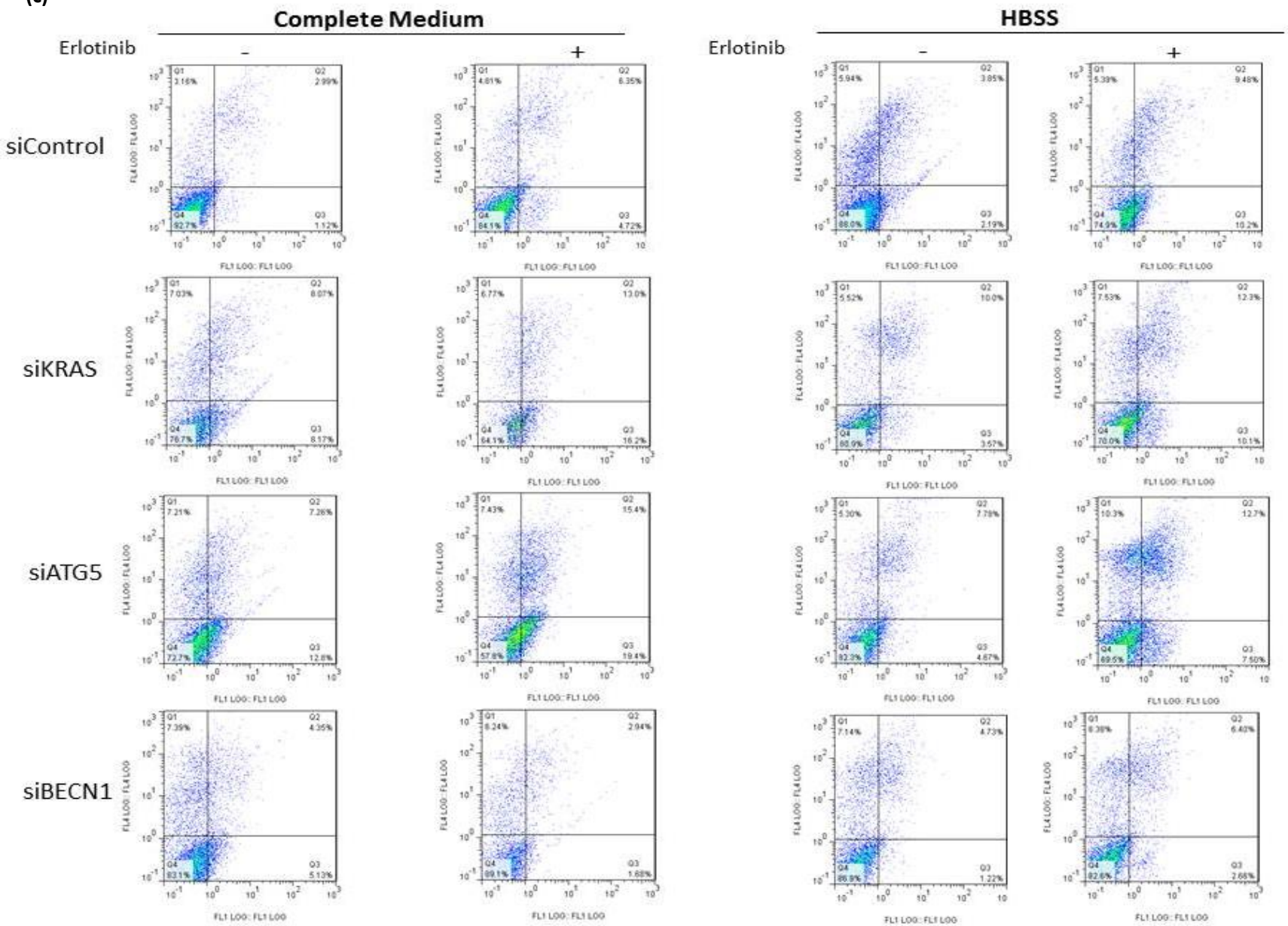
Our previous results on *KRAS* or autophagy inhibition showed that such inhibition resulted in increased cell death determined by Annexin V/PI assay (SFig.VI.3). Consequently, following the determination of the effect of erlotinib alone on cellular proliferation, our next approach was to investigate if we could potentiate the effect of this TKI activity by combining it with autophagy inhibition, by silencing *BECN1* or *ATG5*, or by KRAS inhibition (Fig. III.10a). Our group has already demonstrated that KRAS and autophagy have crucial roles in SW480^{KRASG12V} cell survival (Alves et al. submitted.). We selected the dose of erlotinib (10 μM) that caused a decrease of about 20 per cent in cellular proliferation in SW480^{KRASG12V} cell line after 48h of incubation.

After silencing the targeted genes we performed the Annexin V/PI assay, in order to evaluate if erlotinib in combination with *KRAS*, *ATG5* or *BECN1* inhibition increased cell death, both under nutrient-rich (complete medium) and nutrient-depleted (HBSS) conditions. We observed that erlotinib resulted in an increase in the percentage of Annexin V positive stained cells both in complete medium and in HBSS. Moreover, when combined with KRAS or Atg5 protein depletion (both in complete medium and in HBSS) it led to a greater increase in cell death when compared with erlotinib alone. *BECN1* silencing has less impact on cell viability in comparison with *ATG5* silencing in both nutrient-rich and starvation medium (Fig III.10c,d).

Morphologic alterations 48h after *KRAS* and *BECN1* silencing in complete and HBSS medium are identical to those observed in Fig. III. 7a. *ATG5* silencing in complete medium did not affect cellular morphology as markedly as *KRAS* or *BECN1* knockdown, in comparison with the control. In all experimental conditions in complete medium, erlotinib caused great morphological changes: cells increased in size, forming aggregates and in some cases vacuoles appeared. In starvation medium, erlotininb did not cause such dramatic modifications in cellular morphology, when compared to nutrient-rich medium (Fig. III.10b).


(a)
Blank
sicontrol
siKras
siAtg5
siBeclin-1
KRAS
Atg5
Beclin-1
Actin
SW480
Expression (%)
100
80
60
40
20
0
siBlank
siControl
siKras
siAtg5
siBeclin-1
(b)
0h
48h
Untreated
Erlotinib 10µM
48h
HBSS
HBS + Erlotinib 10µM
siControl
siKRAS
siATG5
siBECN1

(c)


Complete Medium
HBSS
Erlotinib
-
+
siControl
siKRAS
siATG5
siBECN1
FL1 LOG: FL4 LOG
FL1 LOG: FL1 LOG

(d)

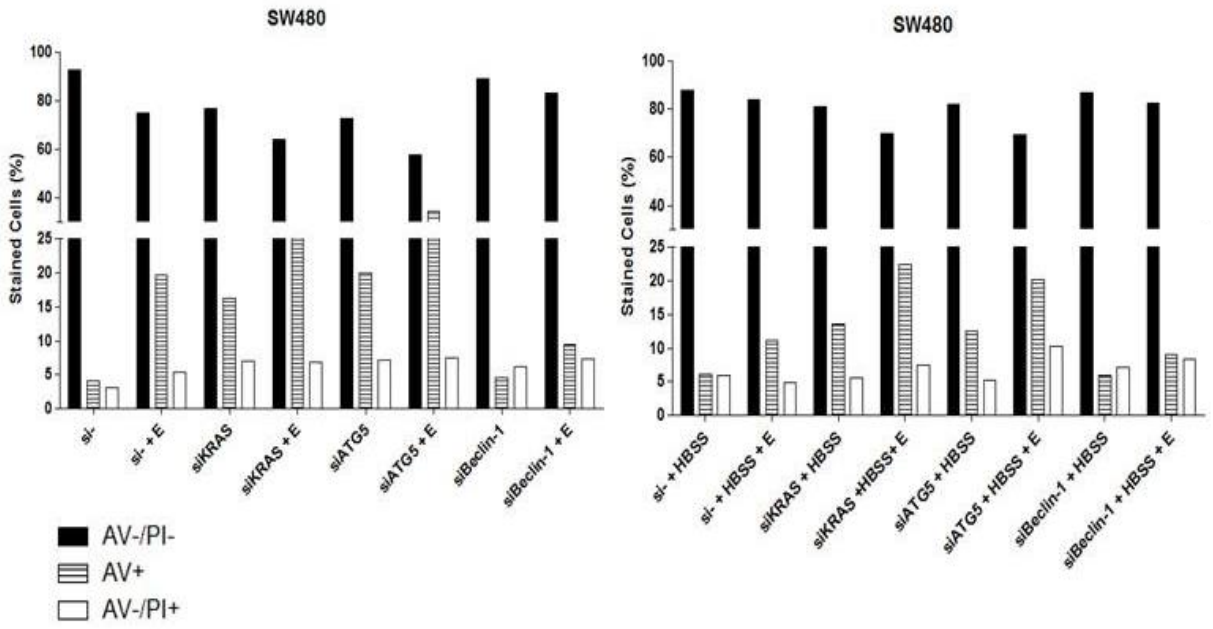


Fig. III.10 **– Erlotinib effect on cell death is potentiated by the silencing of *KRAS* or *ATG5*. (a)** Western blot of *KRAS*, *ATG5* and *BECN1* silencing. **(b)** Representative phase contrast photos of the morphological alterations in SW480^{KRASG12V} cells following the silencing of *KRAS*, *ATG5* and *BECN1* in complete medium in the presence or absence of erlotinib – phase contrast 10x magnification. **(c)** Annexin V/PI dotplots of control, *KRAS*, *ATG5* s and *BECN1* siRNA in complete and HBSS medium with or without erlotinib. Cytometry data was analysed using *Flowjo* software **(d)** Graphical representation of the AnnexinV/PI dotplots results. All of the Annexin V + stained cells (quadrants 2 and 3) were summed.

IV. DISCUSSION

IV-DISCUSSION

Colorectal cancer (CRC) remains an important and frequent mortality cause worldwide despite the significant advances in screening, diagnosis and treatment (Marisa et al., 2013; Stefano & Carlomagno, 2014). One of the most relevant genetic alterations occurring in CRC is the oncogenic activating mutation of *KRAS* gene (Oliveira et al., 2007; Vaughn et al., 2011). Among many other distinctive features, several reports showed that KRAS mutations are associated with elevated basal autophagy levels, being this adaptive characteristic a promoter of cell survival (Kim et al., 2011; Guo et al., 2011; Wang et al., 2012; Zhai et al., 2013; Alves et al., submitted). Indeed, autophagy is able to provide cells with nutrients resulting from the degradation of macromolecules, which can be highly advantageous in nutrient-deprivation conditions, a recurrent scenario in solid tumours like CRCs (Apel et al., 2009; Mancias & Kimmelman, 2011).

Activating *KRAS* mutations (*KRAS*^{MUT}), such as *KRAS*^{G13D}, *KRAS*^{G12D} and *KRAS*^{G12V} are undoubtedly important to CRC carcinogenesis, how and to what extent these mutations regulate the autophagic process in the colon model has been the focus of study of Ana Preto´s lab in the last years. This work was designed as a follow-up project of a previous study developed by our group (Alves et al., submitted). Alves and co-workers studied the role of KRAS^{MUT} in autophagy regulation in starvation conditions using two main cellular models: the yeast *S. cerevisiae* and the colon model (non-cancer colon and CRC derived cell lines). Our group results unravelled several aspects concerning KRAS role in autophagy, namely that KRAS^{MUT} up-regulates autophagy in both models, and accordingly that *KRAS*^{MUT} silencing impairs autophagy in CRC derived cells. Our data also showed that KRAS^{MUT} promoted autophagy up-regulation though MEK/ERK pathway and that in the CRC context autophagy has a pro-survival role (Alves et al., submitted).

A “humanized” yeast model, was constructed using a *S. cerevisae ras2*Δ strain transformed with the pCM184 plasmid harbouring *KRAS*^{WT} or one of three mutants (*KRAS*^{G13D}, *KRAS*^{G12D}, *KRAS*^{G12V}) under the control of a *tet-off* promoter (SFig. VI.1). In this model, the induction of autophagy under nitrogen starvation conditions led to an increase in the levels of KRAS^{WT} and KRAS^{MUT}, being that increase related to increased autophagic flux. In addition, when transcription was repressed (by the addition of

doxycycline) $KRAS^{MUT}$ stabilized their levels, but free GFP, resulting from degradation of ATG8-GFP and an indicator of autophagy, still increased.

This issue had not been addressed in the non-cancer colon cells NCM460 infected with FLAG-$KRAS^{WT}$ and FLAG-$KRAS^{MUT}$ and in CRC cells. In this work we aimed to understand whether the levels of KRAS also changed upon nutrient starvation. We showed that in both NCM460 cells and CRC cells, no significant increase in endogenous KRAS or FLAG-KRAS levels were observed in starvation conditions opposite to what was shown in the yeast model (Fig. III.1 and III.2a). In spite of this, in NCM460 parental, $KRAS^{WT}$ and $KRAS^{G13D}$, the levels of endogenous KRAS presented a tendency to increase upon starvation induction. Even though this tendency was consistent, variability was high and did not reach statistical significance. Pro-survival autophagy has been directly and indirectly correlated with increased KRAS protein levels. Indeed, in a study using MCF10-A breast cancer cells in which $KRAS^{G12V}$ was inserted using a retroviral vector, KRAS expression levels were monitored and consistently increased for 72 h, being that increase connected with increased autophagy levels (Kim et al., 2011). Conversely, using 4-dehydroxy-tamoxifen (OHT), Kohli and colleagues have drawn an association between pro-death autophagy and KRAS degradation in metastatic and recurrent malignant peripheral nerve sheath tumors cells (Kohli et al., 2013). These studies indicate that KRAS levels are important in autophagy regulation, being KRAS increased expression is associated with pro-survival autophagy (Kim et al. 2011)and pro-death autophagy with decreased levels of this protein (Kohli et al., 2013). However, we might speculate that in the cases of $KRAS^{MUT}$, these mutations are constitutively active (Sasaki et al., 2012) and, therefore continuously activating its downstream targets, such as MEK/ERKS proteins that have been proved to be involved in autophagy promotion in our models (Alves et al. submitted). As a consequence, an up-regulation of KRAS proteins translation, as suggested by our results, might be unnecessary and somewhat redundant for its function in the cell.

Simultaneously to the assessment of the expression levels of KRAS upon autophagy induction by nutrient starvation in SW480^{KRASG12V} and HCT116^{KRASG13D} cell lines, we also studied the protein stability through the use of the protein synthesis inhibitor cycloheximide (CHX) (Fig. III.2). Additionally, NCM460 parental cell line was used as a comparison since it harbours $KRAS^{WT}$ (Fig. III.2a). We aimed to unravel

whether the $KRAS^{WT}$ was less stable than $KRAS^{MUT}$, given that $KRAS^{MUT}$ contributes more actively to autophagy in our models.

In the literature $KRAS^{G12V}$ half-life was described to vary from 12- 14 h (Elad et al., 1999; Rabien et al., 2012; Shukla et al., 2014). This is in accordance to the fact that for the three cell lines used there was a decrease in protein level at 12 h when cells were incubated in HBSS medium, nonetheless this decrease was rescued by the addition of CHX and the levels were maintained after 18 h, with the exception of NCM460 parental cells, whose KRAS protein levels diminished after 18 h. The decrease at 12 h, the recovery of the protein levels after the addition of CHX at 12 h and at later time points (for $HCT116^{KRASG13D}$ and $SW480^{KRASG12V}$) seemed contradictory. The decrease of the protein levels at 12 h might be due to an increase in protein degradation associated with KRAS half-life and with the fact that in starvation conditions it is more active. As for the recuperation effect induced by CHX at 12 h, we hypothesize that this might be a side-effect of CHX protein synthesis inhibition, which might be blocking the translation of proteins involved in autophagy and, given that KRAS is the only RAS isoform that can be degraded in the lysosome (Lu et al. 2009;Shukla et al. 2014) the levels of KRAS might be increasing by inhibition of its degradation. On the other hand $KRAS^{WT}$ protein appears less resistant to starvation induction than its mutated counterparts, being this outcome probably related to this form not exerting such a preponderant role in autophagy regulation or to the fact that $KRAS^{MUT}$ suffer additional post-translational processing that result in more stable proteins (Mustachio et al., 2014). Another hypothesis is that there might be a degradation complex that has specificity to $KRAS^{MUT}$ and represses its degradation, having the existence of such a complex already been documented in $HCT116^{KRASG13D}$ cell line. The complex described is formed by Smad ubiquitination regulatory factor 2 (SMURF2) and UBCH5. Loss of SMURF2 resulted in KRAS degradation, whereas the overexpression of SMURF2 increased KRAS stability (Shukla et al., 2014). Also in $HCT116^{KRASG13D}$ cells, OHT-induced autophagy did not concomitantly induced KRAS degradation (like it happened in other cell lines), meaning that KRAS maintained its levels throughout time and increased drug concentration (Kohli et al., 2013). This suggests that in this cell line autophagy induction does not affect KRAS stability, which is in accordance with our results.

These results despite being very preliminary, suggests that $KRAS^{MUT}$ has increased stability in comparison to $KRAS^{WT}$, what might support the view that the loss or degradation of an oncoprotein provides a robust and durable antitumor effect compared to inhibition of the oncogene function (Shukla et al., 2014). A $KRAS^{MUT}$-targeted ubiquitin ligase has been developed and it has been shown to suppress cell growth and decrease independent anchorage-cell in pancreatic cancer cells (Ma et al., 2013), thus providing evidence that KRAS degradation may be an effective therapeutic approach.

Furthermore, in complete medium the levels of KRAS were maintained suggesting that the half-life is increased in those conditions and in comparison to what has been described (Fig.III.2b). It is worth-noticing that the half-life of KRAS protein has not been studied in any of our CRC models and that the only mutation that we have studied and whose half-life has been investigated elsewhere is $KRAS^{G12V}$. Therefore, we may hypothesize that the mutation *per se* may differently impact protein stability, which is in line to what happens with the determination of other tumour characteristics like aggressiveness (Guerrero et al., 2000; Smith et al., 2010) or therapy response (Messner et al., 2013), being these features also differentially affected by specific $KRAS^{MUT}$, and, consequently explaining the results we obtained with $KRAS^{G13D}$. In the case of $KRAS^{G12V}$ mutation, which has been reported to have a half-life of 12-14 h, we can argue that in addition to the mutation subtype, the cell line might influence KRAS protein stability, being the genetic background important in the determination of KRAS half-life, since in SW480 cell line $KRAS^{G12V}$ displayed a half-life superior to 14 h.

Though the concentration of CHX we applied (100 µg/mL) is within the range of the concentrations reported in human cell lines (Ma et al. 2013; Ré et al. 2012; Shukla et al. 2014), since we were not observing any differences in the half-life of $KRAS^{MUT}$ we decided to confirm the inhibitory effect of CHX in both complete and HBSS medium by performing a SRB assay (Fig. III.2c). In complete medium and in the presence of CHX a progressive decrease in cell growth was registered along time (Fig. III2c). In HBSS medium the effect of CHX was not constant, nonetheless further experiments are required to reinforce these results. Even so, we could theorize that the effect of protein synthesis inhibition is more deleterious to cells that are actively proliferating

(complete medium) in comparison to cells whose proliferation may be conditioned, due to nutrient limiting conditions.

We proceeded to understand if upon autophagy induction in NCM460 cells KRAS re-localized and to which cellular compartment. Our group had already shown that KRAS suffered a change in localization - losing its plasma membrane distribution and acquiring a more punctuate and sub-membranous disseminated pattern - when autophagy was induced in this model (Fig. III.4a). Since KRAS has been reported to localize to the mitochondria (Bivona et al., 2006; Hu et al., 2011) and to the lysosome (Lu et al., 2009), we aimed to verify if upon starvation induction KRAS could co-localize with any of these organelles. Nevertheless, the co-localization assay failed, due to antibodies incompatibilities (Fig. III.5). Specifically, the combination of antibodies (primary and secondary) and incubation (duration and sequence) were done as follows: primary antibodies rabbit anti-TOM20 or LAMP2 were incubated overnight and on the next day its secondary antibody goat anti-rabbit was incubated for 30 minutes, followed by the incubation of mouse anti-FLAG antibody for 1h and finally the secondary antibody FITC conjugated rabbit anti-mouse antibody was added for 30 minutes. We performed these sequential incubations to minimize the interaction between the anti-rabbit primary antibodies and FLAG secondary antibody, which was produced in rabbit. Despite of this fact, a cross reaction happened between the primary antibody raised in rabbit and the secondary antibody produced in that same species (Frisch et al., 2011). In the case of FLAG and TOM20 co-staining with FLAG-KRAS it is clearly visible that only TOM20 signal is discernable, having FLAG-KRAS staining been completely masked (Fig. III.5b). In the case of FLAG-KRAS/LAMP2 double-staining, the specific pattern of each antibody disappeared and the signal of both antibodies is identical (Fig. III.5b).

We also wanted to examine the role of Bcl-2-related proteins and understand if its expression was modified under starvation inducing conditions as well as if *KRAS* or *BECN1* regulated the expression of some of the members of this family of proteins. The interaction between the autophagic protein Beclin-1 and the members of the anti-apoptotic Bcl-2 family (e.g., Bcl-2, Bcl-xL and Mcl-1) constitutes a key mechanism of autophagy modulation (Levine et al., 2008), being these proteins generally reported to have an anti-autophagic action (Maiuri, et al., 2007; Pattingre et al., 2005). In contrast

with this view, Priault *et al.,* using HCT116^{KRASG13D} CRC cell line, established a pro-autophagic role for both Bcl-2 and Bcl-xL proteins, being Bcl-2 pro-autophagic activity dependent on Beclin-1 (Priault et al., 2010). Accordingly to its pro-apoptotic activity, Bax has also been described to induce pro-death autophagy (Yee et al., 2009)and to block autophagy with the concomitant promotion of apoptosis (Luo & Rubinsztein, 2010). We analyzed the endogenous levels of some members of this family and did not find any significant variation upon autophagy induction by starvation in both HCT116^{KRASG13D} and SW480^{KRASG12V} cell lines. In both cell lines the levels of Bax remained similar to the control (Fig. III.6), this is in agreement with the fact that increased Bax levels have been associated with pro-death autophagy (Li et al. 2013), what gives way to the extrapolation that in the cellular context of pro-survival autophagy, this protein expression should continue fairly unchanged. As for the anti-apoptotic and in the majority of cases anti-autophagic proteins, only Mcl-1 (in HCT116^{KRASG13D} cell line) exhibited a greater decrease, being the levels of all the other proteins studied, Bcl-xL and Bcl-2, unaffected (Fig. III.6). These results suggest that in order for these proteins to play their role in autophagy regulation an increase in the levels of expression at the protein level is not necessary. This lack of variation is in accordance with previous reports conducted in cortical neurons showing that, under glucose deprivation, only Mcl-1 levels varied, remaining all the other Bcl-2 family members studied in this work unaffected (Germain et al., 2011).

We questioned whether *KRAS* and *BECN1* inhibition by RNAi would affect these Bcl-2-family proteins, as these genes and their protein products have been proved to be crucial in our models (Priault et al., 2010; Alves et al., submitted) (SFig. VI.3). Our results, despite being very preliminary and in need of further confirmation, point in the direction of *KRAS* and *BECN1* having opposite regulatory effects on this family of proteins (Fig. III.7b). In fact, depletion of Beclin-1 in complete medium induced an increase in the levels of Bcl-xL, Mcl-1 and although to a lesser extent of Bax. In HBSS medium, *BECN1* silencing only affected the levels of Mcl-1 and Bax proteins. As for Bcl-2 expression, it did not suffer any alteration when *BECN1* was silenced, whilst *KRAS* silencing abrogated Bcl-2 expression, suggesting that in SW480^{KRASG12V} cell line KRAS exerts a regulatory action in Bcl-2 protein. Hypothetically, in this cell line, similarly to what was demonstrated by Priault and co-workers, Bcl-2 protein may have a pro-

autophagic role (Priault et al., 2010), and, as a consequence of autophagy inhibition mediated by *KRAS* knockdown, the expression of this protein could also suffer a negative regulation. As for Mcl-1, in addition to its expression being modulated by both *KRAS* and *BECN1*, it is also positively controlled by nutrient starvation itself, given that the levels of this protein are increased under nutrient-depletion conditions in comparison to complete medium even in controls (Fig. III7b). Mcl-1 is considered to be a stress sensor (Germain et al., 2011) and is known to be involved in metabolism regulation (Coloff et al., 2011). In our model, under nutrient depletion conditions this protein levels are overexpressed, in contrast to what was suggested by a previous study. In such study the models used were post-mitotic neurons and Mcl-1 degradation was related to autophagy induction under starvation conditions (Germain et al., 2011). However, our results are concordant with a report in which autophagy induction by starvation is compatible with Mcl-1 elevated expression levels in mice cardiac myocytes models (Thomas et al., 2013). Bax increased expression upon *BECN1* downregulation, both under starvation and nutrient-rich conditions may indirectly be explained by an increase in apoptosis detected when nutrient starvation-induced autophagy is inhibited by *BECN1* silencing (Boya et al., 2005). As for Bax increase after *KRAS* knockdown in HBSS it is in accordance with an increase in apoptosis reported by our group (Alves et al. submitted) (SFig.VI.3). Even though these results are preliminary and require further validation, they are primary indicators that Bcl-2 and Mcl-1 might be important players in KRAS-autophagy modulation in SW480^{KRASG12V} cell line.

KRAS^{MUT} constitutes a marker of resistance to anti-EGFR therapeutic agents. In this study we aimed to unravel the potential therapeutic application of KRAS and/or autophagy inhibition in combination with EGFR inhibitors and thus establish if a double targeted approach, i.e., targeting KRAS directly or blocking autophagy, would be a new efficient therapeutic strategy in CRCs. We used two anti-EGFR drugs – monoclonal antibody (mAb) cetuximab and the tyrosine kinase inhibitor erlotinib. The mAb Cetuximab, had no effect on our experimental condition, though we used the appropriate concentration range according to literature and even tested much higher concentrations, therefore we proceeded to study the effect of erlotinib. This TKI affected cellular proliferation in both CRC cell lines (Fig. III.9).

Actually, HCT116^{KRASG13D} cells were the most sensitive to this inhibitor, although this cell line harbours two genetic alterations that are conducing with anti-EGFR therapies resistance: PIK3CA^{H1047R} and KRAS^{G13D} mutations (Jhawer et al., 2008). In our view, these results may be a consequence of the experimental procedure, more specifically of the concentration of cells we used for each cell line. Since we intended to perform a siRNA assay following the SRB experiment, we had to maintain the same cell density, already optimized for the siRNA experiment. Consequently, we had to conserve this previously optimized parameter, being the plating density of HCT116^{KRASG13D} cell line much inferior to that of SW480^{KRASG12V}. We believe that this aspect influenced our results, as cellular density greatly impacts the access of the drug to cells. Nevertheless, this is only one putative explanation, given that this TKI has been reported to have other cellular targets, besides EGFR (Conradt et al., 2011; Weickhart et al., 2012). Indeed, in a panel of CRC cell lines, erlotinib treatment inhibited phosphorylation and subsequent activation of STAT3, whereas the anti-EGFR mAB cetuximab had no effect on STAT3 activation (Weickhart et al., 2012), thus indicating that erlotinib possesses other cellular targets other than EGFR and that this might be the underlying cause for HCT116^{KRASG13D} increased sensitivity to this drug.

To study if the erlotinib anti-proliferative activity was accompanied by an increase in cell death, as well as to infer whether this inhibitor activity could be enhanced by the combinatorial approach of *KRAS* or direct autophagy (*BECN1* and *ATG5*) targeting we performed an Annexin V/PI assay. Our results, despite needing further corroboration, are very encouraging, showing that both in complete and HBSS medium, *KRAS* or *ATG5* silencing in combination with erlotinib potentiate the effect of erlotinib alone leading to a greater percentage of cell death (Fig. III.10 c,d). Therefore, our approach reinforces KRAS or autophagy inhibition in combination with EGFR targeting as a new promising multi-target therapeutic strategy for CRC effective treatment.

As a whole, our work suggests that KRAS^{MUT} is more stable than KRAS wild-type which suggests the existence of a PTM process or of another molecular mechanism, probably related to autophagy regulation and KRAS degradation that renders KRAS^{MUT} more stable. Moreover, KRAS seems to control the Bcl2-family of proteins important in the autophagy-apoptosis loop regulation. The results obtained throughout this work

strongly suggest that a combination of autophagy or *KRAS* inhibition and the existing targeted therapies, namely anti-EGFR drugs might be a novel approach to be explored in clinical trials for the treatment of CRC tumors harbouring $KRAS^{MUT}$ resistant to those therapies.

V. Final Remarks and Future Perspectives

V.1 **Final remarks**

The main objective of this project was to further explore and elucidate the role of $KRAS^{MUT}$ in autophagy modulation in the colon model. As shown by the previous study in which this project was based, KRAS induced autophagy though MEK/ERK signalling pathway, thus affecting many aspects of cell biology namely cell survival. Therefore, we wanted to deepen the understanding of how KRAS-induced autophagy (in response to nutrient starvation) could influence KRAS stability and localization. Moreover, we wanted to address the effect of KRAS or autophagy inhibition in the regulation of the Bcl-2 family and its impact as a new therapeutic approach in combination with the classical anti-EGFR drugs.

The results obtained in this project could be summarized as follow:

- KRAS expression and stability under nutrient starvation conditions: Our results showed that neither in the non-cancer colon model nor in CRC cell lines $KRAS^{MUT}$ expression or stability suffered any alterations. This suggested that higher levels of KRAS protein are not required for KRAS autophagy regulation in these models. In terms of protein stability, $KRAS^{WT}$ appeared to be less stable in HBSS medium when compared to $KRAS^{MUT}$, what might indicate that $KRAS^{MUT}$ possesses additional PTMs or a different regulation of its degradation pathways that make this protein more stable.

- KRAS localization upon starvation induction: In the NCM460 colon model we were able to observe a re-localization of this protein upon starvation induction, however, no definable conclusion about the intracellular organelle or cellular compartment to which KRAS localizes could be drawn, due to technical problems.

- Autophagy, *KRAS* and *BECN1* modulation of the levels of Bcl-2 family proteins: Our results suggest a differential regulation of *KRAS* and *BECN1* over some members of Bcl-2 family, being KRAS presence a determinant factor for the expression of Bcl-2 anti-apoptotic members (Bcl-2, Mcl-1 and Bcl-xL), whereas Beclin-1 depletion led to an up-regulation of Bcl-xL and Mcl-1 proteins. In the

case of Mcl-1, nutrient-starvation seemed to work as a modulator of this protein expression.

- Impact of *KRAS* and/or autophagy inhibition on anti-EGFR targeted therapies: *KRAS* and/or autophagy blockade in combination with the anti-EGFR inhibitor erlotinib had a greater effect on cell death, than the silencing of *KRAS* and *ATG5*, or erlotinib alone. These results indicate that KRAS and autophagy are relevant for CRC cells survival and that they could constitute novel targets with therapeutic value in CRC.

Our results provide new insights towards a more comprehensive and extensive view of how autophagy, KRAS and KRAS-mediated autophagy influence CRC cell fate and about the molecules implicated in that regulation. We believe that this work may open the possibility to further explore KRAS and autophagy inhibition in therapeutic and clinical applications. As a matter of fact, multi-targeted therapies are being increasingly considered as the most efficient therapeutic strategy in cancer treatment. In the case of *KRAS*-driven tumours, both autophagy and KRAS itself might be therapeutically relevant targets to be tested in new CRC therapeutic approaches.

V.2 **Future perspectives**

Various other aspects underlying KRAS role in colorectal carcinogenesis, mainly by autophagy control, require clarification and study. Although this work gave some new indications about the extent to which KRAS influences autophagy, and interferes with cell fate, many more aspects need further exploration, including:

- Unveiling the reason why $KRAS^{MUT}$ is not up regulated under autophagy inducing conditions, through the study of its putative constitutive activation by performing RAS pull-down assay. It would also be important to examine if $KRAS^{MUT}$ suffers additional PTMs, like ubiquitination or sumoylation, that cause its protein product to have higher stability and affinity for downstream targets, as it has been described in pancreatic cancer (Mustachio et al., 2014). Furthermore, since KRAS may

be degraded in the lysosome, it would be worth exploring if/how autophagy influences KRAS turnover, for example by using the lysosomal alkalinizing agent chloquine (CQ). In that way, we will be able to ascertain whether KRAS expression is increased upon autophagy induction and if such increase could be masked by its increased lysosomal degradation during the autophagic process;

- Explore the role of other signalling pathways, such as JNK, p38 MAPK (Kim et al., 2011) and AKT1-GLI3-VMP1 (Ré et al., 2012) pathways, which have been suggested in literature to regulate $KRAS^{MUT}$ autophagy in breast and pancreatic cell lines, respectively. Also understand the mechanism underlying KRAS-induced autophagy through ERK1/2. As a consequence, it would be interesting to investigate whether these pathways also govern KRAS induced-autophagy in our $HCT116^{KRASG13D}$, $SW480^{KRASG12V}$ and NCM460 cell lines. This could be achieved by assessing the levels of expression and the activation status of the proteins involved in these pathways by western blotting analysis, by the use of specific siRNAs and/or chemical inhibitors targeting the effectors of these pathways.

- Determine KRAS localization in NCM460 cells following nutrient starvation by solving the technical problem of antibody cross reactivity and, for that purpose use a secondary antibody against FLAG produced either in goat, donkey, rat or swine.

- Improve the understanding of how KRAS regulates Bcl-2-related proteins, namely Bcl-2 itself. For instance, by silencing *BCL-2* and monitoring KRAS levels in order to understand if this protein also affects KRAS expression, therefore creating a co-regulation mechanism. Furthermore, the levels of Bcl-2 could be assessed after inhibition of KRAS downstream pathways, namely by using small molecule inhibitors or siRNAs to block p38 MAPK (Hui et al., 2014) or AKT (Mortenson et al., 2007), which are KRAS downstream targets that have been shown to control and also to be controlled by Bcl-2 protein.

- Assess if/how the expression of the proteins belonging to Bcl-2 family change in other colon models, namely in NCM460 cells, which will allow a more straightforward comprehension of the role of $KRAS^{MUT}$ in the regulation of these proteins. Also, as it was performed in $SW480^{KRASG12V}$ cell line, in $HCT116^{KRASG13D}$ cells *KRAS* and *BECN1* could be silenced, in order to see if *KRAS* and *BECN1* also control the expression of some members of the Bcl-2 family.

- Since KRAS promoted-autophagy has been linked to metabolic changes, including a shift towards glycolysis (Kim et al., 2011), it would be pertinent to evaluate the effect of KRAS inhibition by siRNA on HCT116^{KRASG13D} and SW480^{KRASG12V} and the impact of different KRAS mutations using NCM460 cells, on glucose uptake, lactate production, oxygen consumption and ROS production to comprehend the effect of KRAS^{MUT} on glucose metabolism.
- Characterize in more depth the molecular mechanisms associated with the double-targeted approach of KRAS or autophagy and erlotinib. It would be interesting to determine the basal levels of EGFR in our CRC cell lines and to verify if erlotinib diminishes these receptor phosphorylation levels as well as to study the phosphorylation status of KRAS downstream effectors. Additionally, it would be relevant to see if that signalling inhibition is potentiated by the combinatorial approach of KRAS and/or autophagy and this anti-EGFR drug. Describing the effects of this combinatorial strategy on cell cycle profile would also be important to address.

In summary, the role of KRAS in colorectal carcinogenesis, particularly in autophagy modulation, is an intricate subject and its deeper understanding is most likely to have tremendous implications in CRC therapeutic strategies.

VI. SUPPLEMENTARY MATERIAL

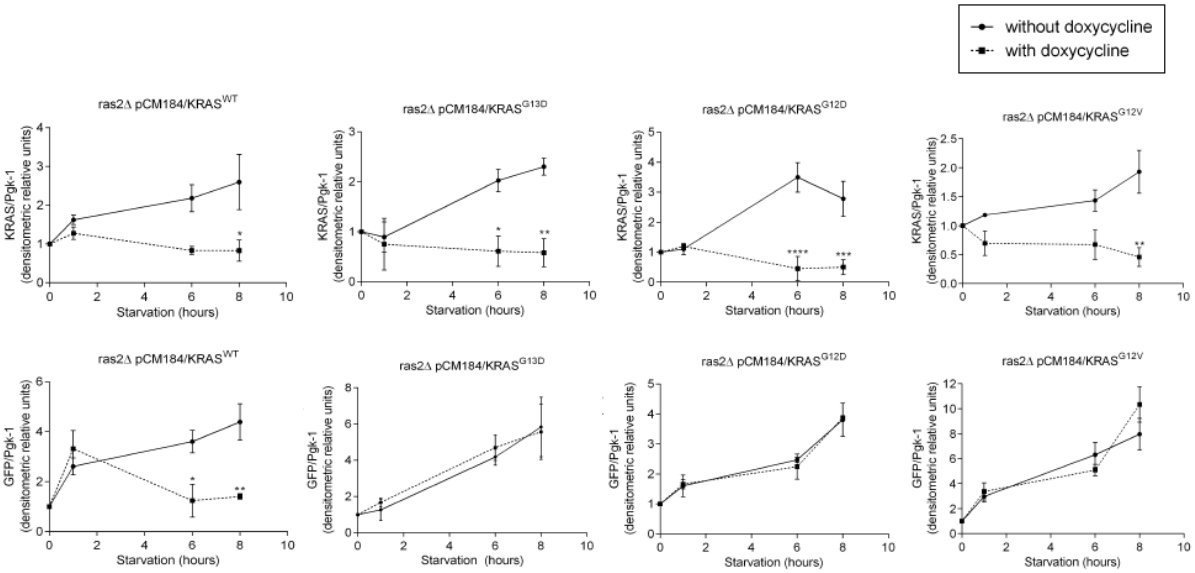


Fig. VI.1 - **Starvation-induced autophagy is dependent on KRAS^{WT} overexpression in the yeast *S. cerevisiae***. The expression of KRAS was assessed in nitrogen starvation medium in the presence and absence of doxycycline and found to be increased when transcription was not repressed (upper panel). In the case of KRAS^{WT} the increase of the autophagic flux (determined by the level of free-GFP) is dependent on the amount of protein, whereas KRAS^{MUT} do not require KRAS overexpression to modulate autophagy (lower panel). The level of free GFP generated and KRAS expression were assessed by immunoblot, at the indicated times and normalized for Pgk-1.

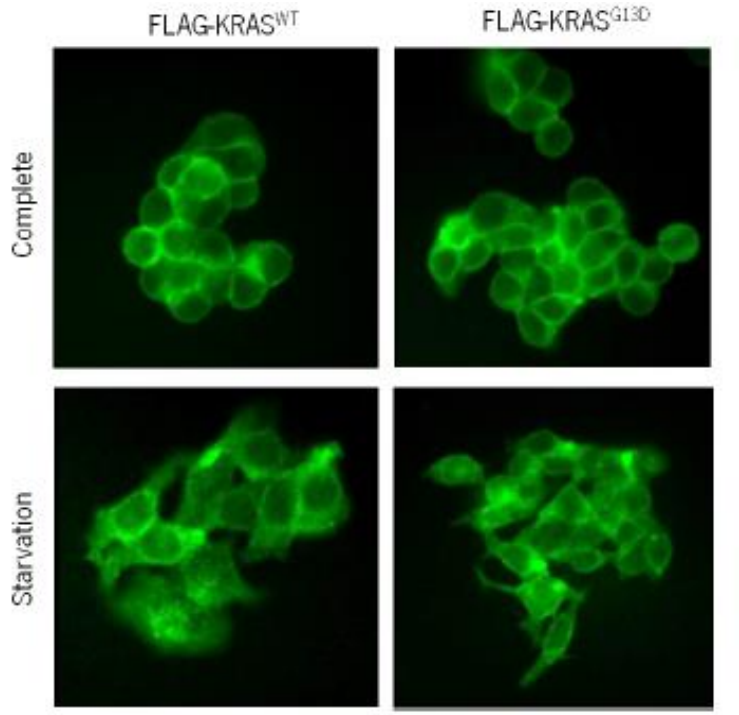


Fig. VI.2 **– KRAS changes its intracellular localization upon autophagy induction.** Following an incubation of 6 h in starvation medium (HBSS), KRAS acquires a more punctuate and sub-membranous distribution both in $KRAS^{WT}$ and $KRAS^{G13D}$ cells.

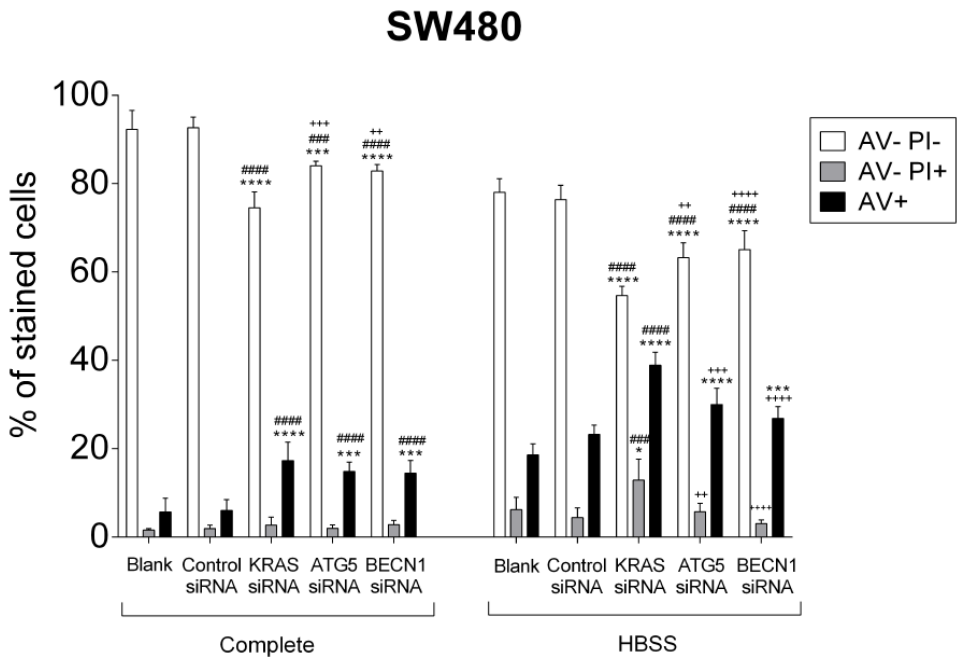


Fig.VI.3 **– KRAS and autophagy inhibition increase cell death in CRC cell line.** siRNA targeting *KRAS*, *ATG5* and *BECN1* lead to an increase in cell death (AV^+ cells) in $SW480^{KRASG12V}$ cell line.

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