Universidade do Minho
Escola de Ciências

Ana Filipa Martins de Almeida

The role of oncogenic KRAS in colorectal cancer: from subcellular localization upon autophagy induction to new therapeutic drugs

Fevereiro de 2018

The role of oncogenic KRAS in colorectal cancer: from subcellular localization upon autophagy induction to new therapeutic drugs

Ana Filipa Martins de Almeida

UMinho | 2018

Universidade do Minho

Escola de Ciências

Ana Filipa Martins de Almeida

The role of oncogenic KRAS in colorectal cancer: from subcellular localization upon autophagy induction to new therapeutic drugs

Master´s Thesis

Master´s in Molecular Genetics

Supervisor: Professora Doutora Ana Preto

Co-supervisor: Professora Doutora Maria João Sousa

Fevereiro 2018

Declaração

Nome: Ana Filipa Martins de Almeida

Endereço eletrónico: anafmalmeida@gmail.com **Telefone:** 912 271 564

Número do Bilhete de Identidade: 14620399

Título da dissertação:

The role of oncogenic KRAS in colorectal cancer: from subcellular localization upon autophagy induction to new therapeutic drugs

Supervisor:

Professora Doutora Ana Arminda Lopes Preto de Almeida

Co-supervisor:

Professora Doutora Maria João Marques Ferreira Sousa Moreira

Ano de conclusão: 2018

Designação do Mestrado: Mestrado em Genética Molecular

Universidade do Minho, 28 de fevereiro de 2018

Assinatura: _____

Agradecimentos

Prestes a terminar mais uma jornada importante na minha vida não posso deixar de expressar o meu agradecimento a todos os que me apoiaram, das mais variadas formas, nesta caminhada.

Primeiramente quero agradecer á minha orientadora e coorientadora, Professora Doutora Ana Preto e Professora Doutora Maria João Sousa, respetivamente, que abraçaram este projeto com muito afinco e me acolheram como aluna para o concretizar. O meu sincero obrigada pela oportunidade e por todos os conselhos, sugestões e atenção dada ás várias questões que me foram surgindo ao longo do trabalho. Quero dedicar um agradecimento especial á Professora Doutora Ana Preto por ter sido uma orientadora exemplar e incansável. Por todas as palavras de apoio e pelos momentos de convívio (após o trabalho, claro) que proporcionou á "Preto Team". Além de nos ter dado valiosos ensinamentos no que toca ao rigor científico e de nos ter orientado sob princípios de brio, rigor e competência, ensinou-nos que a interajuda entre um grupo de trabalho é essencial na investigação. Um gigante obrigada pelo acompanhamento neste percurso, pelas oportunidades que me deu e acima de tudo por ter acreditado em mim. Foi um prazer ter trabalhado consigo e com este grupo espantoso!

Ainda falando do extraordinário grupo de trabalho em que estive inserida neste último ano, também aqui tenho agradecimentos importantes a fazer.

Tenho obrigatoriamente de começar pela pessoa que mais me aturou no laboratório, e claro que me refiro á minha Rita Brás. Estiveste sempre pronta e disponível para me ajudar, em qualquer ocasião e circunstância. Admiro a tua força, empenho e dedicação a tudo e a todos por isso orgulho-me da tua valiosa amizade, a qual quero manter por muitas décadas. Mereces tudo de bom na vida, e ficarei sempre feliz por ti e pelas tuas vitórias, que com certeza serão bastantes. Muito obrigada por este ano fantástico que sem dúvida não teria sido o mesmo sem a tua companhia, boa disposição e amizade!

Claro que também tenho de agradecer á minha "amiga dos westerns", Sara Gomes, por toda a amizade e disponibilidade que sempre mostrou. Obrigada pelas conversas e saídas, que sempre me animaram e me deram motivação para terminar esta etapa. És uma excelente companhia e adoro particularmente a calma e positividade com que enfrentas os desafios da vida! Espero levar a tua amizade comigo para onde quer que eu vá, pois a minha levas para onde fores. Desejo-te toda a sorte do mundo com a certeza que terás um futuro promissor.

Agradeço também á Flávia Pereira, que apesar de ter estado mais distante por estar a trabalhar fora de Braga, mostrou-se sempre disponível para ajudar e isso mostra o grande carácter que ela tem. Obrigada pela amizade, conselhos e presença nesta etapa tão importante. Desejo-te muito sucesso para todos os desafios e oportunidades que estão para vir!

Não posso deixar de agradecer á Professora Doutora Ana João Rodrigues do ICVS e ás suas alunas Carina Cunha e Bárbara Coimbra pelos conselhos na elaboração dos protocolos e aquisição das fotos de imunofluorescência. A vossa ajuda foi essencial!

Agradeço ainda aos meus colegas de mestrado e ao pessoal do LBA e LCCA por todo o companheirismo, boa disposição e partilha de conhecimentos. Sempre me senti bem-vinda a esta casa e isso criou um ambiente de trabalho motivador que se refletiu no meu trabalho. Muito obrigada!

Quero ainda agradecer a amizade da Estela, Teresa, Bea e Rita. Obrigada pelas conversas, pelos cafés e por todos os momentos de descontração e brincadeira que me proporcionaram nestes últimos tempos! Ainda, não seria justo deixar de referir os meus amigos Vila Realenses, que mesmo á distância, me deram força para enfrentar este desafio.

Um agradecimento muito especial ao Carlos, o meu melhor amigo, pelo apoio incondicional, pelas palavras e pela força dadas em todas as horas, mesmo á distância. Tenho muito orgulho na pessoa que és e no que atingiste na vida e espero que tenhas muito sucesso. Obrigada por teres estado ao meu lado nos últimos tempos, foste o meu pilar, a minha calma e muitas vezes, a minha motivação! “Haja o que houver eu estou aqui”, certo?

Agora, e este é o agradecimento mais difícil de fazer pois estas pessoas acompanharam bem de perto esta caminhada, quero agradecer aos seres humanos mais extraordinários que conheço, os meus Pais. Obrigada pelas palavras de força e motivação, pela preocupação constante e pelo amor e amizade dados não só durante os últimos tempos, mas ao longo de uma vida. Acima de tudo, obrigada pelos valores que me transmitiram, que contribuíram muito para o meu crescimento e sucesso e, portanto, que se refletiram neste trabalho. Obrigada por serem o meu alicerce, a minha direção e os meus guias! E pai, um especial agradecimento, por partilhares toda a tua sabedoria e ensinamentos de forma a equilibrar o melhor da minha vida. Nem todos os reis usam coroa e a prova disso és tu! Agradeço ainda ao meu irmão que teve um papel muito importante nesta cruzada e a quem tenho muito apreço e desejo todo o sucesso e sorte do mundo!

A todos vós, o meu mais sincero agradecimento.

Financiamento

Este trabalho teve o apoio do Programa Estratégico UID/BIA/04050/2013 (POCI-01-0145-FEDER-007569) financiado por fundos nacionais através da Fundação para a Ciência e a Tecnologia e pelo Fundo Europeu de Desenvolvimento Regional (FEDER) através do COMPETE 2020 – Programa Operacional Competitividade e Internacionalização (POCI).

The role of oncogenic KRAS in colorectal cancer: from subcellular localization upon autophagy induction to new therapeutic drugs

Abstract

Colorectal cancer (CRC) is the third most common cancer worldwide. KRAS mutations ($KRAS^{MUT}$) are among the most frequent alterations in CRC supporting a role in colon carcinogenesis. Our group showed that KRAS is involved in the regulation of autophagy in CRC. Indeed, we showed that under nutrient starvation conditions $KRAS^{MUT}$ up-regulates autophagy in the colon model, which is required for CRC cell survival. Autophagy is an important degradation pathway which regulates catabolic processes to sustain cellular metabolism and homeostasis. Differences in subcellular localization of RAS or interaction with other proteins may explain the complexity of their signaling outputs. Whether starvation induced-autophagy stimulus leads to relocalization of KRAS in CRC cells is still unknown. In this project, we aimed to understand if under autophagy induced conditions there were changes in wild-type and mutated KRAS subcellular localization and in interaction with partner proteins. Our results showed that KRAS co-localize with mitochondria and lysosomes regardless of autophagy induction and that $KRAS^{MUT}$ may influence its mitochondria localization. Moreover, our data suggest that Gal-3 and KRAS co-localize in colon cells and that autophagy does not interfere with the co-localization. We also observe that KRAS might interact with actin cytoskeleton, and that autophagy may influence actin remodeling possibly in a KRAS-dependent manner.

KRAS mutations are a predictive biomarker of resistance in CRC, thus new therapeutic approaches are needed. MSG-111-cd3 is a phenoxazine derivative which had shown to induce cell death in yeast with the intervention of the vacuolar protease Pep4p, an orthologue of human Cathepsin-D (Cat-D). This project also aimed to study the effect of MSG-111-cd3 compound in CRC cells with different mutations. Our results showed that MSG-111-cd3 decrease cell viability more efficiently in CRC cells comparing with normal colon cells and that Cat-D seems to have a protective role in its effect.

Overall, our data increased our understanding on KRAS localization and protein interaction in response to autophagy stimulus in CRC, which is crucial to understand KRAS signaling regulation. Furthermore, our data suggest that the new phenoxazine tested might be a promising candidate for CRC therapy.

O papel do oncogene KRAS no cancro colorretal: da localização subcelular em resposta á indução da autofagia a novas moléculas terapêuticas

Resumo

O cancro colorretal (CCR) é o terceiro cancro mais comum a nível mundial. As mutações no KRAS ($KRAS^{MUT}$) são das mais frequentes no CCR suportando a sua função na carcinogénese do cólon. O nosso grupo mostrou que o KRAS está envolvido na regulação da autofagia no CCR. Mostramos que sob condições de privação de nutrientes as $KRAS^{MUT}$ aumentam a autofagia no modelo do cólon que é necessária á sobrevivência. A autofagia é uma importante via de degradação que regula processos catabólicos através da sustentação do metabolismo e homeostasia celular. Diferenças na localização subcelular do RAS ou interação com outras proteínas, podem explicar a complexidade da sua sinalização. Desconhece-se se a autofagia leva á relocalização do KRAS nas células de CCR. Neste projeto, tínhamos como objetivo perceber se a indução de autofagia levava a mudanças na localização subcelular do KRAS e na interação com proteínas. Os nossos resultados mostraram que o KRAS co-localiza com a mitocôndria e os lisossomas independentemente da indução de autofagia e que as $KRAS^{MUT}$ parecem influenciar a sua co-localização com a mitocôndria. Os nossos resultados também sugerem que a Gal-3 e o KRAS co-localizam nas células do cólon e a autofagia não parece interferir. Ainda, observamos que o KRAS pode interagir com a actina, e que a autofagia pode influenciar a sua remodelação, provavelmente por um mecanismo dependente do KRAS.

As mutações no KRAS são um biomarcador de resistência nos CCR, e dai serem necessárias novas abordagens. O composto MSG-111-cd3 é um derivativo da fenoxazina, que mostrou induzir morte celular na levedura, com a intervenção da protéase vacuolar Pep4p, ortóloga da Catepsina-D humana (Cat-D). Tivemos também como objetivo estudar o efeito do composto MSG-111-cd3 em células de CCR com diferentes mutações. Os nossos resultados mostraram que o MSG-111-cd3 diminuiu a viabilidade celular mais eficazmente nas células de CCR comparando com as células normais de cólon e que a Cat-D parece ter um papel protetor no seu efeito.

No geral, os nossos dados contribuíram para o conhecimento acerca da localização e interações proteicas do KRAS em resposta ao estímulo autofágico no CCR, o que pode ser crucial para perceber a regulação da sinalização do KRAS. Para além disso, os resultados sugerem que o novo agente pode ser um candidato promissor para a terapêutica do CCR.

Scientific output

Articles:

1. Giulia Cazzanelli, Flávia Pereira, Sara Alves, Rita Francisco, Luísa Azevedo, Patrícia Carvalho, **Ana Almeida**, Manuela Côrte-Real, Maria José Oliveira, Cândida Lucas, Maria João Sousa, Ana Preto. "The yeast Saccharomyces cerevisiae as a model to understand RAS proteins and their role in human tumorigenesis". ***Cells***. 2018 Feb 19;7(2). pii: E14. doi: 10.3390/cells7020014. Review.

2. João C. Ferreira, Carla Lopes, **Ana Almeida**, Ana Preto, M. Sameiro T. Gonçalves, Maria João Sousa. "Molecular pathways involved in the cell death induced by a novel Nile Blue analogue". *(In preparation)*

3. João Azevedo Silva, **Ana Almeida**, Young Hee Ko, Peter L. Pedersen, Ana Preto, Margarida Casal. "Therapeutic implications of the cytoskeleton disruption by the glycolytic inhibitor 3-Bromopyruvate". *(In preparation)*

Table of contents

Declaração ii

Agradecimentos iii

Abstract viii

Scientific output xi

Table of contents xiii

List of figures xv

List of tables xvi

List of abbreviations xvii

1. Introduction 1

1.1 Cancer: a heterogeneous genetic disorder 1

1.1.1. Colorectal cancer overview 2

1.1.1.1. Colorectal cancer associated mutations: role and significance 4

1.2. RAS at a glance 7

1.2.1. RAS effector signaling 7

1.2.2. Differential trafficking and localization of RAS proteins 9

1.2.2.1. Galectin-3 and actin cytoskeleton as modulators of KRAS signal output 11

1.2.2.2. RAS localization matters: are lysosomes and mitochondria important signaling platforms? 12

1.3. Autophagy: a double edge sword in cancer 13

1.3.1. Molecular mechanism of autophagy 13

1.3.2. Role of autophagy in cancer 15

1.3.3. Relevance of actin cytoskeleton in autophagy 17

1.3.4. Autophagy and its impact on colorectal cancer 18

1.3.5. KRAS and autophagy regulation in colorectal cancer: a tight bond 18

1.4. Therapy in colorectal cancer 19

1.4.1. KRAS: a marker of therapy resistance in colorectal cancer 19

1.4.2. New phenoxazine derivatives in cancer 21

1.4.2.1. MSG-111-cd3: a promising anticancer agent against colorectal cancer? 22

2. Rationale and aims 25

3. Material and methods 27

3.1. Cell lines and culture conditions 27
3.2. Immunofluorescence assays and confocal microscopy 27
3.2.1. Co-localization analysis 29
3.3. Sulphorhodamine B (SRB) assay and determination of MSG-111-CD3 IC_{50} in RKO, SW480 and NCM460 cell lines 31
3.4. RNA interference assay 32
3.4.1. RNA interference controls 33
3.4.1.1. Sample preparation for western blotting: cell lysis and protein extraction 33
3.4.1.2. Sample preparation for western blotting: protein quantification 34
3.4.1.3 Western blotting 34
4. Results 37
4.1. KRAS cellular localization in NCM460 cells upon autophagy induction 37
4.1.1 $KRAS^{WT}$ but not $KRAS^{G12V}$ co-localize with mitochondria 41
4.1.2. KRAS seem to co-localize with lysosomes 47
4.1.3. Galectin-3 co-localize with KRAS 50
4.1.4. KRAS mutation might influence actin polymerization 53
4.2. Effect of MSG-111-cd3 compound in colorectal cancer cells 57
4.2.1. MSG-111-cd3 decreased cell growth in colorectal cancer cells but not in normal cells 57
4.2.2. Cathepsin-D might have a protective role in response to MSG-111-cd3 compound 59
5. Discussion 61
6. Conclusions and future perspectives 69
6.1 Conclusions 69
6.2 Future perspectives 70
7. References 73

List of figures

Figure 1.1. The hallmarks of cancer..... 2

Figure 1.2. Adenocarcinoma sequence: the proposed model for colorectal cancer progression.... 3

Figure 1.3. Mutations incidence among the main RAS isoforms..... 7

Figure 1.4. Signaling pathways influenced by RAS proteins..... 9

Figure 1.5. RAS proteins primary structure..... 10

Figure 1.6. RAS modifications and localization..... 11

Figure 1.7. Major stages of the autophagic process..... 15

Figure 1.8. Molecular structure of Oxazine, Phenoxazine and Benzo[a]phenoxazines..... 21

Figure 1.9. N-(5-((4-ethoxy-4-oxobutyl) amino) -10-methyl-9H-benzo[a]phenoxazin-9-lidene) (MSG-111-cd3) structure..... 23

Figure 4.1. Optimized dilutions for TOMM20, LAMP2, and FLAG antibodies in NCM460 FLAG-$KRAS^{WT}$ cell line in complete and HBSS medium..... 39

Figure 4.2. Secondary antibodies negative controls of the different immunofluorescence assays performed..... 40

Figure 4.3. Starvation leads to morphological alterations in mitochondria and causes KRAS relocalization, in NCM460 FLAG – $KRAS^{WT}$ cell line..... 42

Figure 4.4. Starvation leads to morphological alterations in mitochondria and causes KRAS relocalization, in NCM460 FLAG – $KRAS^{G12V}$ cell line..... 43

Figure 4.5. Co-immunofluorescence of FLAG-KRAS with TOMM20 in NCM460 cell lines in complete and HBSS medium..... 45

Figure 4.6. Graphical representation of the overlap coefficient between TOMM20/FLAG-KRAS in NCM460 cell lines, under complete and incomplete nutrient conditions..... 46

Figure 4.7. Co-immunofluorescence of FLAG-KRAS with LAMP2 in NCM460 cell lines in complete and HBSS medium..... 48

Figure 4.8. Graphical representation of the overlap coefficient between LAMP2/FLAG-KRAS in NCM460 cell lines, under complete and incomplete nutrient conditions..... 49

Figure 4.9. Co-immunofluorescence of FLAG-KRAS with Gal-3 in NCM460 cell lines in complete and HBSS medium..... 51

Figure 4.10. Graphical representation of the overlap coefficient between Gal-3/FLAG-KRAS in NCM460 cell lines, under complete and incomplete nutrient conditions..... 52
Figure 4.11. Co-immunofluorescence of FLAG-KRAS with Phalloidin 647 in NCM460 cell lines in complete and HBSS medium..... 54
Figure 4.12. Graphical representation of the overlap coefficient between Phalloidin/FLAG-KRAS in NCM460 cell lines, under complete and incomplete nutrient conditions..... 55
Figure 4.13. Effect of MSG-111-cd3 on cell proliferation of RKO, SW480 and NCM460 cell lines, determined by SRB assay..... 58
Figure 4.14. RKO and SW460 have an IC_{50} value significantly lower than NCM460..... 59
Figure 4.15. Cat-D silencing effects in RKO and SW460 cell lines together with MSG treatment. 60

List of tables

Table 3.1. Some specifications of the antibodies used in the immunofluorescence assays..... 29
Table 3.2. Variations between the immunofluorescence protocols performed..... 30
Table 3.3. RNAi assay Mix I and Mix II composition..... 33
Table 4.1. Dilutions tested for the antibodies TOMM20, LAMP2, and FLAG..... 38
Table 4.2. Values of TOMM20/FLAG-KRAS co-localization analysis in both NCM460 cell lines, under complete and incomplete nutrient conditions..... 46
Table 4.3. Values of LAMP2/FLAG-KRAS co-localization analysis in both NCM460 cell lines, under complete and incomplete nutrient conditions..... 49
Table 4.4. Values of Gal-3/FLAG-KRAS co-localization analysis in both NCM460 cell lines, under complete and incomplete nutrient conditions..... 52
Table 4.5. Values of Phalloidin/FLAG-KRAS co-localization analysis in both NCM460 cell lines, under complete and incomplete nutrient conditions..... 55

List of abbreviations

AKT/PKB Protein Kinase B

APC Adenomatous polyposis coli

ARF ADP ribosylation factor

Atg Autophagy-related genes

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

Cat-D Cathepsin-D

CIMP CpG island methylator phenotype

CIN Chromosomal instability

CRC Colorectal cancer

DAPI 4',6-diamidino-2-phenylindole

DMEM Dulbecco's Modified Eagle's Medium

dsRNA small double stranded RNA

EDTA Ethylenediamine tetraacetic acid

EGF Epidermal growth factor

EGFR Epidermal growth factor receptor

ER Endoplasmic reticulum

ERK Extracellular regulated MAP kinase

FAP Familial adenomatous polyposis

FBS Fetal bovine serum

FIP200 FAK family-interacting protein of 200 kDa

FoxO Forkhead box

FRET Fluorescence resonance energy transfer

GAPs GTPase activating proteins

GDP Guanosine diphosphate

GEFs Guanine nucleotide exchange factors

Grb2 Growth factor receptor-bound protein 2

GTP Guanosine triphosphate

GTPase Guanosine triphosphatase

HBSS Hank's buffered salt solution

HDAC6 Histone deacetylase 6

HER Human epidermal growth factor Receptor

HPNCC Hereditary nonpolyposis colorectal cancer

HRAS Harvey rat sarcoma virus oncogene

HVR Hypervariable region

ICMT Isoprenylcysteine carboxyl methyltransferase

IP3Rs Inositol triphosphate receptors

JNK c-Jun N-terminal kinase

KRAS Kirsten rat sarcoma viral oncogene

LAMP Lysosomal-associated membrane protein

LC3 Microtubule-associated protein light chain 3

Les Late endosomes

LOH Loss-of-heterozygosity

mAbs Monoclonal antibodies

MAPK Mitogen-activated protein kinase

Mdm2 Mouse double minute 2 homolog

MEK Mitogen-associated extracellular signal-regulated kinase

MLH1 MutL homolog 1

MMR Mismatch repair

MOC Mander's Overlap Coefficient

MSH2 MutS protein homolog 2

MSI Microsatellite unstable

MSS Microsatellite stable

mTOR Mammalian target of rapamycin

NRAS Neuroblastome rat sarcoma virus oncogene

NSCLC Non-small cell lung cancer

OC Overlap coefficient

PBS Phosphate buffer saline

PCC Pearson's correlation coefficient

PDK1 Phosphatidylinositol dependent kinase 1

PFA Paraformaldehyde

PFT Protein farnesyl transferase

PIP2 Phosphatidylinositol-4,5-diphosphate

PIP3 Phosphatidylinositol-3,4-triphosphate

PI3K Phosphatidylinositol 3-kinase

PLA Proximity ligation assay

PI3P Phosphatidylinositol-3-phosphate

PM Plasma membrane

PTEN Phosphatase and tensin homolog deleted on chromosome 10

PTMs Posttranslational modifications

RAB RAS-related GTP-binding protein, alternative splice

RAC RAS-related C3 botulinum toxin

RAF Rapidly accelerated fibrosarcoma

RALGDS RAL guanine nucleotide dissociation stimulator

RAN RAS-related nuclear GTP binding protein

RAS Rat sarcoma

Rce1 RAS converting enzyme 1

RHO Rhodopsin

RNAi RNA interference

RPMI Roswell park memorial institute

RT Room temperature

RTK Receptor tyrosine kinases

SDS Sodium dodecyl sulfate

siRNA Small interfering RNA

SOS 1 Son of sevenless 1

SOS 2 Son of sevenless 2

SMAD4 Mothers against decapentaplegic homolog 4

TGN Trans-Golgi network

Tiam1 T-cell lymphoma invasion and metastasis protein 1

TOMM20 Mitochondrial import receptor subunit TOM20 homolog

TP53 Tumor protein p53

ULK1 Serine/threonine-protein kinase ULK1

UVRAG UV radiation resistance-associated gene protein

Vsp15 Vesicular protein sorting 15

WASH Wiskott–Aldrich syndrome protein and SCAR homologue

WHAMM WASP homolog associated with actin, membranes and microtubules

5FU Fluorouracil

1. Introduction

1.1 Cancer: a heterogeneous genetic disorder

According to Centelles (2012) "Tumors are more than insular masses of proliferating cells. They are complex tissues of multiple cell types interacting with one another"(Centelles, 2012).

Cancer arises because of a classical three-step process involving initiation, promotion, and progression. A single mutation is not enough to develop cancer, that results from an accumulation of genetic and epigenetic changes compromising vital functions such as proliferation, survival, and differentiation (Centelles, 2012; Schmidt, 2007). Initiation comprises the formation of a malignant cell due to gene mutations arising spontaneously or induced by exposure to exogenous carcinogenic agents. When DNA remains unrepaired the uncontrolled growth and accumulation of cells with mutated genes are stimulated in the so-called promotion phase. Eventually, a benign tumor becomes evident contributing to tumor progression (Centelles, 2012).

The genes of interest that are involved in genetic alterations can be divided into three groups: oncogenes, tumor suppressor genes, and DNA repair genes. Under normal conditions, oncogenes stimulate appropriate cell growth, when mutated there is a 'gain of function' and the resulting mutated protein is constitutively activated and induces cell growth even in the absence of growth signals. Tumor suppressor genes normally arrest the cell cycle or promote programmed cell death (apoptosis), but when their expression is absent there is a loss of normal inhibitory control. Finally, DNA repair genes are involved in controlling the rate of gene mutations. When mutated, repair genes are incapable to repair errors leading to mutations accumulation at a fast rate (Bertram, 2000; Leslie, Carey, Pratt, & Steele, 2002).

To survive, proliferate and disseminate, tumors must acquire biological capabilities that were firstly described in 2000 by Hanahan and Weinberg, as Hallmarks of Cancer (Hanahan & Weinberg, 2000). The Hallmarks include traits such as self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion from programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis capacity, deregulated cellular energetics, ability to avoid the immune response, tumor-promoting inflammation, and genome instability and mutation (Figure 1.1.) (Hanahan & Weinberg, 2000, 2011).

These alterations can happen in the germline cells, resulting in hereditary tumors or more commonly in somatic cells, where their accumulation gives rise to sporadic tumors (Centelles, 2012; Schmidt, 2007; Souglakos, 2007).

Considering the molecular features and capabilities of cancer cells, it is not surprising that cancer is a leading cause of death worldwide having accounted for 8.2 million deaths in 2012 and that predictions point to an increase of 19.3 million new cancer cases by 2025 (Ferlay et al., 2015).

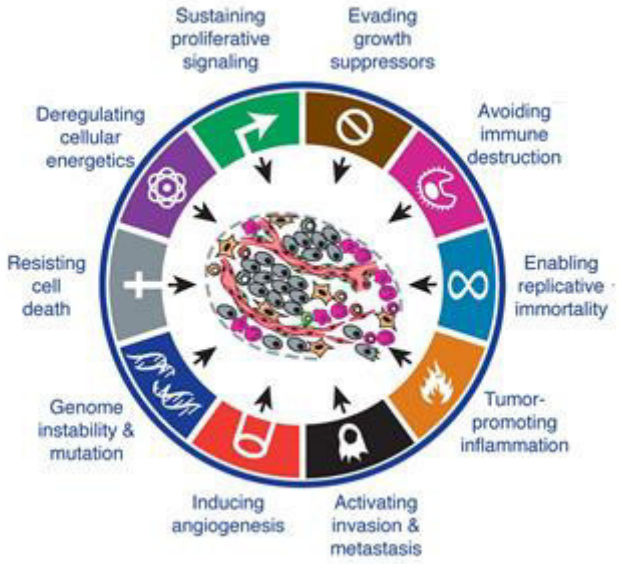


Figure 1.1.The hallmarks of cancer. Charactheristics acquired by tumor cells essential for tumor cell progression and survival (Adapted from (Hanahan & Weinberg, 2011).

1.1.1. Colorectal cancer overview

Colorectal cancer (CRC) is a malignancy that affects the large intestine wall with a worldwide prevalence of about 1.4 million individuals. CRC accounts for over 10% of all cancer incidence and is the fourth commonest cause of cancer-related mortality with 693,900 deaths reported in 2012 (Armaghany, Wilson, Chu, & Mills, 2012; De Stefano & Carlomagno, 2014; Y. Guo, Bao, & Yang, 2017; Jia, Zhang, Li, & Li, 2017; Marisa et al., 2013; Tariq & Ghias, 2016).

It is generally accepted that the adenocarcinoma sequence is the process by which most, if not all, colorectal cancers emerge (Leslie et al., 2002). The first model of colorectal carcinogenesis that supports this hypothesis was proposed by Fearon and Vogelstein (1990) who emphasized that rather than a single event, a sequence of events is the leading cause of colonic mucosal changes (Al-Sohaily, Biankin, Leong, Kohonen-Corish, & Warusavitarne, 2012; Fredericks,

2015). In this model, the authors proposed that first, an inactivation of the adenomatous polyposis coli (APC) tumor suppressor gene occurs in the normal colonic mucosa, followed by activating mutations in the KRAS gene and further mutations that result in adenocarcinoma formation with several genetic mutations (Fredericks, 2015; Roper & Hung, 2013). Alterations in APC, KRAS, SMAD4, TP53, and in the mismatch repair (MMR) genes, MLH1 and MSH2 are the genetic changes predominantly found in CRC (Figure 1.2.) (Arends, 2013).

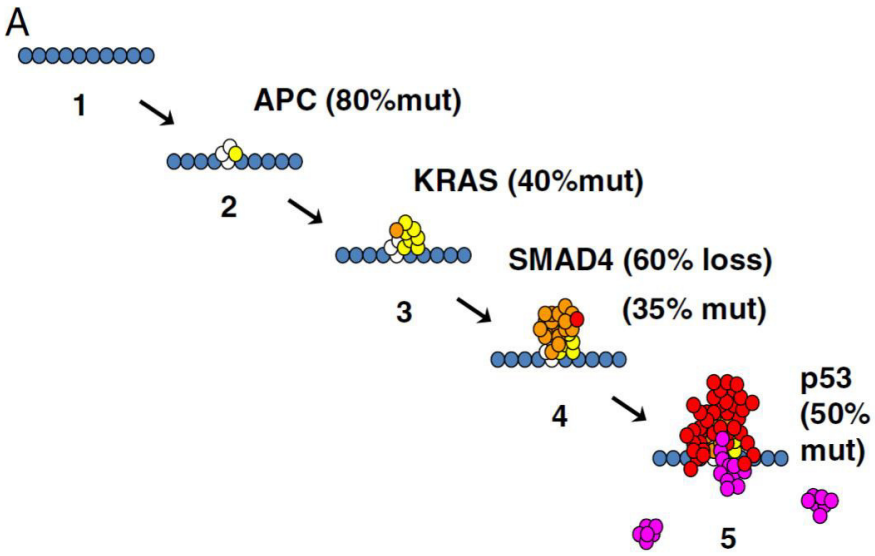


Figure 1.2. Adenocarcinoma sequence: the proposed model for colorectal cancer progression. The most frequent genetic alterations that occur as a very early event occur in APC, mutated in around 80% adenomas and carcinomas. KRAS and TP53 appear to occur as late events and are seen in 40% and 50%, respectively. Other genetic or epigenetic changes can happen during this sequence, including loss of a large part of chromosome 18, containing the SMAD4 and SMAD2 genes, occurring in around 60% CRC, although SMAD4 mutations can be found in up to 35% CRC (Arends, 2013).

Two subtypes of colorectal cancers can be distinguished: a sporadic subtype, normally without family history involved, and an inherited subtype emerging in the context of hereditary syndromes (Stigliano, Sanchez-Mete, Martayan, & Anti, 2014). Most of the patients (around 70% of the cases) have sporadic CRC (nonhereditary) being age and other factors such as a sedentary life and a poor diet the most likely causes for this high incidence (Al-Sohaily et al., 2012; Souglakos, 2007). Concerning CRC types that have an inherited predisposition, they can be subdivided according to whether or not colonic polyps are present. Polyposis syndromes account for about 1% of colorectal carcinomas and the most common is the familial adenomatous polyposis coli (FAP) (Ellis, 2005; Grande Pulido, 2011; Jasperson, Tuohy, Neklason, & Burt, 2010; Rustgi, 2007).

Hereditary nonpolyposis colorectal cancer (HPNCC) or Lynch syndrome is associated with 5% to 8% of colorectal cancers (Ellis, 2005; Jasperson et al., 2010).

There are at least three major molecular pathways to CRC including chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP) pathway (Worthley & Leggett, 2010).

CIN tumors constitute not only most of the sporadic tumors (85%) but also involve FAP cases associated with germline mutations in the APC gene (Fearon & Vogelstein, 1990; Walther et al., 2009). The CIN pathway is characterized by widespread imbalances in chromosome number resulting in aneuploid karyotype, frequent loss-of-heterozygosity (LOH) and chromosomal rearrangements (Stigliano et al., 2014). Moreover, CIN tumors are distinguished by the accumulation of mutations in specific oncogenes and tumor suppressor genes (e.g., APC, KRAS, PIK3CA, BRAF, SMAD4, and TP53), thereby activating pathways critical for carcinogenesis (Worthley & Leggett, 2010; Yamagishi, Kuroda, Imai, & Hiraishi, 2016).

Another important type of genomic instability is MSI pathway caused by dysfunction of DNA MMR genes (essential to maintain genomic stability). MSI is found in only 15% of sporadic CRCs and in contrast, MSI is the hallmark of Lynch syndrome (Al-Sohaily et al., 2012; Armaghany et al., 2012) and is seen in more than 95% of these patients. According to defined markers, MSI CRC are classified as microsatellite high (MSI-H), microsatellite low (MSI-L) or microsatellite stable (MSS). Inactivation of MMR enzymes can result either from abnormal methylation of promoter CpG islands or from point mutations in MMR family members (Armaghany et al., 2012; Imai & Yamamoto, 2008).

The CIMP pathway is the second most common pathway to sporadic CRCs accounting for approximately 15% of sporadic cases (Yamagishi et al., 2016). It is characterized by a vast hypermethylation of promoter CpG island sites (methylation of repetitive sequences between cytosine and guanine), resulting in the inactivation of several tumor suppressor genes (e.g. p16, MINT1, NEUROG1, MLH1) (Grande Pulido, 2011; Nazemalhosseini Mojarad, Kuppen, Aghdaei, & Zali, 2013).

1.1.1.1. Colorectal cancer associated mutations: role and significance

Several mutations in key regulatory genes have been implicated in the progression of colorectal cancer. Modifications in genes such as EGFR, BRAF and KRAS, have been ascertained to be present in colorectal cancer and will be briefly discussed next (Sameer, 2013).

The epidermal growth factor receptor (EGFR) is a cell-surface receptor that belongs to the ErbB family of receptors, a family of four tyrosine kinase receptors, EGFR (ErbB-1), HER2 (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4) (Oh, Lee, Chung, & Kim, 2011). EGFR is involved in cell growth and proliferation, metastasis and cell death through its role in the RAS/ RAF/MAPK pathway and the PI3K/AKT pathway (Markman, Javier Ramos, Capdevila, & Tabernero, 2010). Several epithelial tumors are known to express high levels of the EGFR, including CRC, where its overexpression is found in about 65-75% of mCRC patients and is related to progression, differentiation and recurrence of colorectal cancer (Markman et al., 2010; Oh et al., 2011). Currently, two different monoclonal antibodies (mAbs), Cetuximab and Panitumumab, are being used to prevent EGFR activation by binding to its extracellular domain (Metzger et al., 2011). However, this clinical approach presents low response rates that aside from the KRAS mutational status (discussed hereafter), could be explained by the variation of the EGFR receptor copy number (Metzger et al., 2011). Unlike non-small-cell lung carcinomas (NSCLC), where a correlation with the EGFR mutation has been reported, for colorectal cancer, only the incidence rate of the mutation has been demonstrated (Nagahara et al., 2005).

BRAF is a serine/threonine protein kinase activated by the RAS small GTPase that plays a critical role not only in the (EGFR)-mediated-activated protein kinase (MAPK) signaling pathway that strictly controls cell proliferation, growth and differentiation but also in other vital cellular processes namely apoptosis (through the regulation of Bcl-2) and cell migration (through RHO small GTPases). Therefore, it is not surprising that activating mutations in this gene are found in about 15% of all human known types of cancer (Barras, 2015). The most widespread activating mutation found in this gene is characterized by GTG > GAG substitution at position 1799 of exon 15, which results in the V600E amino acid change leading to sustained MAPK signaling pathway. Oncogenic $BRAF^{V600E}$ is prevalent in about 10% of CRCs and is related to poor prognosis and treatment resistance in mCRC patients (Korphaisarn & Kopetz, 2016).

KRAS mutations are found in approximately one third of all human cancers, including up to 50% of CRCs (Hisamuddin & Yang, 2006) and mostly appear during early stages of tumor progression supporting a causative role of KRAS in human tumorigenesis (Cox, Fesik, Kimmelman, Luo, & Der, 2014; Fernandez-Medarde & Santos, 2011). Interestingly, the frequency of KRAS mutations in CRC varies worldwide suggesting an influence of ethnicity and geographical distribution in its occurrence (J. Zhang et al., 2015). In fact, the effect of geographic area on KRAS mutation rate in CRC cases has been previously described and it was reported that patients from

Western Europe had the highest frequency of mutation (44.7%) followed by Eastern Europe (35.8%) while those from outside Europe had the lowest (19.5%) (Zekri, 2016).

Oncogenic mutations of all RAS family members are concentrated within 3 hotspots (around codons 12, 13 and 61) of the primary nucleotide sequence. However, the incidence of mutation in these sites varies among the RAS isoforms (Figure 1.3.). In KRAS, clear majority of mutations are marked by the substitution of glycine by aspartate/valine at codon 12 ($KRAS^{G12D}$ / $KRAS^{G12V}$) and aspartate at codon 13 ($KRAS^{G13D}$) accounting for about 99% of the mutations detected (86% and 13%, respectively), whereas mutations affecting Glutamic acid 61 (Q61), the other possible hotspot in RAS proteins, account for the remaining 1% (Fernandez-Medarde & Santos, 2011; Rajasekharan & Raman, 2013; Szpon et al., 2016). Codon 12 GGT and codon 13 GGC are responsible for incorporation of glycine into KRAS protein formed during translation (a biogenic amino-acid with no side chain). Glycine at position 12 is situated in a tight loop to which the GAP protein binds. Any mutation in this position will result in the incorporation of a side-chain amino-acid and thus prevent, in terms of spatial architecture, the transition of the protein to inactive status. KRAS mutations result therefore in a continuous activation status of this protein and consequently, this leads to uncontrolled cell growth and cell division rates (Szpon et al., 2016).

KRAS mutations in colon cancers were associated with a lower survival rate, compared with wild-type KRAS patients, increased tumor aggressiveness and cancer recurrence (Cabrera-Mendoza, Gainza-Lagunes, Castaneda-Andrade, & Castro-Zarate, 2014; Dinu et al., 2014). Additionally, KRAS mutations are established biomarkers for predicting the poor efficacy of anti-EGFR monoclonal antibodies in patients in advanced stages of CRC (H. S. Kim et al., 2016).

The determination of KRAS mutations development may be supplementary to existing diagnostic methods in early-stage CRC, and particularly may be an additional parameter for evaluation of the degree of malignancy in CRC (Szpon et al., 2016). This makes KRAS mutations a special case, that draw the attention of the scientific community (Cox et al., 2014; Matallanas et al., 2011).

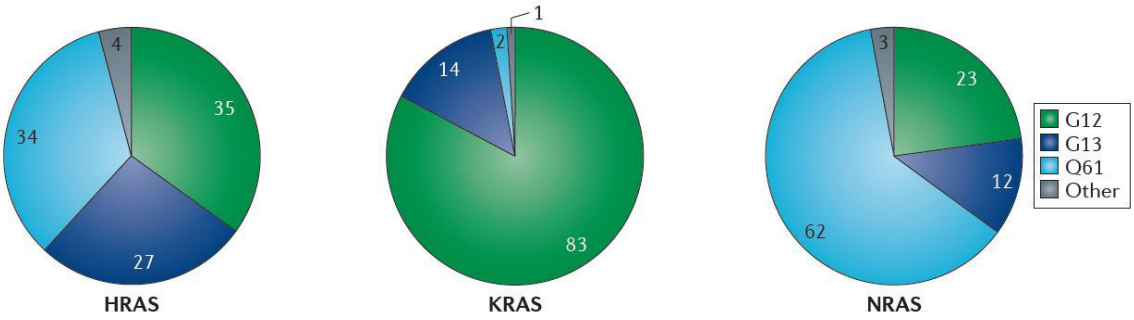


Figure 1.3. Mutations incidence among the main RAS isoforms. RAS genes mutations associated to cancers are characterized by single base missense mutations, 98% of which are found at residues G12 (at green), G13(at dark blue) or Q61(at light blue). G12 mutations are predominant in KRAS and HRAS whereas Q61 mutations are predominant in NRAS (Adapted from (Cox et al., 2014)).

1.2. RAS at a glance

The three canonical members of the RAS gene family, HRAS, NRAS and KRAS, were identified more than 30 years ago because of their frequent oncogenic activation in human tumors (Castellano & Santos, 2011). They are broadly conserved across eukaryotic species and play functionally significant roles in numerous cellular key roles. Their expression is nearly ubiquitous, although there are quantitative and qualitative differences of expression depending on the tissue and/or developmental stage under consideration (Castellano & Santos, 2011). RAS mutations are involved in several cancers with RAS isoforms exhibiting different distributions among them; For example, KRAS mutations are enriched in pancreatic (70%-90%) and colon cancer (35%–50%) as well as lung cancer (20%–35%), while NRAS mutations are most common in hematopoietic/lymphoid (10%) and skin cancers and HRAS mutations dominant in cervical (9%) and salivary gland tumors (15%) (Hunter et al., 2015).

The biochemical and structural basis as well as the pathways that RAS promotes are well known, however, the precise mechanisms by which RAS proteins function are less clear. Thus, understanding RAS interactions within the cell and its action is essential to intervene in RAS-driven diseases (Gysin, Salt, Young, & McCormick, 2011).

1.2.1. RAS effector signaling

RAS oncoproteins are the founding members of RAS superfamily of small GTPases and have been the subject of intense research mainly because of their critical roles in human

oncogenesis (Wennerberg, Rossman, & Der, 2005). Conventionally, RAS superfamily has been divided into five subfamilies – RAS, RAN, RHO, ARF, and RAB - on the basis of sequence and functional similarities (Bos, Rehmann, & Wittinghofer, 2007; Vigil, Cherfils, Rossman, & Der, 2010). Whereas RAN family is best known for its function in nucleocytoplasmic transport of proteins and RNA, RHO family regulates cytoskeleton dynamics, gene expression and cell cycle progression. Finally, ARF and RAB families are involved in vesicular transport (Cazzanelli et al., 2018).

Small GTPases are molecular switches capable of alter between an inactive GDP-loaded to an active form that binds GTP (Boland, Sinicrope, Brenner, & Carethers, 2000). This process is regulated by guanine nucleotide exchange factors (GEFs), namely SOS1 and SOS2 (recruited via the adaptor Grb2), that stimulate nucleotide exchange and by GTPase activating proteins (GAPs) that accelerate the intrinsic GTP hydrolysis activity of RAS (Zeitouni, Pylayeva-Gupta, Der, & Bryant, 2016). Once activated, RAS-GTP interacts with a spectrum of catalytically diverse downstream effectors that then regulate a myriad of cellular functions such as cell proliferation, differentiation, survival, cell cycle entry and cytoskeletal dynamics (Fernandez-Medarde & Santos, 2011; Hancock & Parton, 2005). Upon stimulation of receptor tyrosine kinase (RTK) among which the most studied is the epidermal growth factor receptor (EGFR), GEFs are recruited leading to RAS activation, through the process described above. Activated KRAS can bind at least to 20 effectors, among which RAF (rapidly-accelerated fibrosarcoma) kinases and phosphatidylinositol 3-kinase (PI3K) are the best characterized (Johnson & Chen, 2012; Prior & Hancock, 2012). Other effectors are RAL guanine nucleotide dissociation stimulator (RALGDS), p120GAP, T lymphoma invasion and metastasis-inducing 1 (Tiam 1), PLCε and PKCζ (Figure 1.4.) (Cazzanelli et al., 2018).

Interaction of activated RAS with RAF initiates the RAF→ MEK→ ERK kinase (also called MAPK) cascade. Activated ERK can then phosphorylate many substrates, that control cell cycle progression, proliferation and survival (e.g. autophagy) (Fernandez-Medarde & Santos, 2011).

In the other pathway, RAS activation of PI3K triggers production of the PIP3 (phosphatidylinositol-3,4-triphosphate) by phosphorylating PIP2 (phosphatidylinositol-4,5-diphosphate), process that can be reversed by the phosphatase PTEN (phosphatase and tensin homolog deleted in chromosome ten) (Rajasekharan & Raman, 2013). PIP3 activates phosphatidylinositol dependent kinase 1 (PDK1), which promotes activation of AKT (Protein Kinase B (PKB)). AKT main downstream is mTOR (mammalian target of rapamycin), a serine/threonine kinase, that through its downstream effectors (e.g. S6K), controls several aspects related to cell growth and metabolism (Porta, Paglino, & Mosca, 2014). In cells, mTOR can be divided in two

distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) and is mTORC1 that appear to play critical roles in cell growth in response to nutrients (Kang, Zeh, Lotze, & Tang, 2011).

AKT-mediated phosphorylation also inhibits some pro-apoptotic proteins (Bad, FoxO), and stimulates others (Mdm2) that promote cell survival (Fernandez-Medarde & Santos, 2011; Hancock, 2003; Jancik, Drabek, Radzioch, & Hajduch, 2010; Overmeyer & Maltese, 2011; Rajasekharan & Raman, 2013).

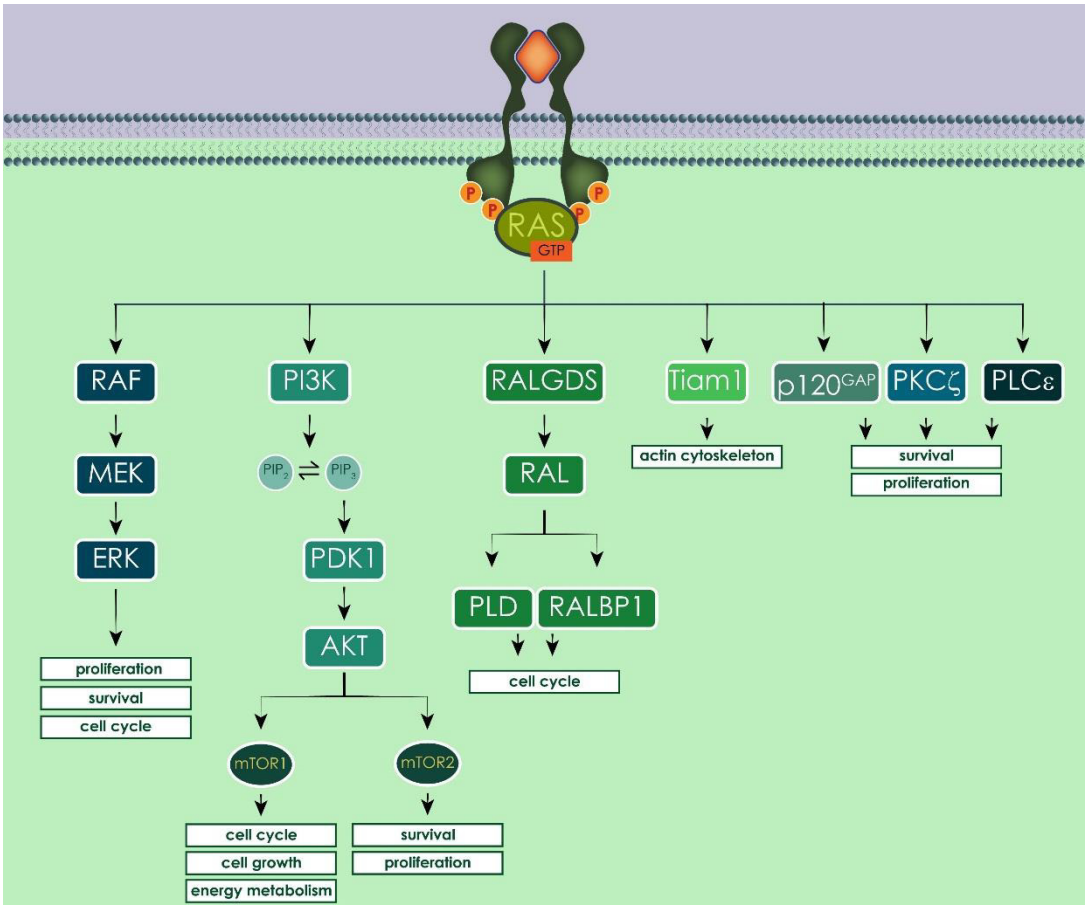


Figure 1.4. Signaling pathways influenced by RAS proteins. Following RAS activation, multiple effectors can be modulated, dictating different signal outputs. Among the most studied downstream RAS effectors, RAF-mediated MAPKs and PI3K/AKT-mediated cascades promote pro-growth signaling whereas RALGDS stimulate cell cycle progression (Cazzanelli et al., 2018).

1.2.2. Differential trafficking and localization of RAS proteins

The RAS family includes three genes: HRAS, NRAS, and KRAS. These three loci encode four different protein isoforms: HRAS, NRAS, KRAS4A, and KRAS4B. The two KRAS isoforms differ due to the alternative splicing of exon 4 in the KRAS locus, with KRAS4B being the predominant

isoform expressed in most tissues (Zeitouni et al., 2016). All isoforms are similar in their primary amino acid sequence (~85%) in the G-domain, which is responsible for GTP/GDP binding with major differences concentrated in the hypervariable region (HVR). This region incorporates a crucial signal, at the extreme C terminus, for localizing RAS to the inner surface of the plasma membrane, the CAAX box (C = cysteine, A = aliphatic amino acid, and X = serine/methionine) (Figure 1.5.) (Cazzanelli et al., 2018; Matallanas et al., 2003).

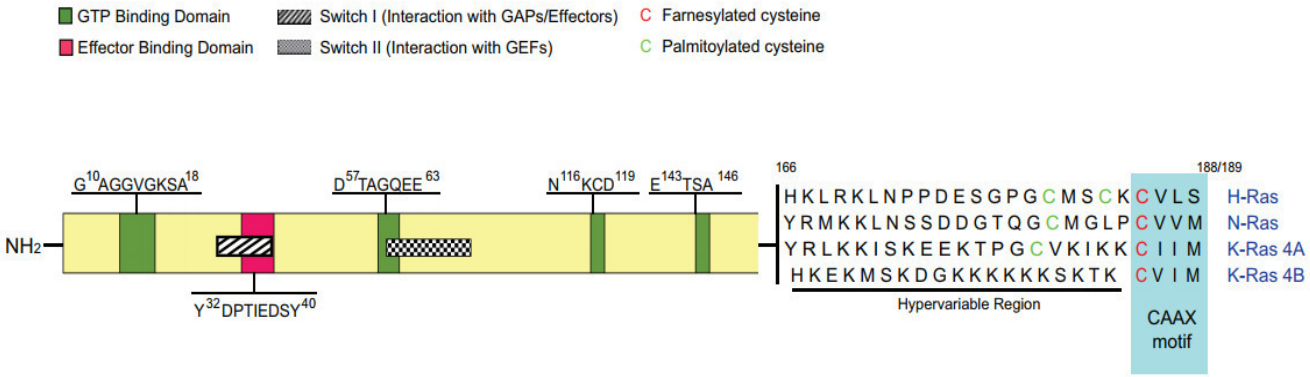


Figure 1.5. RAS proteins primary structure. Green boxes refer to the epitopes responsible for interaction with guanine nucleotides. The red box represents the area of interaction with downstream effectors. The areas corresponding to the striped box (switch I) and the squared box (switch II) undergo conformational changes depending on RAS binding to GDP or GTP. The unique cysteine residue located in the CAAX box (red) is farnesylated, whereas the immediately upstream cysteine residues (green) located in the hypervariable region can be palmitoylated (Castellano & Santos, 2011).

For proper function and oncogenic potential RAS proteins must be associated with the inner leaflet of plasma membrane (Niv, Gutman, Kloog, & Henis, 2002). For that, the newly synthesized, cytosolic, inactive RAS precursors need to bear several postransductional modifications to attain proper activation (Castellano & Santos, 2011). The C-terminal CAAX motif is the target of the first set of three modifications that are common to all RAS isoforms. First, the cysteine residue of CAAX motif is farnesylated by the cytosolic enzyme protein farnesyl transferase (PFT). Next, the AAX sequence is removed by Rce1. Finally, the now C-terminal farnesyl cysteine is carboxymethylated by ICMT (Figure 1.6.) (Abankwa, Gorfe, & Hancock, 2007). However, for proper targeting of RAS to membranes each of the proteins H-, N-, and KRAS suffer further modifications. Whereas HRAS is palmitoylated on C181 and C184, NRAS is palmitoylated on C181 and KRAS4B does not have any palmitoylation at all (Rajalingam, Schreck, Rapp, & Albert, 2007). Instead, KRAS4B is constituted by a polybasic region (a stretch of lysines) that enables an

electrostatic interaction with the negatively charged plasma membrane phospholipids (Agudo-Ibanez, Herrero, Barbacid, & Crespo, 2015).

Once CAAX processing is complete, RAS isoforms have different traffickings. Palmitoylated isoforms visit the Golgi where they are acylated and thereby trapped in its' membranes from where they traffic, via vesicular transport, to the plasma membrane (Ahearn, Haigis, Bar-Sagi, & Philips, 2011). Alternatively spliced KRAS4A is believed to follow this traffic as this splice variant does not have the polybasic domain and it is palmitoylated at C180 (Rajalingam et al., 2007). In contrast, KRAS4B cannot be trapped on the Golgi and is directly routed from the endoplasmic reticulum to the plasma membrane by a still poorly understood delivery system that could involve cytosolic chaperones (Figure 1.6.) (Ahearn et al., 2011; Cazzanelli et al., 2018).

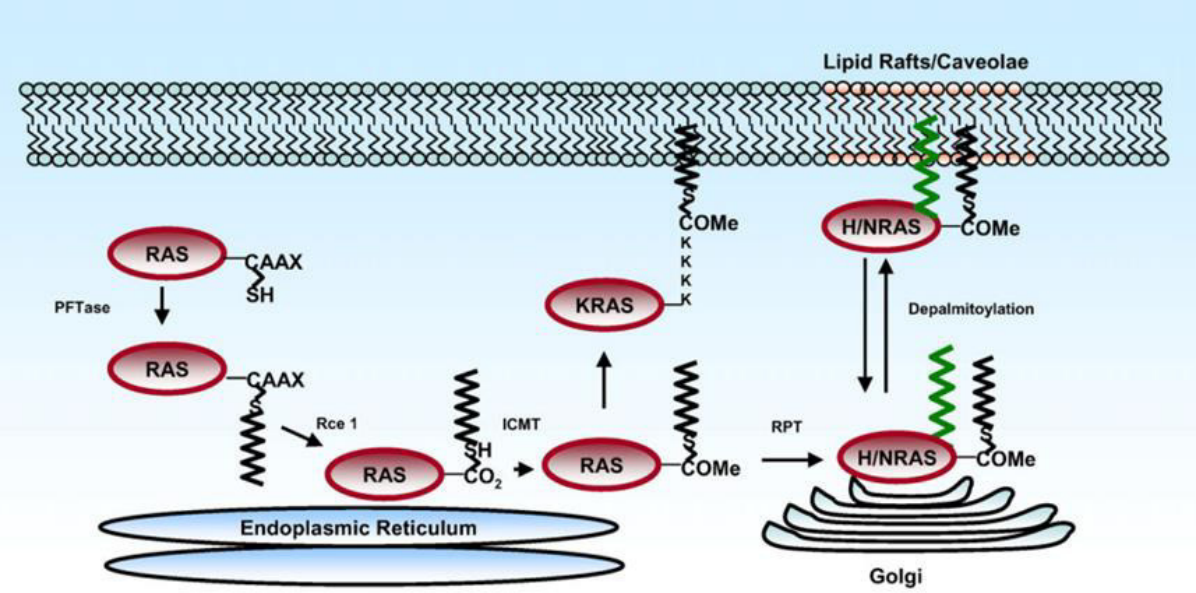


Figure 1.6. RAS modifications and localization. Cytosol is where RAS proteins are synthesized; Here, RAS proteins are farnesylated by protein farnesyl transfeRASe (PFT) at the cysteine residue of the CAAX motif (A is an aliphatic amino acid and X is any amino acid). Then, RAS converting enzyme 1 (Rce 1) and Isoprenylcysteine carboxylmethyl transferase (ICMT) mediate the cleavage of AAX tripeptide and methylation, respectively, at the cytosolic surface of the ER. H- and NRAS further undergo palmitoylation in the hypervariable region (HVR) on the Golgi to reach the plasma membrane via vesicular transport, while KRAS reaches the plasma membrane by a yet uncharacterized pathway. Depalmitoylation of H- and NRAS will lead to the localization of these two isoforms in the Golgi (Rajalingam et al., 2007).

1.2.2.1. Galectin-3 and actin cytoskeleton as modulators of KRAS signal output

Due to their different posttranslational modifications (PTMs), the palmitoylated and polybasic-targeted RAS isoform proteins are conducted to different localizations in the plasma

membrane, where they incorporate into nanoclusters, unique locations for effector recruitment and activation, thus essential for signal propagation (Castellano & Santos, 2011). Approximately 40% of RAS proteins are organized into nanoclusters, whereas the remainder freely diffuse as monomers in the cytoplasm (Ahearn et al., 2011). These nanoclusters display different features: HRAS GDP-loaded nanoclusters are recruited to cholesterol-rich microdomains (lipid rafts and caveolae) in the plasma membrane but are absent from rafts when are GTP-loaded. Apparently, exchange of GDP for GTP causes HRAS migration into disordered plasma membrane regions in a Galectin-1 dependent manner (Ahearn et al., 2011). In contrast, NRAS nanoclusters localize preferentially into the lipid rafts when they are active, but when GDP-loaded they reside on the nonraft domain (Roy et al., 2005). In the case of KRAS, its nanoclusters are actin-dependent, since early experiments have shown that perturbing actin cytoskeleton inhibits KRAS nanoclustering and abrogates its signaling pathways (Hancock & Parton, 2005). Moreover, it is described that , when mutated, KRAS is capable to maintain the cytoskeleton organization through the MEK-ERK pathway (Pollock, Shirasawa, Sasazuki, Kolch, & Dhillon, 2005). Also, available data show that actin cytoskeleton has an active role in membrane organization, regulating its lipids distribution and thus determining the proteins distribution at the membrane with subsequent influence in their signaling outputs (Chichili & Rodgers, 2009; Gomez-Llobregat, Buceta, & Reigada, 2013).

Furthermore, KRAS nanoclusters are not localized in cholesterol-rich microdomains, meaning that are cholesterol-independent and seem to be stabilized by Galectin-3 (Gal-3) (Heo et al., 2006; Kenworthy, 2007; Tian, Plowman, Parton, Kloog, & Hancock, 2010). Gal-3 is a unique chimeric member of β-galactosidase binding protein family that stabilize KRAS in its active state (by decreasing the efficiency of GAP-mediated GTP hydrolysis) thus promoting its association with several effectors, apparently by activation of RAF and PI3K signaling pathways (Elad-Sfadia, Haklai, Balan, & Kloog, 2004; R. Levy, Biran, Poirier, Raz, & Kloog, 2011). Thus, through this mechanism, Gal-3 is capable to determine the strength, duration, and selectively of the KRAS signal (Elad-Sfadia et al., 2004).

1.2.2.2. RAS localization matters: are lysosomes and mitochondria important signaling platforms?

Like the partitioning of RAS into membrane microdomains, its trafficking between organelles is also regulated by PTMs. In addition to plasma membrane, as a platform from which RAS proteins activate their effectors, increasing evidences show that when located on the membranes of endomembranous organelles these proteins are still capable of activating their

signaling pathways (Choy et al., 1999; Hancock, 2003; Jiang & Sorkin, 2002). It was reported that KRAS translocate onto early and late endosomes through a clathrin-dependent pathway independently of CaM and protein kinase C (PKC) phosphorylation (Lu et al., 2009). From the early endosomes, KRAS but not HRAS nor NRAS, was transported to late endosomes (Les) and eventually targeted to lysosomes, confirmed by confocal microscope images where was possible to observe co-localization between GFP-KRAS and both LAMP1 and LAMP2, which are lysosomal markers (Lu et al., 2009). Using fluorescent probes, it was shown that KRAS was active on Les since it elicited a signal output through RAF to promote MAPK signaling cascade (Lu et al., 2009). Moreover, KRAS was found to dissociate from the plasma membrane and relocate to ER, Golgi and, mitochondria after action of PKC maintaining the capacity to induce MAPK pathway after EGF stimulation (Bivona et al., 2006; Cazzanelli et al., 2018; Prior & Hancock, 2012).

Hereupon, RAS is present in different cellular compartments and plasma membrane microdomains, and the same isoform activates different signaling networks according to its specific localization (Fey, Matallanas, Rauch, Rukhlenko, & Kholodenko, 2016). Therefore, understanding how RAS is targeted to cellular compartments can give us valuable information about RAS-mediated signaling and, ultimately, about how RAS is involved in the tumorigenic process (Castellano & Santos, 2011).

1.3. Autophagy: a double edge sword in cancer

Autophagy, the process of cellular self-eating, has long been recognized as an important protein degradation pathway, particularly during starvation or stress. It refers to a collection of tightly regulated catabolic processes, all of which deliver damaged cytoplasmic components to the lysosome for degradation, sustaining cellular metabolism and homeostasis (Kaur & Debnath, 2015; Mah & Ryan, 2012). Thus, autophagy exerts opposing and context-dependent roles in cancer, since it confers stress tolerance, limits damage and sustains viability under adverse conditions acting either as a tumor suppressor and tumor promoting mechanism (Mathew, Karantza-Wadsworth, & White, 2007).

1.3.1. Molecular mechanism of autophagy

Autophagy describes the process by which cellular proteins, organelles, and pathogens are sequestered within a membranous crescent that elongates to form a double membrane-bound structure called autophagosomes which subsequently fuses with the lysosome, where their

contents are degraded and recycled into metabolic precursors (Greenfield & Jones, 2013; Mizushima, 2007; Mizushima & Klionsky, 2007).

The molecular mechanism of autophagy can be broken down into several stages: initiation and nucleation, elongation, maturation and cargo degradation (Figure 1.7.). Each step involves several Atg (autophagy-related) proteins, most of which were first identified in yeast, suggesting that autophagy is a highly conserved pathway through evolution (Badadani, 2012; He & Klionsky, 2009).

Phagophore initiation is triggered by inactivation of mammalian target of rapamycin (mTOR) by certain signals including rapamycin and nutrient deprivation (Greenfield & Jones, 2013; Yang, Chee, Huang, & Sinicrope, 2011). Under the control of mTORC1 is the ULK complex (ULK1, Atg13, Atg101 and FIP200). When mTOR is inactivated, Atg13 and FIP200 are phosphorylated by ULK1 and the entire complex is relocated to the phagophore (Hale, Ledbetter, Gawriluk, & Rucker, 2013). The nucleation phase involves the formation of a phospholipid bilayer membrane known as the isolation membrane or phagophore that will be the precursor to the mature autophagosome membrane. It is a highly orchestrated process relying on class III phosphatidylinositol 3-kinase (PI3K) complex, consisting of Vps34, Beclin 1, Vps15 and Atg14L (Hale et al., 2013). This complex serves to produce phosphatidylinositol-3-phosphate (PtdIns3P or PI3P) that recruit's complexes and lipids from several membrane sources including endosomes and mitochondria (Kruppa, Kendrick-Jones, & Buss, 2016) to elongate the phagophore membrane (Hale et al., 2013; Wong, Cheung, & Ip, 2011).

The elongation of the phagophore involves two ubiquitin-like conjugation pathways (Badadani, 2012). The first is the Atg5– Atg12–Atg16L1 complex and the second is the microtubule-associated protein light chain 3 (LC3)–phosphatidylethanolamine complex (Greenfield & Jones, 2013). LC3 is required for the elongation and remains associated with the completed autophagosome (Y. K. Lee & Lee, 2016; Wong et al., 2011).

Following elongation, maturation of the autophagosome and degradation of the cargo occurs. These steps involve fusion of the outer membranes of the autophagosome and lysosome to form the autophagolysosome, which becomes acidified and delivers lysosomal hydrolases, including cathepsins, into the compartment, ultimately degrading its contents (Greenfield & Jones, 2013).

Aside from the Atg's and the mTOR pathway, other proteins also participate in the process of autophagy such as some of RAS downstream substrates, including JNK and ERK (Schmukler, Kloog, & Pinkas-Kramarski, 2014).

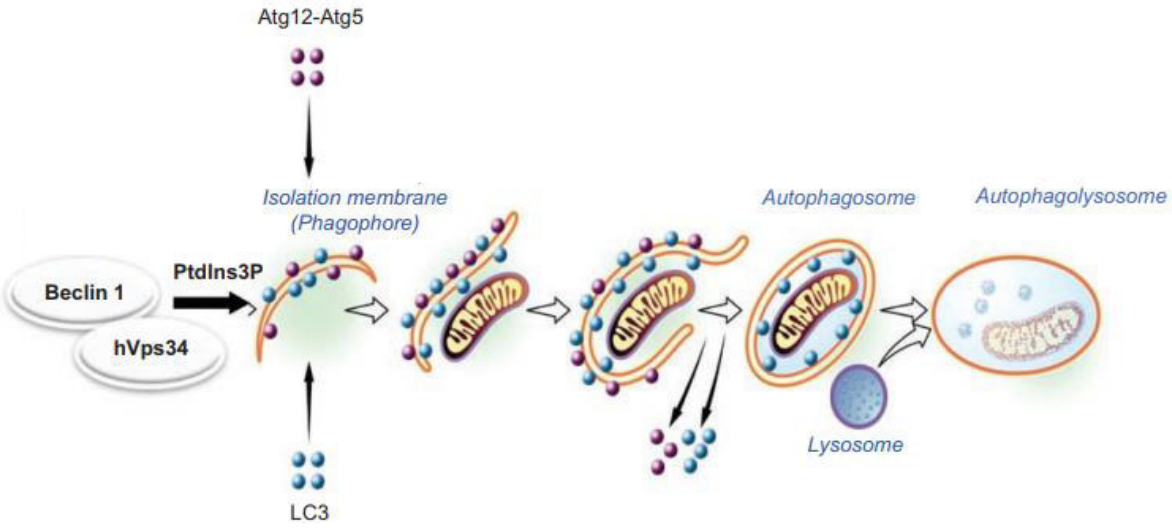


Figure 1.7. Major stages of the autophagic process. Autophagic process follows several stages: autophagosome initiation and nucleation, where Beclin 1 association with the class III phosphatidylinositol 3-kinase/Vps34 is essential; Elongation, involving the two ubiquitin-like conjugation pathways Atg12-Atg15 and LC3, and maturation and cargo degradation, characterized by fusion of the outer membranes of the autophagosome and lysosome to form the autophagolysosome, which becomes acidified and delivers lysosomal hydrolases into the compartment, ultimately degrading its contents (Adapted from (Meijer & Codogno, 2009).

1.3.2. Role of autophagy in cancer

The findings of Yoshinori Ohsumi about the mechanism of autophagy, that award him the Nobel Prize for Physiology or Medicine, highlighted the importance of autophagy in health and disease (J. M. M. Levy, Towers, & Thorburn, 2017). Arguably, the disease in which the functional status of autophagy has been investigated the most is cancer in which is thought to prevent its progression as well as to enable tumor cell survival showing a controversial role (J. M. M. Levy et al., 2017). Evidence suggests that autophagy may prevent cancer promotion in pre-malignant lesions but contributes to tumor survival during cancer progression (Choi, 2012). Furthermore, tumor cells are capable to use autophagy to escape from several anti-cancer therapies (Chen & Karantza-Wadsworth, 2009). Thus, an important question arises in cancer therapy: autophagy should be enhanced or inhibited?

Cancer cells experience higher metabolic demands and stresses comparing to normal cells, due to their rapid proliferation and altered glycolytic metabolism, therefore, autophagy plays a similar role in tumor cells as it does in normal cells, but because the inherent stress tumor cells encounter is greater, the dependence on autophagy may be more substantial (White & DiPaola, 2009).

Autophagy has been proposed to suppress tumorigenesis by preserving genomic stability, disposing endogenous sources potentially mutagenic, maintaining normal bioenergetic functions and degrading oncogenic proteins (Galluzzi et al., 2015). The most important evidences supporting the role of autophagy in tumor suppression come from studies involving the essential autophagy gene *beclin 1* (Choi, 2012). Allelic loss of *beclin 1* is frequent in human breast, ovarian and prostate cancers (White & DiPaola, 2009) and its impaired function is described to lead to higher incidence of lymphoma, liver and lung cancer in mice (Qu et al., 2003; Yue, Jin, Yang, Levine, & Heintz, 2003). Also, the constitutive activation of the PI3K/AKT/mTOR pathway, that is frequently altered in cancers, is known to suppress autophagy, thus promoting tumor cell proliferation and survival (Martelli et al., 2007). Moreover, autophagy may protect against malignant transformation through limiting necrosis and chronic inflammation (Z. J. Yang et al., 2011).

On the other hand, once malignant transformation is established, autophagy is believed to promote tumor progression and resistance to therapy because under metabolic stress, cells seek an alternative source of energy and metabolites, that autophagy is capable to satisfy (Degenhardt et al., 2006). It is described that autophagy is able to promote tumor progression by improving the resistance of cancer cells to endogenous conditions that normally induce cell death, ensuring the maintenance of the transdifferentiation complex epithelial-to-mesenchymal transition (EMT) and sustaining the survival of cancer cells that enter a state of dormancy or senescence in response to therapy (Galluzzi et al., 2015). In a number of studies where established tumors were subjected to genetic or pharmacological inhibition of autophagy, were demonstrated high sensitivity of cancer cells to exogenous stimuli (Amaravadi et al., 2007; Boya et al., 2005; Kroemer, Marino, & Levine, 2010). Accordingly, tumors with impaired autophagy are usually less resistant to a wide range of therapeutic agents as well as to radiotherapy comparing to their autophagy-proficient counterparts (Janku, McConkey, Hong, & Kurzrock, 2011; Ko et al., 2014; J. M. Levy et al., 2014).

Summing up, autophagy has competing and context-dependent effects that are not completely understood, however, the pharmacological modulation of this mechanism seems to have significant clinical potential (Choi, 2012). Therefore, unraveling the context-dependent effects

of autophagy on cancer is essential and should enable tailor interventions to specific situations (J. M. M. Levy et al., 2017).

1.3.3. Relevance of actin cytoskeleton in autophagy

Actin, the major component of the cytoskeleton, is a 42 KDa globular protein (G-actin) that polymerizes to form filaments (F-actin) in a reversibly manner. The assembly of actin filaments is essential for correct performance of the multiple biological processes where actin is involved such as cell motility and contraction, vesicle trafficking, intracellular organization and autophagy (Desouza, Gunning, & Stehn, 2012). Actin dynamics is mainly regulated by the RHO family of GTPases (namely RHOA, RAC1 and, CDC42) which play important roles in shaping the actin cytoskeleton. Because voluntary actin filament assembly is difficult and is limited by the nucleation of actin dimers and trimers, cells use the ARP2/3 complex and nucleation promoting factors such as WHAMM and WASH to nucleate actin and overcome this limitation (Kruppa et al., 2016).

The general importance of the actin cytoskeleton in autophagy was demonstrated when actin polymerization abolishment lead to impaired autophagosome formation, showing that polymerization of actin is important at certain steps during the autophagic process after starvation induction (Aguilera, Beron, & Colombo, 2012). Molecules such as Atg14 and Beclin 1 were shown to co-localize with actin during starvation induced autophagy, in contrast with LC3 that co-localized only in some occasions leading the authors to conclude that actin is involved in very early stages of autophagosome conception, likely at the omegasome (platforms that manufacture autophagosomes) (Aguilera et al., 2012). Supporting these results, recently reports demonstrated that upon autophagy induction, the actin nucleator WHAMM recruits ARP2/3 complex to omegasomes providing the necessary conditions for phagophore expansion and subsequent autophagosome formation (Kast, Zajac, Holzbaur, Ostap, & Dominguez, 2015). Together the data support a role for WHAMM in autophagosome formation through an Arp2/3-dependent actin comet tail mechanism (Coutts & La Thangue, 2016).

Interestingly, evidence suggests that in addition to its role in the initial stages of autophagosome formation, the actin cytoskeleton is also required for the fusion of autophagosomes with lysosomes. Lee and colleagues have demonstrated that during selective autophagy (not in starvation-induced autophagy) histone deacetylase 6 (HDAC6)-mediated actin remodeling is required for fusion of autophagosomes to lysosomes. Apparently HDAC6 recruits the actin remodeling factor cortactin to autophagosomes thus promoting actin polymerization and

reorganization which stimulates F-actin network assembly enabling fusion of the autophagosome with the lysosomes and subsequent cargo degradation (J. Y. Lee et al., 2010).

1.3.4. Autophagy and its impact on colorectal cancer

The autophagic process is regulated by important signaling pathways that are frequently altered in CRC (Burada et al., 2015). Activating mutations in class I PI3K, AKT and RAS, or inactivating mutation in PTEN, have been shown to decrease autophagy through activation of mTORC1 (Maiuri et al., 2009; Morselli et al., 2009). Moreover, it was reported that inactivating mutation in PTEN impairs tight junction integrity, and consequently, stimulates tumor growth and metastatic potential of colon cells (Langlois et al., 2010).

In advanced CRC cases, a high activity of mTOR has been observed, which is associated with increased *in vitro* and *in vivo* cell growth, demonstrating the importance of mTOR pathway in CRC progression and the possible role of autophagy in CRC (Y. J. Zhang et al., 2009).

In addition, other proteins and genes involved in the autophagy machinery may demonstrate the impact of autophagy in CRC. For example, LC3-II is overexpressed in CRC compared to normal tissue, especially in advanced stages. Zheng *et al.* reported that LC3-II was overexpressed in cancer cells and that autophagy enhanced the aggressiveness of CRC. Also, BECN1, that encodes the Beclin 1 protein, is overexpressed in most colorectal carcinomas (95%) compared to normal colon mucosa, being especially associated with advanced stages of CRC (Burada et al., 2015; Mokarram et al., 2017). Moreover, mutations in UVRAG leads to the expression of its truncated form in CRC enhancing metastasis through activation of the small GTPase RAC (Mokarram et al., 2017).

1.3.5. KRAS and autophagy regulation in colorectal cancer: a tight bond

Since RAS isoforms have been implicated in autophagy regulation and mutation of the KRAS oncogene is highly frequent in colorectal cancer, the scientific community questioned whether/how mutant KRAS alleles regulate autophagy in CRC and its implications (Alves et al., 2015). Yoo and colleagues have shown that oncogenic $KRAS^{G13D}$ blocks autophagy by down-regulating Beclin 1 in colon epithelial cells (Yoo et al., 2010). However, Alves *et al.* (2015), using colorectal cancer cell lines, have reported that mutated $KRAS^{G13D}$ and $KRAS^{G12D}$ up-regulate autophagy to a higher extent than wild-type KRAS in response to nutrient limitation, by up-regulating ERK pathway and down-regulating the PI3K/AKT pathway (known to activate the autophagy inhibitor

mTOR) (Alves et al., 2015). Also, to understand whether autophagy and/or KRAS are required for CRC cell survival Zhai and colleagues (2013) 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* (Zhai, Song, Xu, Zhu, & Ju, 2013). Moreover, Sakitani and co-workers (2015), demonstrated that mice deficient in Atg5 exhibit increased expressions of caspase 3 (key enzyme during apoptosis) in colon tumors and reduced tumor size (Sakitani et al., 2015). Furthermore, Alves *et al.* (2015), depleted KRAS, Atg5 and BECN1 by siRNA in the colorectal cell line SW480 and observed that KRAS knockout increased the cell death compared to control siRNA- transfected cells, being these results particularly evident under starvation conditions. Depletion of Atg5 or BECN1 had the same effect, though to a slightly lesser extent (Alves et al., 2015).

To sum up, the interplay between RAS and autophagy on the survival of colorectal cancer cells is complex, as it may have both pro- and anti-tumorigenic roles. However, recent data indicate that autophagy mediated by activating KRAS mutations has a pro-survival role in CRC, suggesting that blocking autophagy may be an effective treatment approach (Z. J. Yang et al., 2011).

1.4. Therapy in colorectal cancer

1.4.1. KRAS: a marker of therapy resistance in colorectal cancer

Regardless the fact that 25-30% of human cancers harbor mutations in RAS genes (Fernandez-Medarde & Santos, 2011), and despite the increasing interest in exploring anti-RAS therapeutic strategies and decades of deep investigation and attempts, none anti-RAS therapeutics have reached clinical application (Hobbs, Der, & Rossman, 2016). This is also true for CRC harboring KRAS mutations.

The main curative treatment for patients with CRC is surgery (Boland et al., 2000). However, 50% of the patients with CRC develop metastases, and most patients with metastatic colorectal carcinoma (mCRC) eventually die of their disease. Hence, chemotherapy plays an important role in the treatment of patients with metastatic disease. Drugs that target the synthesis of DNA such as 5-fluorouracil (5FU), oxaliplatin, irinotecan, and monoclonal antibodies (mAbs) that block epidermal growth factor receptor (EGFR) signaling, such as cetuximab and panitumumab, have been used in clinical trials (Hohla et al., 2014).

In the last years, therapies that involve targeting of the EGFR with drugs such as cetuximab and panitumumab, demonstrated effectiveness for patients with RAS^{WT} mCRC, however, only a small percentage of the patients respond to these therapies (Bokemeyer et al., 2011) and even those who initially respond, eventually develop resistance to it (Volker Heinemann et al.).

Although little is known about the mechanism involved, KRAS mutations are a predictive biomarker of resistance to EGFR inhibitors in mCRC and for that reason have been used to plan the appropriate treatment decision (Heinemann, Douillard, Ducreux, & Peeters). Over the last years, several trials have suggested that KRAS mutations in codon 12 or 13 do not benefit from anti-EGFR therapy (Allegra et al., 2009; Van Cutsem et al., 2011). However, conflicting opinions exist about KRAS mutation in codon 13 since some authors reported that patients carrying these mutations might respond to anti-EGFR therapies (De Roock et al., 2010) whereas others showed that patients with these mutations are unlikely to respond to these kind of therapeutic approaches (Gajate et al., 2012).

In normal cells, upon growth factor (EGF) stimulation and the activation of its receptor (EGFR), the inactive RAS protein changes transiently to the active form. However, $KRAS^{MUT}$ sustains the activated RAS-GTP form. This causes constitutive activation of downstream effectors, mainly RAF in the RAF-MEK-ERK pathway and PI3KCA in the PI3K-AKT-mTOR. This phenomenon possible explains why treatments that block EGFR signaling are not efficient since KRAS oncogene is unaffected by EGFR signaling (Tsuchida, Murugan, & Grieco, 2016). Furthermore, in addition of being a key driver of primary resistance, KRAS mutations also participates in acquired resistance since Bouchahda *et al.* (2010) reported that after treatment with cetuximab, patients developed resistance, and when samples were screened for KRAS mutations, that previously did not exist, KRAS mutations were detected, suggesting a late switch in KRAS mutational status after treatment with anti-EGFR drugs (Bouchahda et al., 2010).

Besides KRAS, additional studies have shown that patients with NRAS, PI3KCA, and BRAF oncogene mutations are also resistant to anti-EGFR therapy (Zhao et al., 2017). Because several biomarkers and pathways are associated with resistance to anti-EGFR therapy, currently approaches consist in targeting in a simultaneous or sequential manner different aberrant biomarkers to improve treatment efficiency (Di Nicolantonio et al., 2008).

1.4.2. New phenoxazine derivatives in cancer

Discovery of novel organic compounds for science life applications are currently the focus of intense research because of their biological activity and modes of action. Among these, are the cationic polycyclic phenoxazine derivatives, which are fluorescent markers that have assumed increasing importance not only as probes but also as antiproliferative compounds with potential antimicrobial and antitumoral applications (Abe, Yamane, & Tomoda, 2001; Bolognese et al., 2002). Benzo[a]phenoxazines are derived from the oxazine heterocycle (Figure 1.8 A) and are structurally characterized by the presence of a benzene ring fused with the *a* face of the phenoxazine structure (Figure 1.8. B and C) (Jose, 2006). Besides the central structure of benzophenoxazine, the major similarity between them, is the presence of an amine function at 9-position (Jose, 2006). Furthermore, benzo[a]phenoxazines can have a functional group (amine, carboxyl or hydroxyl group) at position 5 (Figure 1.8. C) that is essential for covalent labeling of molecules as well as to enable other chemical modifications (V. Frade, Sousa, M.J., Moura, J., and Gonçalves, M.S., 2007).

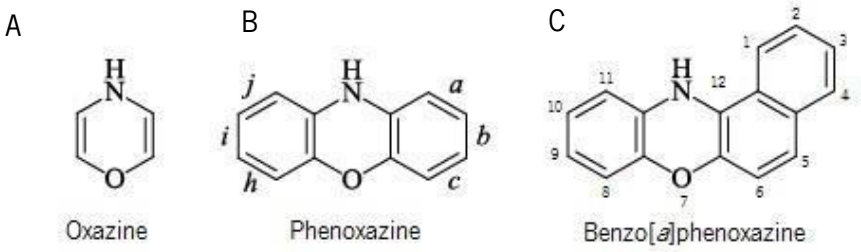


Figure 1.8. Molecular structure of Oxazine (A), Phenoxazine (B) and Benzo[a]phenoxazines (C).

Due to the presence of an extended aromatic system, it is suggested that their antitumoral activity is exerted through intercalation between specific DNA base pairs, which is known to influence DNA properties (Bolognese et al., 2006). Lewis *et al.* (1949) reported that when oxazine dyes and derivatives, were oral administered in tumor-bearing mice, these compounds had the ability of retarding tumor growth, with benzo[a]phenoxazine derivatives demonstrating high potential from a therapeutic point of view. Moreover, they shown that different substitutions give rise to compounds with significantly differences in toxicity and selectivity (Lewis, 1949). Suzuki and co-workers (2007), compared several phenoxazine derivatives in human normal and cancer cells and among the compounds tested, it was WM7 33 compound (a benzo[a]phenoxazine salt) that

presented the highest affinity and specificity to tumor tissues. The cell death mechanism induced by this compound was also studied and it was found that apoptosis was not involved, since DNA was not fragmented, and apparently autophagosome formation was inhibited (Suzuki, 2007). In contrast, some reports have demonstrated that some phenoxazine derivatives display apoptotic activity against different cell lines (Abe et al., 2001; Kucukkilinc, 2007) showing the huge versatility of this class of compounds.

Importantly, the fact that these compounds tend to accumulate preferentially in tumor cells, makes them potential cancer therapy agents that need further exploitation in order to maximize their use.

1.4.2.1. MSG-111-cd3: a promising anticancer agent against colorectal cancer?

In the past years Professor Maria João Sousa group in collaboration with the group of Professor Sameiro Gonçalves (University of Minho, Portugal) have been exploring the potential use newly synthetized benzo[a]phenoxazine compounds as antifungal agents (V. Frade, Sousa, M.J., Moura, J., and Gonçalves, M.S., 2007; V. H. J. Frade, Sousa, M. J., Moura, J. C. V. P., and Gonçalves, M. S. T., 2008). Among those, one in particular have attracted their interest, due to its high antifungal activity, the N-(5-((4-ethoxy-4-oxobutyl)amino)-10-methyl-9H-benzo[a]phenoxazin-9-lidene), also designated as MSG-111-cd3 (Figure 1.9.).

The studies using *Saccharomices cerevisiae* as a model, shown that MSG-111-cd3 accumulates at the vacuolar membrane with subsequent vacuolar membrane damage and vacuolar permeabilization (Carvalho, 2011). Moreover, several cell death markers such as ROS accumulation, vacuole membrane permeabilization, chromatin condensation and nuclear release of Nhp6Ap (protein that remodels nucleosomes) were tested and it was observed that the compound lead to Nhp6Ap release from the nucleus to the cytosol in yeast cells without loss of plasma membrane integrity (J. C. C. Ferreira, 2014; Lopes, 2015). Furthermore, previous results suggested that MSG-111-cd3 toxic effects may be due vacuolar membrane damage and vacuolar permeabilization (Lopes, 2015). In order to further elucidate the cell death process induced by this compound, Ferreira (2017) studied the consequences of vacuolar permeabilization, mainly the role of Pep4p (vacuolar protease) in the cell death process and also explored the involvement of autophagy. With these experiments, it was shown that MSG-111-cd3 yeast cell death is dependent on Pep4p and that the vacuole permeabilization lead to its translocation from the vacuole to the cytosol. Moreover, autophagy seems not to be involved in the cell death mechanism induced by

MSG-111-cd3. Indeed, the compound appears to block autophagy induction (J. C. C. Ferreira, 2017).

Because this compound has been exhibiting interesting results and arising several questions in the yeast model, we tested for the first time this compound as a potential anticancer agent in CRC derived cells and explore its mechanism based on the discoveries already made in the yeast model.

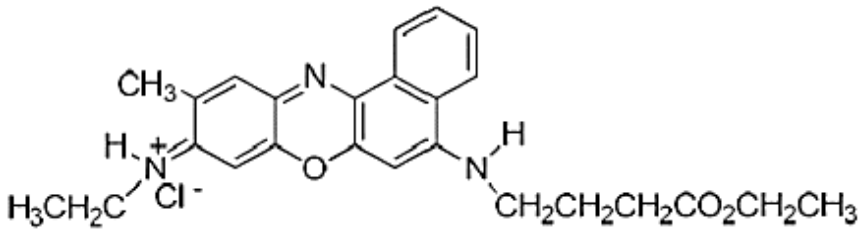


Figure 1.9. N-(5-((4-ethoxy-4-oxobutyl)amino)-10-methyl-9H-benzo[a]phenoxazin-9-lidene) (MSG-111-cd3) structure.

2. *Rationale* and aims

During the thesis project we had **two different purposes**:

Rationale 1: Several data available support a central role of mutated KRAS-signaling pathways in the control of autophagy, which has been proved to have a protective role in colorectal cancer cells (Alves et al., 2015; J. Y. Guo et al., 2011; J. Y. Guo et al., 2013; S. Yang et al., 2011). Differences in subcellular localization of KRAS are able of generate different signal outputs. The localization of $KRAS^{MUT}$ upon autophagy induction is still unknown. Here, we hypothesized that $KRAS^{MUT}$ might regulate autophagy from specific intracellular compartments such as mitochondria and lysosomes, and/or from organized molecular complexes with Gal-3 or actin filaments, that were already described as important KRAS signal output modulators (Elad-Sfadia et al., 2004; Pollock et al., 2005). Understand the interaction between $KRAS^{MUT}$ with different subcellular organelles or molecules can provide valuable insights about $KRAS^{MUT}$ signaling and might lead to the identification of new molecular targets for new therapeutic approaches in CRC.

We **specifically aimed** to answer the following questions:

1. Does autophagy induction by starvation lead to co-localization of KRAS wild-type and mutated proteins to mitochondria or lysosomes?

2. Are the complexes KRAS-Gal-3 and KRAS-actin affected under starvation-induced autophagy?

For this purpose, we performed immunofluorescence assays to assess co-localization and protein-protein interaction. For that matter we used a normal colonic epithelial cell NCM460 model where the wild-type KRAS ($KRAS^{WT}$) and a specific hotspot KRAS mutation ($KRAS^{G12V}$) were individually overexpressed (Alves et al., 2015).

Rationale 2: KRAS mutations are a predictive biomarker of resistance to EGFR inhibitors, thus new approaches to overcome this resistance are needed. The search for the discovery of new drugs has increased all over the years. New phenoxazine derivatives have been synthesized in the Department of Chemistry and have proven to be very promising. A new phenoxazine derivative (MSG-111-cd3) was already tested in *Saccharomyces cerevisiae* and appears to block autophagy induction and to induce cell death by a Pep4p (human Cat-D orthologue) dependent mechanism.

Interestingly, we have previously demonstrated that yeast is an important translational model for colorectal cancer concerning induction of lysosomal membrane permeabilization with Cat-D release in response to acetate, a short chain fatty acid (Marques et al., 2013). Thus, we thought it would be interesting to further investigate the effect of the MSG-111-cd3 agent in CRC cells, as a possible new drug to be potentially used in CRC therapy.

We specifically aimed to answer the following questions:

1. Which is the effect of this newly synthesized compound MSG-111-cd3 in human colorectal cancer cells? Is the mechanism of action in CRC cells similar to yeast? Could this compound be promising anti-cancer agents for treatment of CRC with KRAS mutations?

To achieve our aim, we used the parental normal colonic epithelial cell line NCM460 and CRC-derived cell lines RKO and SW480 harboring different genetic background and performed SRB assay to analyze the effects of this compound in cell proliferation. Furthermore, to confirm if Cat-D (Pep4p homologous) is involved in MSG-111-cd3 effect on CRC cells we silenced Cat-D using a RNA interference (RNAi) approach.

3. Material and methods

3.1. Cell lines and culture conditions

In order to perform the different experiments hereafter described, we used a noncancerous NCM460 cell line and CRC-derived cell lines RKO and SW480.

NCM460 cell line, derived from healthy mucosal epithelium from the human colon and was earlier transfected by our research group with lentiviral vectors (Alves et al., 2015) to express a FLAG-tagged wild-type KRAS ($KRAS^{WT}$) and a FLAG-tagged KRAS hotspot mutation ($KRAS^{G12V}$). RKO and SW480 are colorectal cancer-derived cell lines that harbor a mutation in $BRAF^{V600E}$ and $KRAS^{G12V}$, respectively (Ahmed et al., 2013).

NCM460 and SW480 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with stable glutamine (Biowest) supplemented with 1% penicillin-streptomycin (Biowest) and 10% heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen); RKO cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) High Glucose (Biowest) supplemented with 1% penicillin-streptomycin (Biowest) and 10% heat-inactivated FBS (Gibco, Invitrogen). All cell lines were plated onto 25 cm^3 tissue culture flasks, maintained in a humidified incubator with 5% CO_2 at 37 °C and seeded in sterile test plates for the different assays. For autophagy induction, cells growing in complete medium were washed three times with phosphate buffer saline (PBS) 1x and incubated for 6 hours in Hank's Buffered Salt Solution (HBSS; PAA, Austria) buffered with 2.2 g/l $NaHCO_3$.

To determine whether cells were infected with *Mycoplasma*, cultures were regularly tested using a commercial kit (VenorTM GeM Mycoplasma Detection Kit, Sigma Aldrich), according to the manufacturer's protocol. Cells with positive results for *Mycoplasma* contamination were discarded.

3.2. Immunofluorescence assays and confocal microscopy

To understand if the subcellular localization of KRAS proteins changed under autophagy induction by starvation we performed immunofluorescence assays with antibodies that targeted specific molecules (Table 3.1.), in the NCM460 cell lines stably expressing the different FLAG-KRAS constructs.

NCM460 cells were seeded on glass coverslips at 3 x 10^5 cells/well in 12-well plates. After autophagy induction by nutrient starvation, cells were fixed in 4% paraformaldehyde (PFA) in PBS

7.4 for 12 minutes at room temperature (RT) followed by washes with PBS 1x. Cells were permeabilized with PBS-Triton X-100 0.2% for 7 minutes and blocked with 10% FBS for 30 minutes at RT. Afterwards, the different protocols of immunofluorescence performed vary (Table 3.2.).

In one of the protocols, we used the primary antibodies TOMM20 and FLAG. It was performed a simultaneous incubation of the antibodies, cells were incubated for 3h with TOMM20 and FLAG in a dilution of 1:500 and 1:150, respectively, at RT. On the other hand, in the immunofluorescence protocols with LAMP2 and Gal-3 together with FLAG, we performed a sequential incubation where we incubated one primary antibody at a time. In LAMP2 + FLAG protocol we incubated cells first with LAMP2 antibody overnight (ON) in a concentration of 1:100 at 4°C and in the next day we added FLAG antibody for 3h in a dilution of 1:100, at RT. In Gal-3 + FLAG protocol cells were first incubated with Gal-3 antibody ON at 4°C in a concentration of 1:100 and in the next day FLAG antibody was added during 1h30 in a dilution of 1:100, at RT. Following primary antibodies, the secondary antibodies were added. The secondary antibodies used in these assays were AlexaFluor™ 488 Chicken Anti-Rabbit (Invitrogen™, A21441), AlexaFluor™ 488 Donkey Anti-Goat (Santa Cruz Biotechnology, sc-2020) and, AlexaFluor™ 594 Goat Anti-Mouse (Invitrogen™, R37121). Independently of the immunofluorescence protocol, secondary antibodies were added simultaneously for 1h at RT, in a concentration of 1:2000.

The immunofluorescence protocol performed for phalloidin with FLAG is a different protocol once the AlexaFluor™ 647 phalloidin (ThermoFisher Scientific, A2287) provide fluorescence for itself not being necessary the use of a secondary antibody. In this case, we incubated cells with the primary antibody FLAG for 3h, RT, in a dilution of 1:150, followed by the incubation with the secondary antibody AlexaFluor™ 594 Goat Anti-Mouse for 1h, RT, in a dilution of 1:2000. At last, AlexaFluor™ 647 phalloidin was added to the cells, in a dilution of 1:40 for 20 minutes, in the dark.

At the end, after the immunostaining process and independently of the immunofluorescence protocol that was being followed, cells were stained with DAPI (Invitrogen™, D1306) for 5 minutes and the coverslips were mounted with a mouting solution. Finally, images were acquired in a confocal microscope in a magnification of 60x or 100x and processed with LAS AF Leica Microsystems software.

3.2.1. Co-localization analysis

After immunofluorescence images were obtained, the amount of co-localization between the different probes was measured with the Just Another Co-Localization Plugin (JACop) of Image J software. After set a suitable threshold, JACop plugin generated several coefficients that permitted co-localization evaluation such as the Pearson's coefficient, M1& M2 coefficients and overlap coefficients for each image. All data were collected, analysed and the mean for each condition was obtained and compared for main conclusions. Note that the same threshold was used for each fluorochrome and applied for all conditions.

Table 3.1. Some specifications of the antibodies used in the immunofluorescence assays.

Primary antibody	Temp. (°C)	Target	Biological source	Secondary antibody used
FLAG Sigma Aldrich, F1804	-20	FLAG-KRAS constructs	Mouse	AlexaFluor™ 594 Goat Anti-Mouse
TOMM20 Sigma Aldrich, hpa 011562	-20	Mitochondrial mass	Rabbit	AlexaFluor™ 488 Chicken Anti-Rabbit
LAMP2 Abcam, ab18528	4	Lysosomal membrane	Rabbit	AlexaFluor™ 488 Chicken Anti-Rabbit
Gal - 3 Santa Cruz Biotechnology, sc-19280	4	Galectin-3	Goat	AlexaFluor™ 488 Donkey Anti-Goat

Table 3.2. Variations between the immunofluorescence protocols performed.

Immunofluorescence protocol	Primary antibodies dilution	Simultaneous primary antibodies incubation?	Sequential primary antibodies incubation?
TOMM20 + FLAG	TOMM20 – 1:500 in 5% FBS-PBS/Tween20 FLAG – 1:150 in 5% FBS-PBS/Tween20	Yes TOMM20 is incubated together with FLAG antibody for 3h in a humidified atmosphere.	No
LAMP2 + FLAG	LAMP2 – 1:100 in 1% FBS-PBS/Tween20 FLAG – 1:100 in 1% FBS-PBS/Tween20	No	Yes LAMP2 is incubated ON at 4°C whereas FLAG is incubated for 3h, in the next day. Both are incubated in a humidified atmosphere.
Gal-3+ FLAG	Gal-3– 1:100 in 5% FBS-PBS/Tween20 FLAG – 1:100 in 5% FBS-PBS/Tween20	No	Yes Gal-3 is incubated ON at 4°C whereas FLAG is incubated for 1h30, in the next day. Both are incubated in a humidified atmosphere.
Phalloidin + FLAG	Phalloidin – 1:40 in PBS 1x FLAG – 1:150 in 5% FBS-PBS/Tween20	No	Yes FLAG is incubated for 3h in a humidified atmosphere following incubation with the respective secondary antibody for 1h. At last, Phalloidin is added for 20 min. in the dark.

3.3. Sulphorhodamine B (SRB) assay and determination of MSG-111-CD3 IC_{50} in RKO, SW480 and NCM460 cell lines

MSG-111-cd3 compound is a phenoxazine derivative described as a promising antitumoral agent synthetized by Professor Sameiro Gonçalves group (University of Minho, Portugal). The effects of the MSG-111-cd3 compound on cell proliferation were determined by the SRB assay in RKO, SW480 and NCM460 $KRAS^{WT}$ cell lines. The basis of this assay is the ability of SRB dye to bind in a pH dependent manner with cellular proteins. Because this is a stoichiometric binding, the amount of dye extracted from stained cells is directly proportional to the cell mass.

Cells were plated with a density of $4x10^4$ (RKO), $1,75x10^5$ (SW480), and $3x10^5$ (NCM460) cells/well and allowed to adhere for 24h at 37 °C, with 5% CO_2. On the next day, increasing concentrations (1 μM, 2.5 μM, 5 μM, and 7.5 μM) of the MSG –111–cd3 compound were added to the respectively wells and the plates were incubated for 48h. For the SW480 cell line a 6 μM concentration of MSG-111-cd3 was also tested. Besides the negative control only with cells and growth medium, the highest concentration of the solvent where the compound was dissolved (DMSO 0.1%) was also used as a second negative control, in order to discard any influence of DMSO in the results. Briefly, after the incubation period, medium was discarded, and the cells were fixed with 1 mL of ice-cold methanol in 1% acetic acid for at least 90 minutes at -20 °C. The fixation solution was then removed, and plates were allowed to dry. Afterwards, 250 μL of 0.5% SRB in 1% acetic acid solution were added and plates were incubated in the dark at 37 °C for 90 minutes. After the incubation time, plates were washed with 1% acetic acid, in order to remove unbound dye, and after dry, 1 mL of Tris 10 mM was added. Finally, the absorbance at 540 nm was read in a multi-plate reader (SpectraMax 340PC Molecular Devices). Values of inhibition of cellular proliferation were expressed against the negative control (untreated cells), which was considered as 100% of proliferation.

The results were obtained from at least three independent experiments, each experiment was done in triplicate. The statistical analysis of comparisons of data from different groups *versus* negative control were performed by ordinary one-way ANOVA followed by Dunnett's multiple comparisons test, using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA. The IC_{50} was determined applying a dose *vs* response (variable slope) non-linear regression using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA.

3.4. RNA interference assay

RNA interference (RNAi) is a regulatory mechanism that uses small double stranded RNA (dsRNA) molecules to direct homology-dependent control of gene activity (Aagaard & Rossi, 2007). RNAi pathways are guided by small interfering RNAs (siRNAs), which derive from dsRNAs cleavage by RNaseIII endonuclease Dicer (D. Kim & Rossi, 2008). Each resulting siRNA is loaded into Argonaute 2 (AGO2) and the RNA-induced silencing complex (RISC). The siRNA guide strand recognizes target sites to direct mRNA cleavage through the catalytic domain of AGO2. Finally, the endoribonuclease in the RISC cleaves the targeted mRNA leading to specific gene silencing (Sui & Shi, 2005).

To unravel if Cathepsin-D is directly related to MSG-111-cd3 compound effects in RKO and SW480 cells proliferation we silenced Cathepsin-D in cells treated with MSG-111-cd3 IC_{50} and performed SRB assays. For this assay the siRNA targeting cathepsin-D was purchased from Dharmacon™ (A-003649-16-0005). The sense siRNA target sequence was: 5' – GAAUGGUACCUCGUUUGAC – 3` for Human CTSD (Cathepsin-D). The control (non-silencing) siRNA target sequence was 5' – AATTCTCCGAACGTGTCACGT – 3' and was purchased from Qiagen.

On the first day, RKO and SW480 were plated in 24-well plates at a density of $1.75x10^5$ cells per well in complete DMEM and RPMI medium, respectively. Next day, after a 60% cell confluence reached, cells were washed with PBS 1x and 375 µL of OPTIMEM (Gibco) were added to wells for 1h30. During this incubation time two distinct mixes were prepared under RNase free conditions (Table 3.3.). Mix I was composed by OPTIMEM and siRNA whereas Mix II was composed by Lipofectamine 2000 (Invitrogen) and OPTIMEM. After prepared in separate, Mix II was added to Mix I followed by a 20-minute incubation at RT. Finishing the 1h30 incubation with OPTIMEM the solution with both mixes was added to the cells for 14h. After the 14h of transfection, siRNA was removed from the wells and cells were treated with MSG-111-cd3 IC_{50}, previously determined. In the remaining cells that were not treated with MSG-111-cd3, siRNA was removed and replaced by complete DMEM or RPMI medium. After 48h incubation, SRB assay was performed according to the described above.

The results were obtained from two independent experiments for RKO and from one experiment for SW480, each experiment was done in triplicate. The statistical analysis of comparisons of data from different groups *versus* negative control were performed by ordinary one-

way ANOVA followed by Dunnett's multiple comparisons test, using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA.

Table 3.3. RNAi assay Mix I and Mix II composition. These values are calculated per well, for 24-well plates.

	Mix I		Mix II	
Conditions	siRNA Cat-D (µL) (Stock: 20 µM)	OPTIMEM (µL)	Lipofectamine 2000 (µL)	OPTIMEM (µL)
Negative control (untreated cells)	_____	125	_____	_____
siRNA scramble (100 nM)	2,5	60	3	59,5
siRNA Cat-D (100 nM)	2,5	60	3	59,5

3.4.1. RNA interference controls

Besides the usual controls of siRNA assays consisting of a negative control composed by untreated cells and a non-silencing siRNA control (siRNA scramble), we did an extra control to guarantee that Cat-D is silenced. For that, at the same time we performed the siRNA assay in the 24-weel plates, we plated RKO and SW480 cells in a 6-well plate and did another siRNA assay without MSG-111-cd3 treatment. We follow the protocol described above, however, after the 48h incubation cells were collected, protein extraction and quantification were performed and, finally, samples were run in a polyacrylamide gel by western blotting to detect or not the presence of Cat-D antibody.

3.4.1.1. Sample preparation for western blotting: cell lysis and protein extraction

After performed the siRNA Cat-D silencing protocol, cells in suspension were collected to a 15mL falcon tube which was maintained on ice during all the process. In order to also collect attached cells trypsin 0.05% (v/v) (dissolved in phosphate buffer saline - ethylenediamine tetra acetic acid (PBS-EDTA)) (Gibco) was added to each well and transferred to the same 15 mL falcon tube. Between these steps wells were washed with PBS 1x that was once again placed into the 15mL falcon tube to ensure the collection of all the cells. Cells were then centrifuged at 2000 rpm for 10 minutes at 4°C and the pelleted cells resuspended in PBS 1x and transferred to

microcentrifuge tubes. After another centrifugation at 1200 rpm for 5 minutes at 4°C, pellets were suspended in RIPA Buffer and chilled on ice for 20 minutes. RIPA Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40) disrupt cells enabling the extraction of membrane, cytoplasmic and nuclear proteins and must be supplemented with 20 mM NaF, 20 mM Na_3VO_4, 1 mM PMSF and 50 µL/mL protease inhibitor cocktail (Roche) to prevent proteolysis and maintain protein phosphorylation. After a final centrifugation at 14 000 rpm for 15 minutes, the protein extracts present in the supernatants were transferred to a new microcentrifuge tube and kept at -20 °C until protein quantification.

3.4.1.2. Sample preparation for western blotting: protein quantification

The estimation of protein concentration necessary for assessing the levels of specific protein for western blotting was performed using a Bio-Rad DC Protein Assay Kit. This assay is based on a principle where protein reacts with an alkaline copper tartrate solution and subsequently reduces the folin reagent and leads to color development. The method entails the preparation of five protein standards (from 0.25 mg/mL to 5 mg/mL) in RIPA buffer from a 10% BSA (bovine serum albumin) stock solution. After this preparation, the wells of a 96-well plate were filled with 5 µL of each protein standard, 1 µL of sample + 4 µL of RIPA buffer and 5 µL of RIPA buffer (blank). 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 followed by a 15-min incubation in the dark at RT. After 15 min, absorbances were read at 720 nm (Spectra Max 340PC – Molecular Devices) and the calibration curve was obtained using the values of the BSA standards from which the concentration of the samples was extrapolated.

3.4.1.3 Western blotting

Western blotting is a technique used to visualize proteins that have been separated by gel electrophoresis. Firstly, samples were prepared by dilution in dH_2O, addiction of Laemmli 4x buffer (9.2% SDS, 5% β - mercaptoethanol, 40% glycerol, 0.5% bromophenol blue and 0.25M Tris-HCl) and denaturation by heat (at 95°C for 5 min). After this, samples were put on ice and were prepared to be loaded onto the polyacrylamide gel.

The polyacrylamide gel used is divided into an upper stacking gel (625 µL 4x Tris-HCl pH 6.8, 50 µL SDS 10%, 625 µL 30% Acrylamide:0.8% Bisacrylamide, 50 µL 10% APS and 5 µL

TEMED) of low percentage (with large pore size) and low pH, where the protein bands get squeezed down and a resolving gel at a concentration of 10% (3353 µL 4x Tris-HCl, 70 µL 10% SDS pH 8.8, 1750 µL 30% Acrylamide:0.8% Bisacrylamide, 70 µL 10% APS and 7 µL TEMED) (pH 8.8) with smaller pores that allow separation of the proteins in the sample based on their molecular weight. Equal amounts of protein (25 µg) were loaded into the wells of the polyacrylamide gel, along with molecular weight marker (4 µL) (Precision Plus Protein™ Dual Color Standards, Bio-Rad) and electrophoresis run in a Mini-Protean III electrophoresis system at 90 – 100V for about 1h30. The running buffer 1x used during the run was diluted from running buffer 10x which was composed by 0.25 M Tris base, 1.92 M Glycine and 1% SDS. After electrophoretic separation, proteins were transferred to PVDF (polyvinylidene difluoride; Thermo Scientific) membranes after its' activation with methanol for 5 seconds, with the help of the Mini Trans-Blot System (Bio-Rad) at 100V for 1h30 in a cold environment. The transfer buffer 1x used in this step was diluted from transfer buffer 10x which contains 0.25 M Tris base, 1.92 M and Glycine. As for the blocking step, which consists in eliminating nonspecific binding sites, membranes were blocked for at least 1h at RT, with 5% soy milk in PBST (PBS 1x and 0.1% Tween 20). After this procedure, membranes were briefly washed with PBST and incubated overnight with the recommended dilution of primary antibody at 4°C, using a roller mixer. On the next day, membranes were washed several times with PBST and incubated with the secondary antibody conjugated with IgG horseradish peroxidase, for 1h at RT. Finally, membranes were washed again with PBST and signal was developed and detected using the Immobilon solutions (Millipore, Billerica) under a chemiluminescence detection system, the Chemi Doc XRS (Bio-Rad Laboratories, Inc.).

The primary antibody used was anti- cathepsin-D (Merck chemicals, IM03) in a dilution of 1:100 in 5% soy milk/PBST. The secondary antibody used was peroxidase conjugated AffiniPure goat anti mouse IgG (Jackson ImmunoResearch) in a dilution of 1:1000 in 5% soy milk/PBST.

4. Results

4.1. KRAS cellular localization in NCM460 cells upon autophagy induction

Differences in subcellular localization of RAS isoforms or interactions with other proteins provides one explanation for the apparent complexity of their signaling outputs (Hernandez-Valladares & Prior, 2015; Prior & Hancock, 2012).

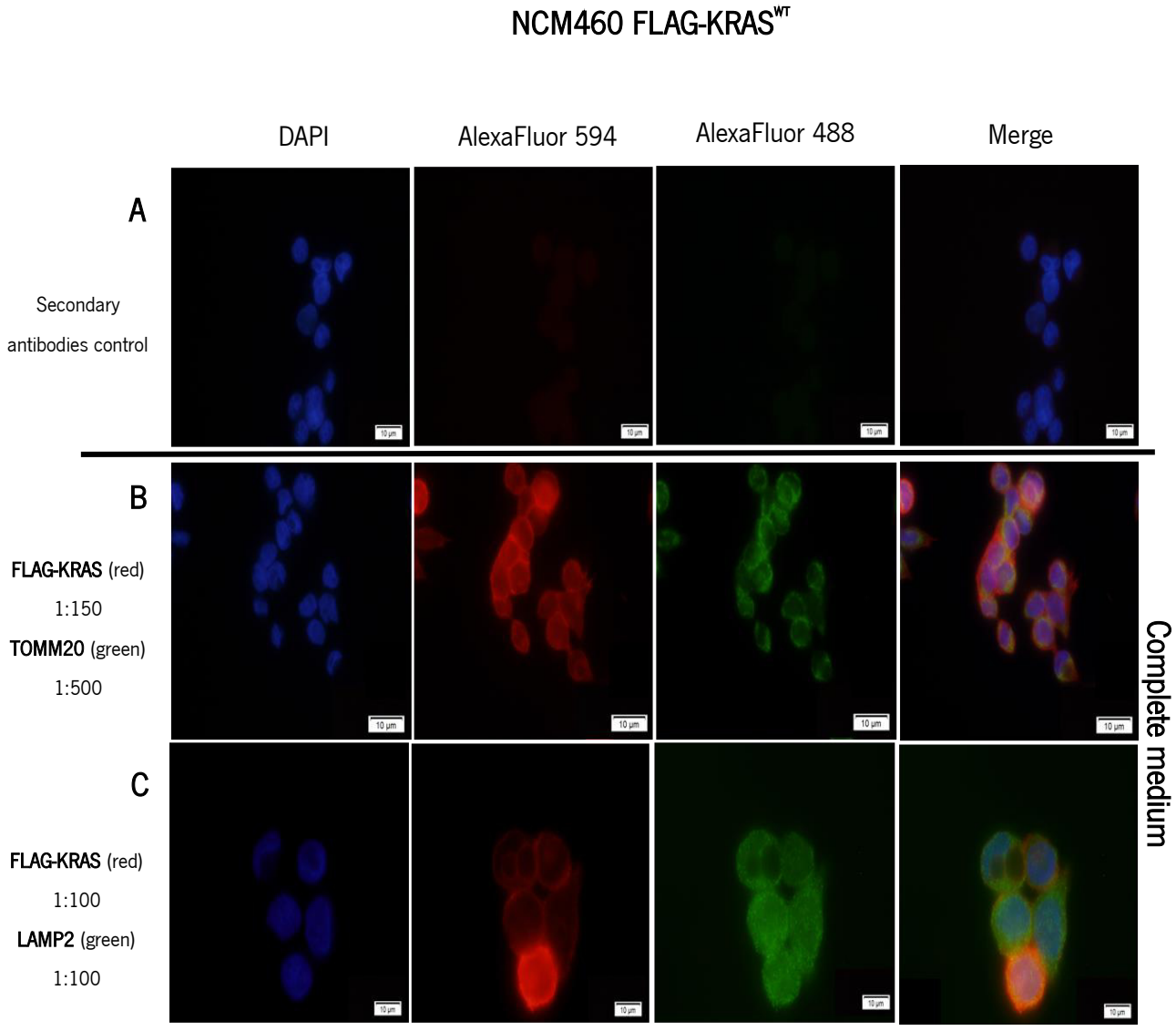
To investigate the possibility of KRAS co-localization with mitochondria, lysosomes, Gal-3 and actin filaments upon autophagy induction by starvation, we performed immunofluorescence assays in a noncancerous NCM460 cell line expressing a FLAG-tagged wild-type KRAS (FLAG - $KRAS^{WT}$) and a FLAG-tagged KRAS hotspot mutation (FLAG - $KRAS^{G12V}$). For that, five antibodies were used: FLAG antibody – which allow us to assess the localization of FLAG – $KRAS^{WT}$ and FLAG – $KRAS^{G12V}$ constructs; a TOMM20 antibody to detect the mitochondrial network; a LAMP2 antibody which is a lysosomal marker; a Gal-3 antibody to assess Galectin-3 protein and Phalloidin used to stain and visualize F-actin.

We needed to optimize the dilution of FLAG, TOMM20 and LAMP2 antibodies (Figure 4.1.) since our group never used these antibodies for immunofluorescence. The optimization assays were performed in the NCM460 FLAG-$KRAS^{WT}$ cell line under complete and starvation conditions. The antibodies dilutions tested are summarized below, in table 4.1. After observation under the fluorescence microscope, the best dilutions of the antibodies were 1:500 for TOMM20, 1:100 for LAMP2 and 1:150 or 1:100 for FLAG antibody, depending on the second antibody used (Figure 4.1.).

For the following immunofluorescence assays performed, the negative controls were constituted by both secondary antibodies (except for the assay of KRAS-FLAG + Phalloidin 647, where only the secondary antibody of FLAG-KRAS was used), under complete medium conditions, and they did not cross-react when incubated together without the primary antibodies (Figure 4.2.).

Table 4.1. Dilutions tested for the antibodies TOMM20, LAMP2, and FLAG.

Primary antibody	Dilutions tested	Secondary antibody	Dilutions tested
TOMM20	1: 200 and 1:500	AlexaFluor™488 Chicken Anti – Rabbit	1:2000
LAMP2	1:100; 1:150 and 1:200	AlexaFluor™488 Chicken Anti – Rabbit	1:2000 and 1:1000
FLAG	1: 100 and 1:150	AlexaFluor™594 Goat Anti – Mouse	1:2000



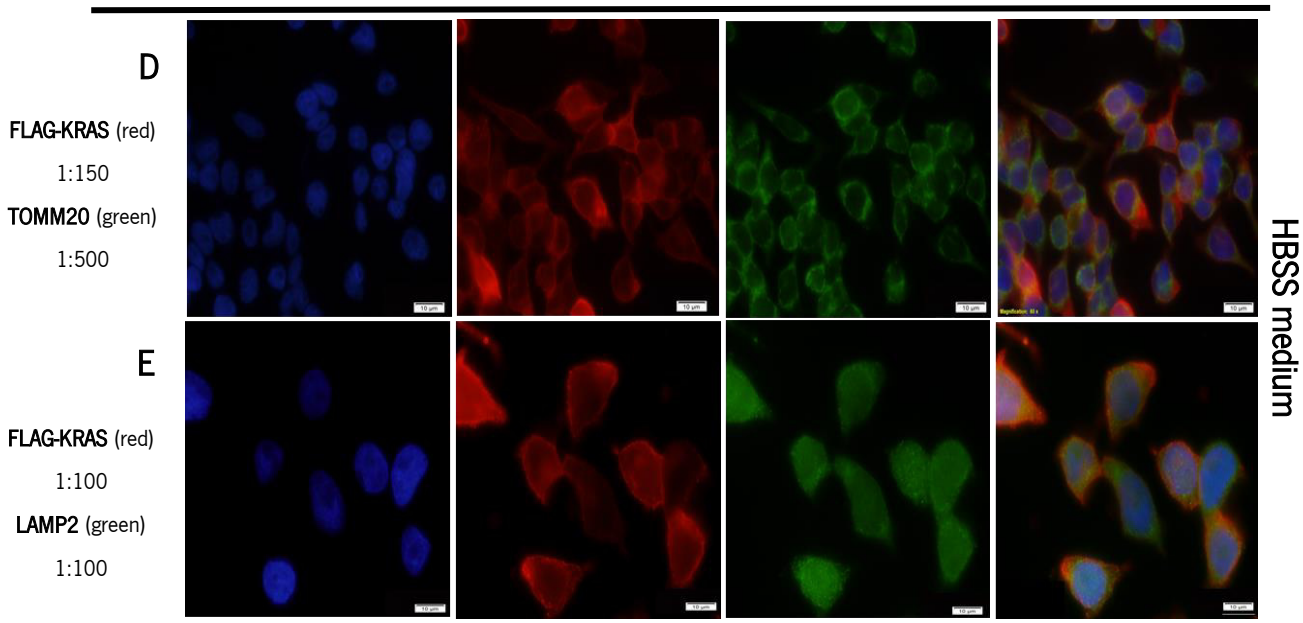


Figure 4.1. Optimized dilutions for TOMM20, LAMP2, and FLAG antibodies in NCM460 FLAG-KRAS^{WT} cell line in complete and HBSS medium. A. Secondary antibodies control Goat Anti-Mouse 594 (red) and Chicken Anti-Rabbit 488 (green). **B.** Fluorescence microscopy images of FLAG-KRAS and TOMM20 antibodies in NCM460 FLAG-KRAS^{WT} in complete medium – 60x magnification. **C.** Fluorescence microscopy images of FLAG-KRAS and LAMP2 antibodies in NCM460 FLAG-KRAS^{WT} in complete medium – 100x magnification. **D.** Fluorescence microscopy images of FLAG-KRAS and TOMM20 antibodies in NCM460 FLAG-KRAS^{WT} in HBSS medium – 60x magnification. **E.** Fluorescence microscopy images of FLAG-KRAS and LAMP2 antibodies in NCM460 FLAG-KRAS^{WT} in HBSS medium – 100x magnification.

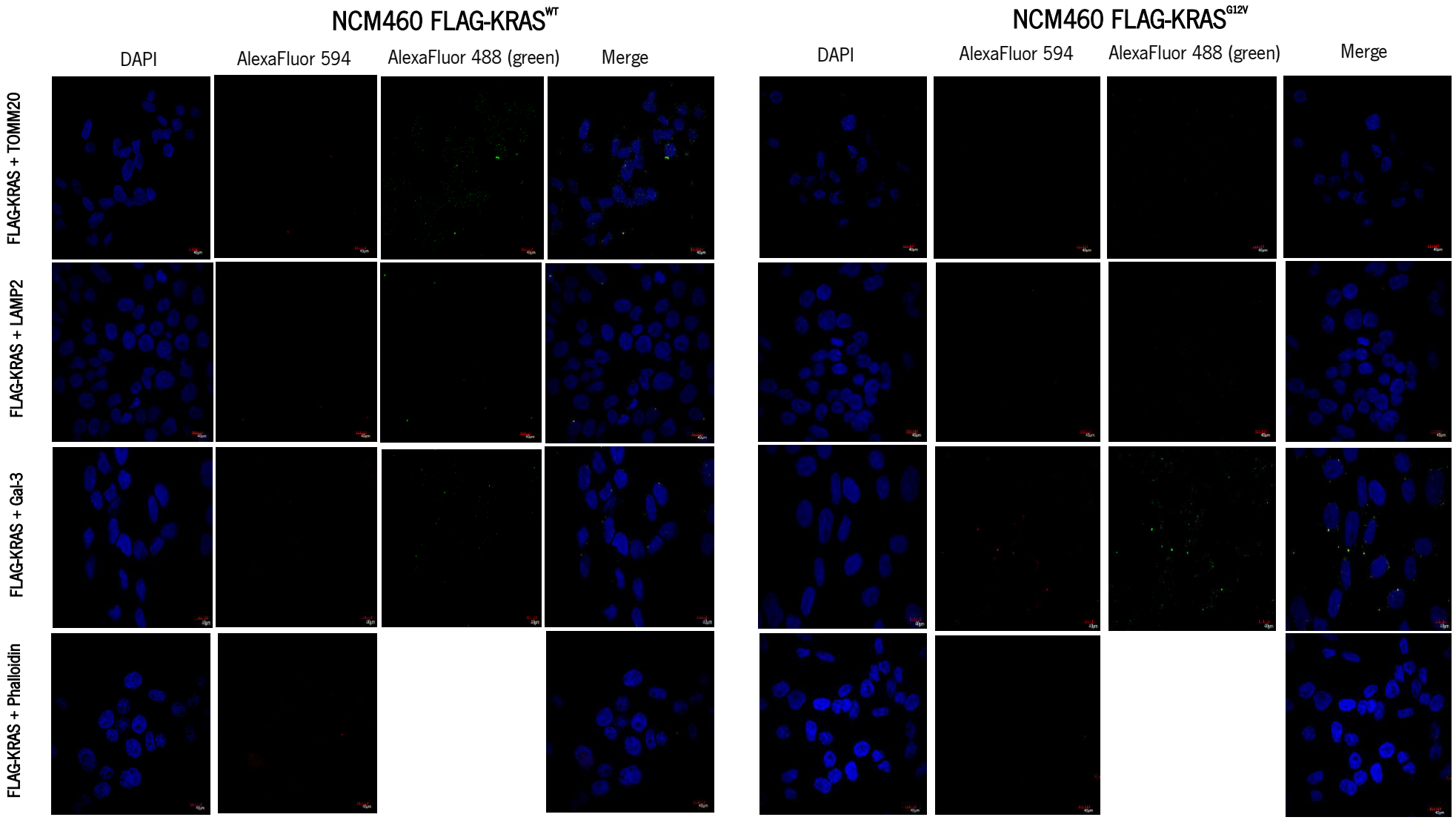


Figure 4.2. Secondary antibodies negative controls of the different immunofluorescence assays performed.

4.1.1 KRAS^{WT} but not KRAS^{G12V} co-localize with mitochondria

Here we wanted to understand if or to what extent mitochondria and KRAS co-localized and if KRAS activating mutations induce changes in the localization. For that matter we used TOMM20 and FLAG antibodies and evaluated if autophagy induction under starvation conditions (upon incubation with HBSS medium) led to mitochondrial network changes or KRAS relocalization within the cell, in the two NCM460 cell lines: NCM460 FLAG – KRAS^{WT} and NCM460 FLAG – KRAS^{G12V}.

We observed that mitochondria have a well-defined network pattern in cells incubated in complete medium in comparison with cells incubated in HBSS medium where an elongated mitochondrial network as well as a more punctuate pattern are observed both in NCM460 FLAG – KRAS^{WT} and NCM460 FLAG – KRAS^{G12V}. Moreover, mitochondria seem to gain a perinuclear localization when cells were exposed to nutrient limitation conditions (Figure 4.3.A and 4.4.A). Also, it was evident that KRAS re-localized within the cell, as it has been already reported by previously studies made by our group (Sara Alves, PhD thesis). In complete medium conditions KRAS distributes along the plasma membrane whereas when cells were incubated with HBSS medium KRAS became also cytosolic (Figure 4.3.B and 4.4.B).

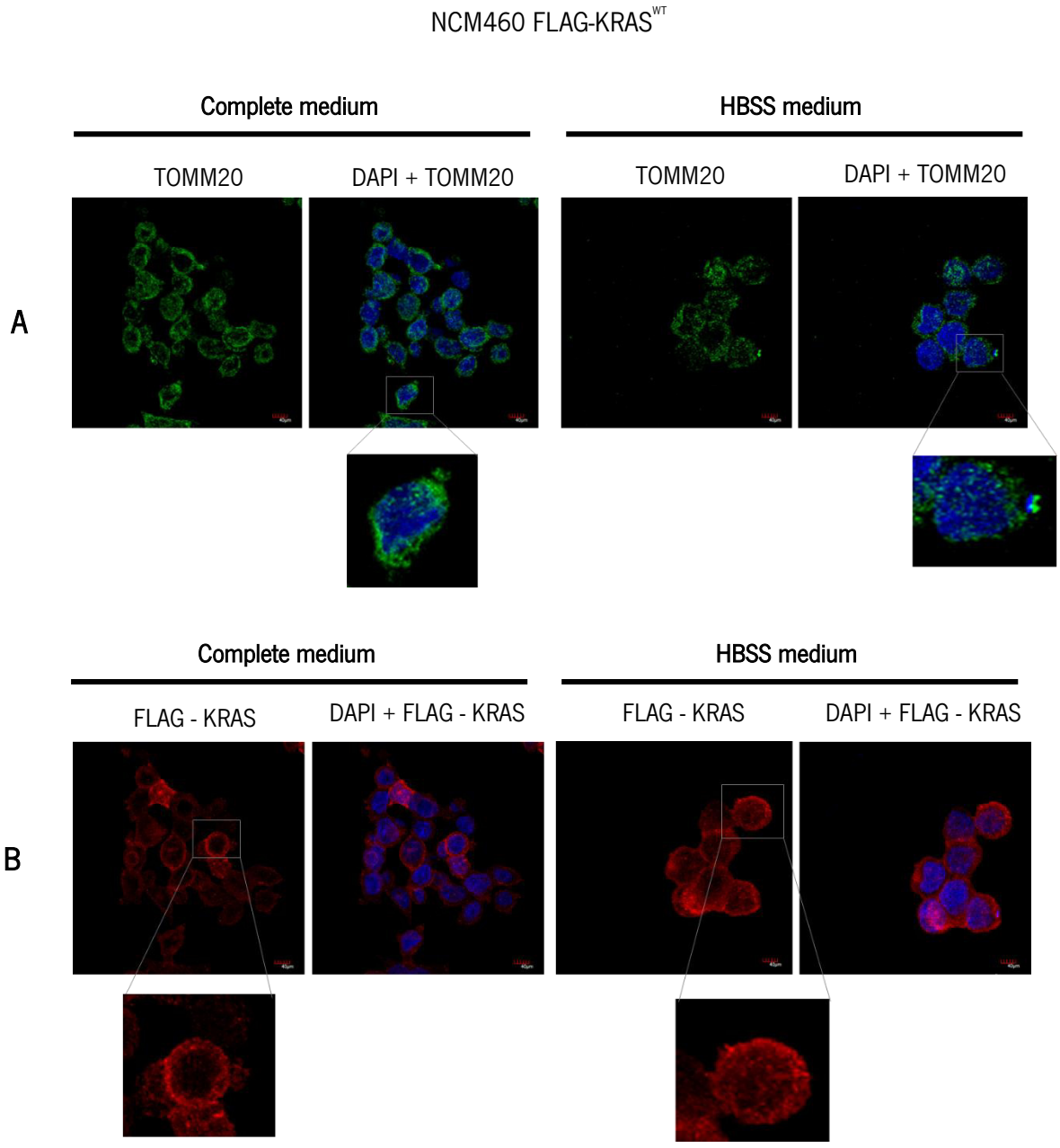


Figure 4.3. Starvation leads to morphological alterations in mitochondria and causes KRAS relocalization, in NCM460 FLAG – KRAS^{WT} cell line. A. After starvation induction by 6h incubation with HBSS medium, mitochondria network has a more punctuate aspect and becomes perinuclear. **B.** KRAS acquires a cytosolic distribution within the cell when starvation is induced instead of maintaining its typical plasma membrane localization – 60x magnification.

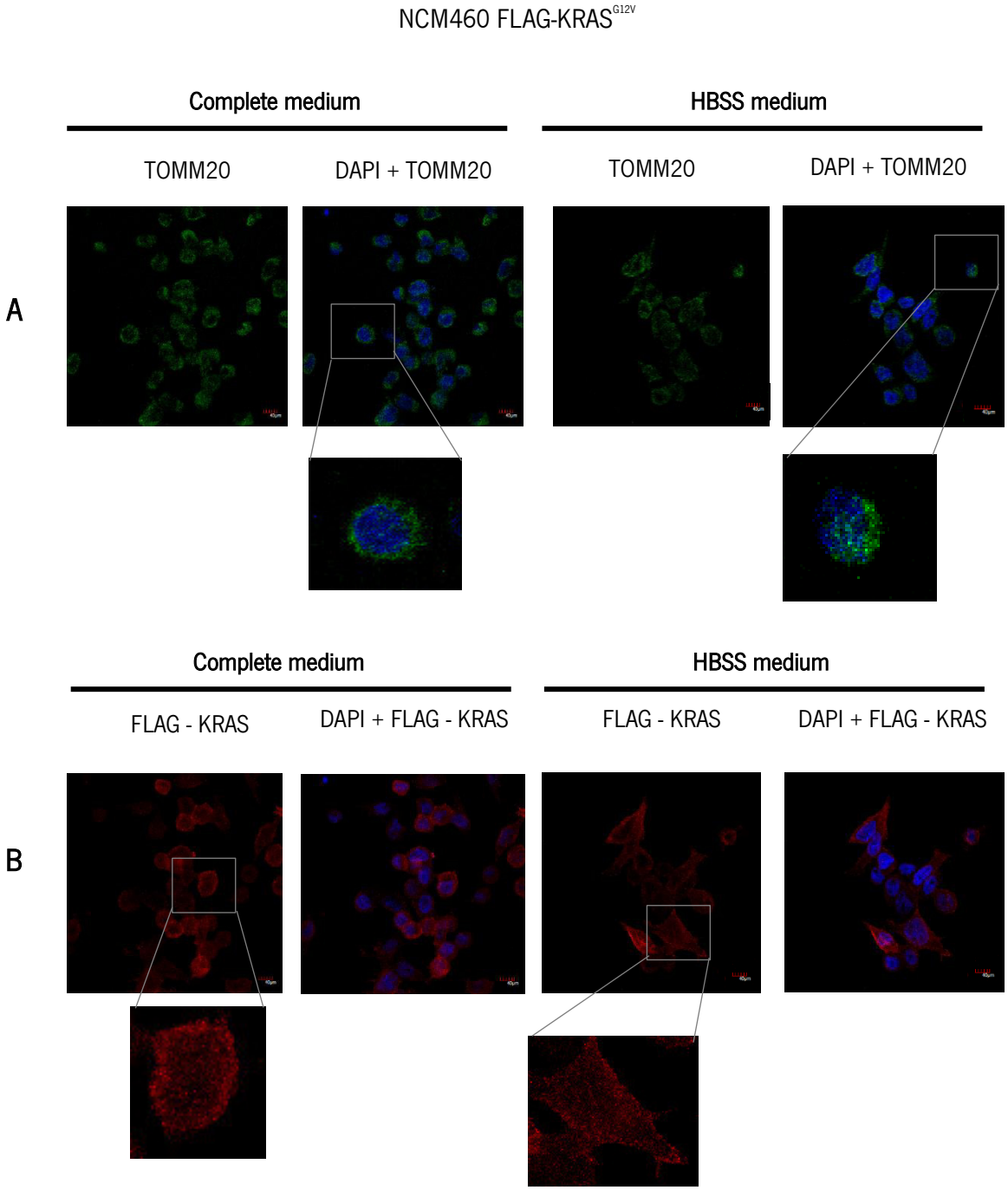


Figure 4.4. Starvation leads to morphological alterations in mitochondria and causes KRAS relocalization, in NCM460 FLAG – KRAS^{G12V} cell line. A. After starvation induction by 6h incubation with HBSS medium, mitochondria network has a more punctuate aspect and becomes perinuclear. **B.** KRAS acquires a cytosolic distribution within the cell when starvation is induced instead of maintaining its typical plasma membrane localization – 60x magnification.

Observation of the double staining microphotographs of KRAS with mitochondria TOMM20 marker (Figure 4.5.), suggest that KRAS does not seem to co-localize with this organelle although in NCM460 $KRAS^{WT}$ in starvation conditions this conclusion was not so clear. Because our eyes are not accurate tools to take this kind of conclusions we performed an analysis using the Just Another Co-Localization Plugin (JACop) of Image J software that uses specific algorithms which calculate several coefficients, such as Pearson's Correlation Coefficient (PCC), Overlap Coefficient (OC) and Mander's Overlap Coefficient (MOC), M1 and M2, in order to evaluate in a quantitative manner, the amount of co-localization between the dyes through pixel intensity measurement.

PCC, ranging from -1 to 1, estimates the association strength between two proteins and for that reason -1 indicates an inverse correlation; 0 no correlation whereas 1 a positive correlation that can suggest partial or complete co-localization. Values between -0.5 and 0.5 do not allow to conclude anything. Although PCC is a good parameter because it is independent of background noise, it is insensitive to differences in signal intensities which is a limitation when working with proteins with very different levels of expression (which is not our case). Thus, looking at PCC values (Table 4.2.) it is possible to say that in both NCM460 cell lines seems to exist a positive correlation between KRAS and mitochondria since we do not have negative results, however, we cannot conclude anything because we have values below 0.5.

OC refers to an upgrade of PCC that considers the differences in intensities between two channels and the background noise. It ranges from 0 to 1 meaning no or full co-localization, respectively. From our OC values (Table 4.2.) we can observe differences among the KRAS subtypes: independently of the nutrient conditions, when wild-type KRAS is present it co-localizes with mitochondria, proven by the fact that the values are close to 1, whereas when mutated KRAS is present it does not, since it presents values below 0.5, the minimum value to consider co-localization (Figure 4.6.).

PCC and OC are useful for comparative analysis, but to estimate the quantity of co-localization we should resort to MOC. M1 and M2 range from 0 to 1 and estimates the amount of co-localization from one channel over another indicating the percentage of overlapping pixels normalized against the total pixel intensity. In contrast with the information given by the OC values, M1 and M2 parameter demonstrate that co-localization is a rare event, since we have values close to zero (Table 4.2.).

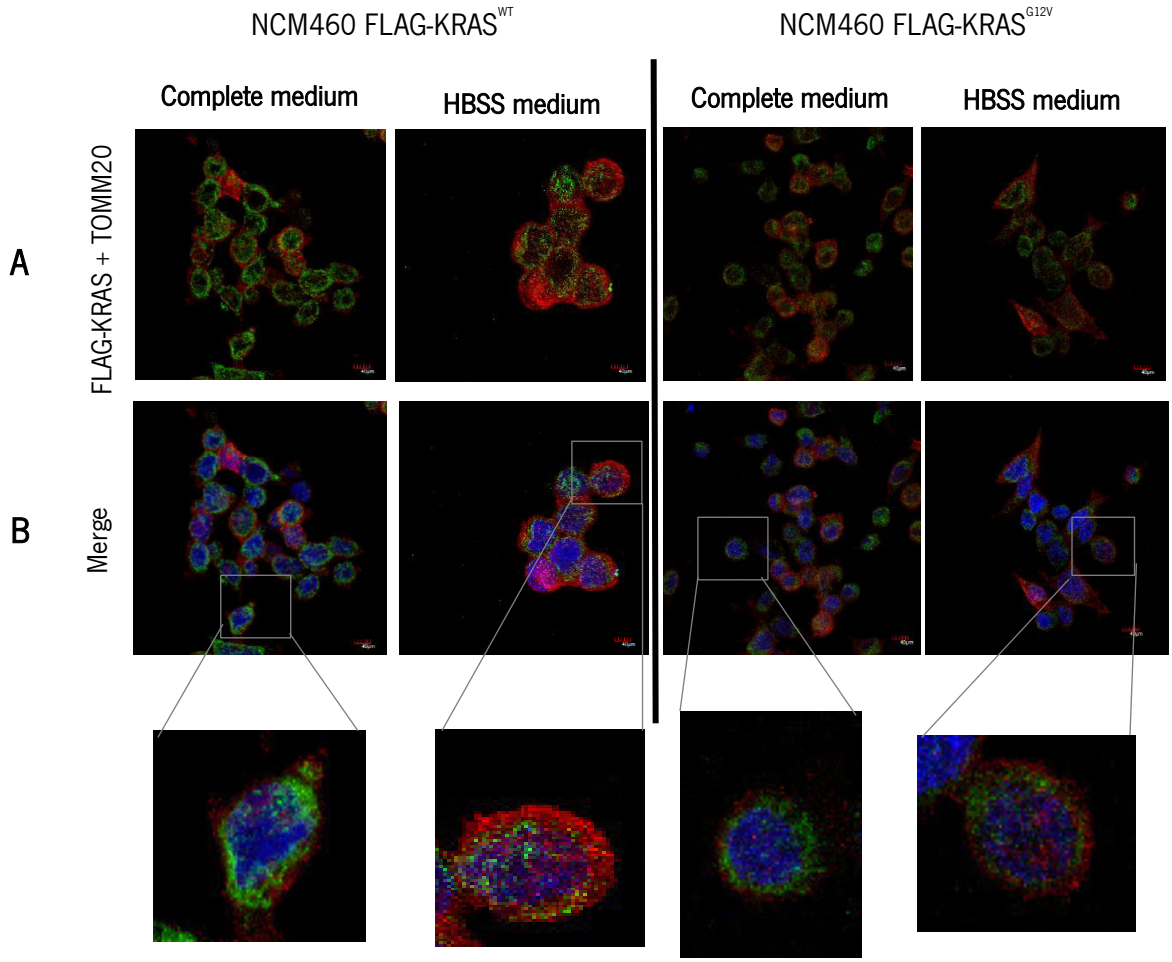


Figure 4.5. Co-immunofluorescence of FLAG-KRAS with TOMM20 in NCM460 cell lines in complete and HBSS medium. A. Confocal images of FLAG-KRAS (red) + TOMM20 (green) in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12} (right) cells in complete and HBSS medium. **B.** Confocal images of DAPI + FLAG-KRAS + TOMM20 (Merge) in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12} (right) cells in complete and HBSS medium– 60x magnification.

Table 4.2. Values of TOMM20/FLAG-KRAS co-localization analysis in both NCM460 cell lines, under complete and incomplete nutrient conditions. Pearson's Correlation Coefficient (PCC), Overlap Coefficient (OC) and Mander's coefficients M1 and M2 were obtained performing the co-localization analysis by JACoP. These values were adjusted to the threshold pre-defined for each fluorochrome and applied for all conditions in order to have comparable results.

NCM460 FLAG – $KRAS^{WT}$	PCC	OC	M1	M2
Complete medium	0,142	0,781	0,037	0,001
HBSS medium	0,243	0,879	0,229	0,000
NCM460 FLAG – $KRAS^{G12V}$				
Complete medium	0,156	0,401	0,081	0,000
HBSS medium	0,124	0,449	0,050	0,000

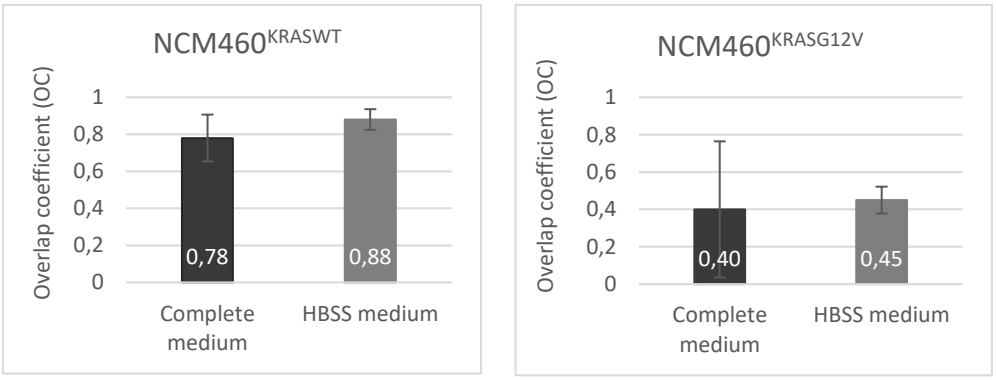


Figure 4.6. Graphical representation of the overlap coefficient between TOMM20/FLAG-KRAS in NCM460 cell lines, under complete and incomplete nutrient conditions. From the OC values, we may infer that only wild-type KRAS (left) co-localizes with mitochondria, in both complete and incomplete medium conditions, once the co-localization values are close to 1. In contrast, $KRAS^{G12V}$ (right) presents co-localization values with mitochondria (TOMM20) above 0.5 which mean that is very likely that these two molecules do not co-localize in any nutrient conditions.

Overall the analysis performed suggest that $KRAS^{WT}$ might have some mitochondria localization which is lost when KRAS is mutated.

4.1.2. KRAS seem to co-localize with lysosomes

In order to assess the possible KRAS localization with lysosome under starvation conditions, we performed immunofluorescence assays with FLAG and LAMP2, which is a lysosomal marker.

From the double staining microphotographs of FLAG-KRAS^{WT} and FLAG-KRAS^{G12V} with LAMP2 (Figure 4.7.) it is possible to observe that in complete medium conditions both NCM460 cell lines present a heterogeneous distribution of lysosomes, however, it seems that lysosomes exist in higher number in the cell line with mutated KRAS^{G12V}, consistent with induced autophagy. When starvation was induced, in both cell lines, the number of lysosomes seemed to decrease and tended to accumulate in the perinuclear region. Moreover, the merged figures suggested co-localization between FLAG-KRAS/LAMP2 in all the conditions, by the presence of yellow and orange (stronger signal) staining resulting from the overlap or proximity between green and red signals (Figure 4.7.).

According to the PCC values (Table 4.3.) of this assay, we could say that these proteins have some affinity in both cell lines because of the positive values exhibited, however, because they are below 0.5 it is inconclusive. The OC values (Table 4.3.) close to 1 indicate that KRAS co-localizes with lysosomes (Figure 4.8.) but once again, M1 and M2 coefficients were inconclusive. Despite M2 values (referent to green signal intensity) proximity to zero means that green channel barely contributes to the co-localization, M1 values (referent to red signal intensity) indicate that red pixels co-localize with green pixels meaning that co-localization is a possibility. This situation can be explained due to background noise, that tends to decrease M2 values to zero.

Our observations and quantification of the signals suggest that both forms of KRAS, KRAS^{WT} and KRAS^{G12V} co-localize with the lysosome.

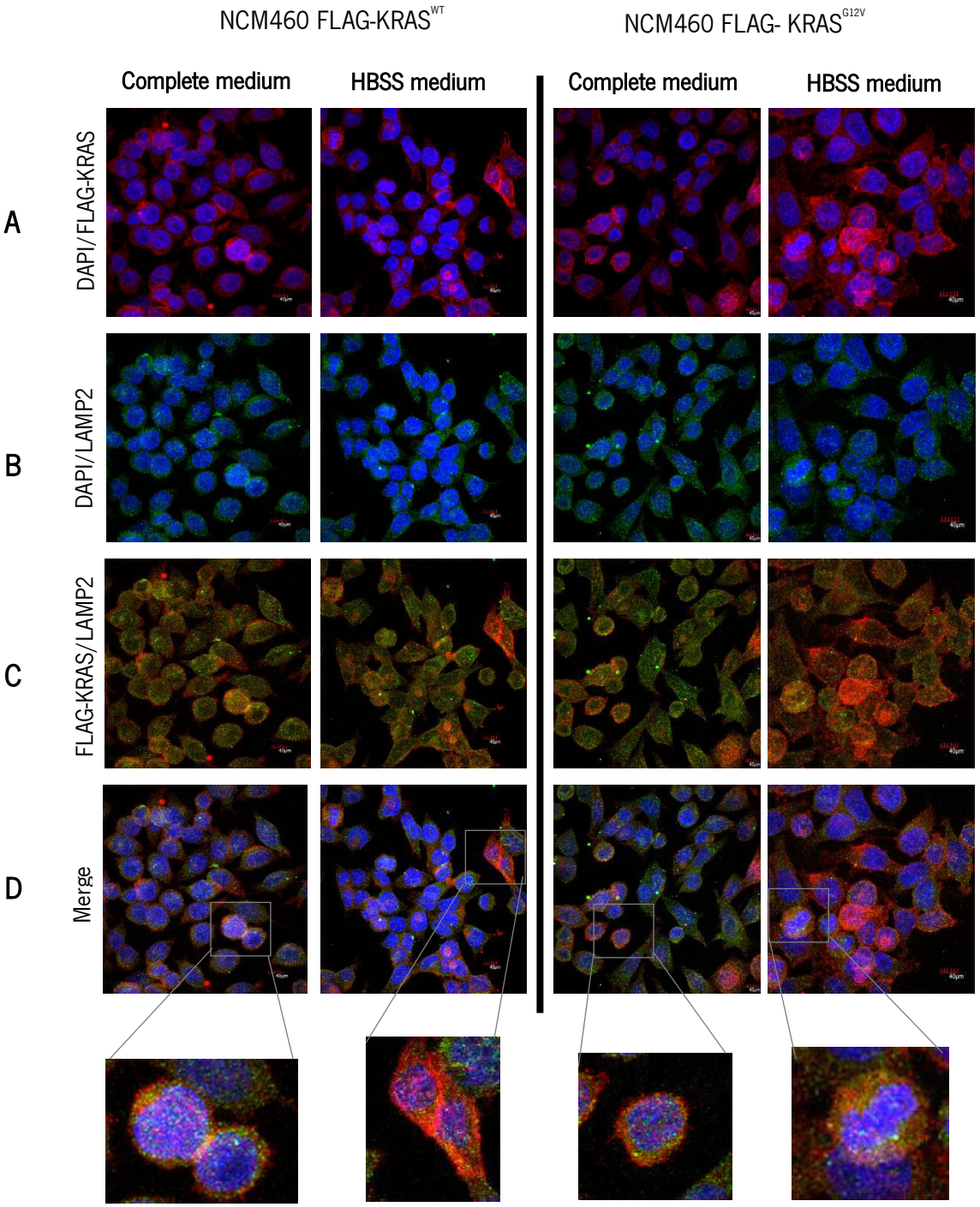


Figure 4.7. Co-immunofluorescence of FLAG-KRAS with LAMP2 in NCM460 cell lines in complete and HBSS medium. A. Confocal images of FLAG-KRAS (red) + DAPI in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12V} (right) cells under complete and HBSS medium. **B.** Confocal images of LAMP2 (green) + DAPI in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12V} (right) cells under complete and HBSS medium. **C.** Confocal images of FLAG-KRAS (red) + LAMP2 (green) in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12V} (right) cells under complete and HBSS medium. **D.** Confocal images of DAPI + FLAG-KRAS + LAMP2 (Merge) in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12} (right) cells under complete medium and starvation conditions – 100x magnification.

Table 4.3. Values of LAMP2/FLAG-KRAS co-localization analysis in both NCM460 FLAG-KRAS cell lines, under complete and incomplete nutrient conditions. Pearson's Correlation Coefficient (PCC), Overlap Coefficient (OC) and Mander's coefficients M1 and M2 were obtained performing the co-localization analysis by JACoP. These values were adjusted to the threshold pre-defined for each fluorochrome and applied for all conditions in order to have comparable results.

NCM460 FLAG – $KRAS^{WT}$	PCC	OC	M1	M2
Complete medium	0,260	0,761	0,415	0,005
HBSS medium	0,350	0,912	0,458	0,013
NCM460 FLAG – $KRAS^{G12V}$				
Complete medium	0,290	0,912	0,376	0,007
HBSS medium	0,297	0,853	0,523	0,004

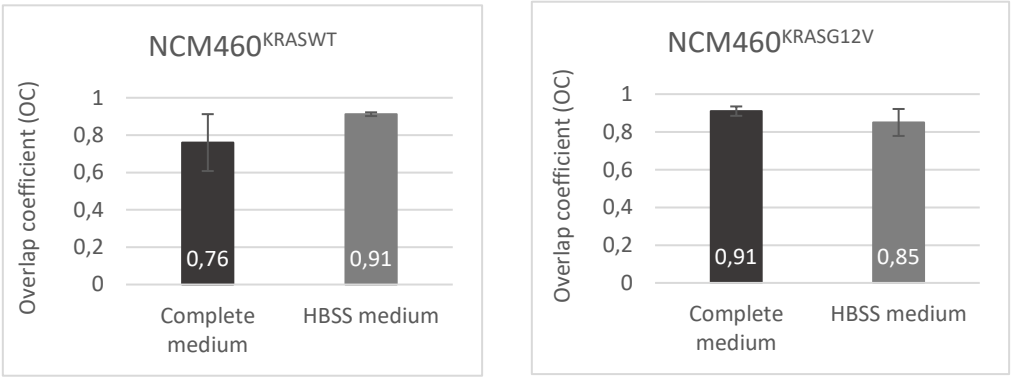


Figure 4.8. Graphical representation of the overlap coefficient between LAMP2/FLAG-KRAS in NCM460 cell lines, under complete and incomplete nutrient conditions. From the OC values we may infer that both wild-type (left) and mutated KRAS (right) co-localizes with lysosomes, under complete and incomplete medium conditions, once the co-localization values are close to 1.

4.1.3. Galectin-3 co-localize with KRAS

Galectin-3 (Gal-3) is a binding protein capable to interact with activated KRAS and control its signal outputs, thus interactions between Gal-3 and KRAS constitute an important subject of research. Taking this into account, we aim to investigate the interaction between Gal-3 and KRAS under autophagy induction by starvation.

From the confocal photos (Figure 4.9.) we can observe that Gal-3 presents a dotted staining more evident in NCM460 FLAG-KRAS^{G12V} cell line, possibly suggesting that this molecule is more expressed in these cells. Furthermore, Gal-3 display a heterogeneous distribution in the cytoplasm and at the perinuclear region. In addition, some nuclear localization is also found. Concerning FLAG-KRAS its localization is at the membrane and at the cytoplasm. Moreover, co-localization between FLAG-KRAS/ Gal-3 is suggested by the evident yellow staining observable in all the conditions. In NCM460 FLAG-KRAS^{G12V} cell line under starvation conditions, instead of a yellow staining it is visible an orange staining suggesting that one signal might be stronger than the other.

By quantifying co-localization levels (Table 4.4.), it is possible to infer from PCC values that these proteins have a positive correlation, since we obtained values between 0.5 and 0.6, in both cell lines. Accordingly, OC values demonstrated high levels of co-localization presenting values between 0.8 and 0.9, meaning almost full co-localization (Figure 4.10.). However, we faced the same problem previously described, when observing M1 and M2 values, which were inconclusive.

In conclusion our data suggest that Gal-3 co-localize with KRAS^{WT} and KRAS^{G12V} in NCM460 cells.

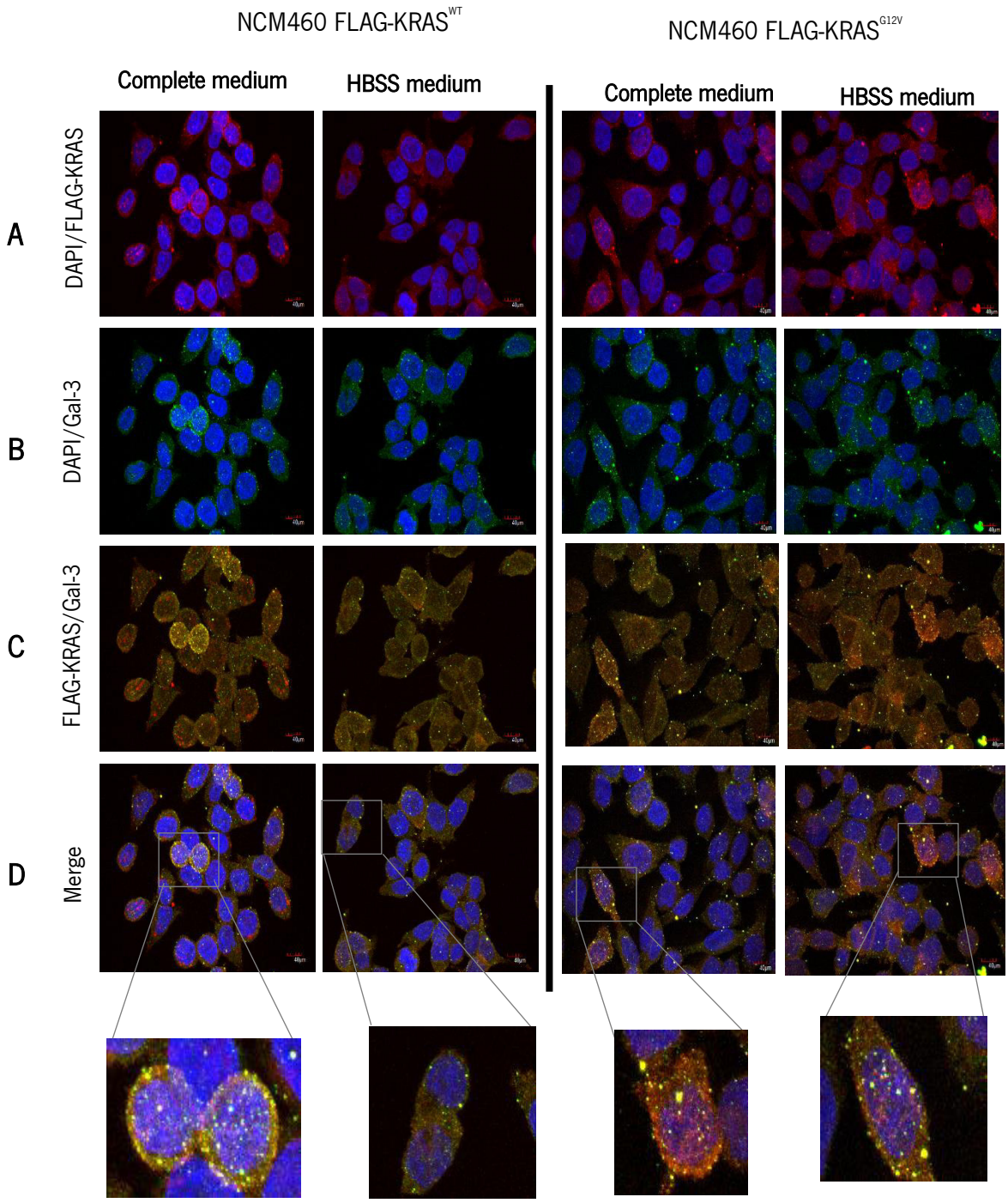


Figure 4.9. Co-immunofluorescence of FLAG-KRAS with Gal-3 in NCM460 cell lines in complete and HBSS medium. A. Confocal images of FLAG-KRAS (red) + DAPI in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12V} (right) cells under complete and HBSS medium. **B.** Confocal images of Gal-3 (green) + DAPI in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12V} (right) cells under complete and HBSS medium. **C.** Confocal images of FLAG-KRAS (red) + Gal-3 (green) in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12V} (right) cells under complete and HBSS medium. **D.** Confocal images of DAPI + FLAG-KRAS + Gal-3 (Merge) in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12V} (right) cells under complete and HBSS medium – 100x magnification.

Table 4.4. Values of Gal-3/FLAG-KRAS co-localization analysis in both NCM460 FLAG-KRAS cell lines, under complete and incomplete nutrient conditions. Pearson's Correlation Coefficient (PCC), Overlap Coefficient (OC) and Mander's coefficients M1 and M2 were obtained performing the co-localization analysis by JACoP. These values were adjusted to the threshold pre-defined for each fluorochrome and applied for all conditions in order to have comparable results.

NCM460 FLAG – $KRAS^{WT}$	PCC	OC	M1	M2
Complete medium	0,602	0,919	0,927	0,015
HBSS medium	0,582	0,921	0,909	0,016
NCM460 FLAG – $KRAS^{G12V}$				
Complete medium	0,590	0,903	0,801	0,026
HBSS medium	0,541	0,891	0,729	0,031

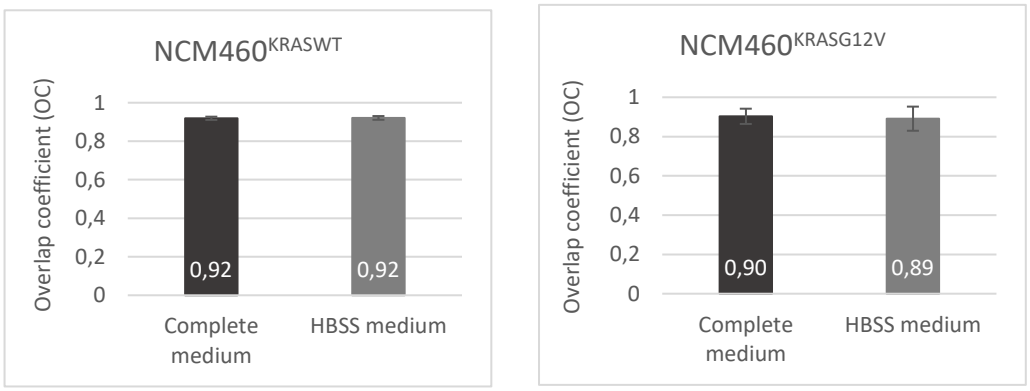


Figure 4.10. Graphical representation of the overlap coefficient between Gal-3/FLAG-KRAS in NCM460 cell lines, under complete and incomplete nutrient conditions. From the OC values we may infer that both wild-type (left) and mutated KRAS (right) co-localizes with Gal-3, under complete and incomplete medium conditions, once the co-localization values are close to 1.

4.1.4. KRAS mutation might influence actin polymerization

Actin filaments of the cytoskeleton have an active participation in events of autophagosome formation during starvation conditions and most aspects of its dynamics are regulated by the RHO family of GTPases (Kruppa et al., 2016; Zientara-Rytter & Subramani, 2016). Thus, we questioned if autophagy induction by starvation might change the structure of actin in NCM460 cells with $KRAS^{WT}$ and $KRAS^{G12V}$ and if the actin structure within the cell differed between the two cell lines. For that we assess the localization between FLAG-KRAS and Phalloidin probe (F-actin) in normal and autophagic conditions.

Confocal microphotographs of FLAG-KRAS and Phalloidin (Figure 4.11.) have shown a clear rearrangement of actin cytoskeleton under limiting-nutrient conditions with differences between the cell lines used. Under complete medium conditions actin exhibits the typical heterogeneous distribution of cytoplasmic projections that support cytoskeletal elements needed for roles in sensing, migration and cell-cell interaction. On the other hand, when autophagy was induced, it was possible to observe membrane ruffling, which contains a meshwork of newly polymerized actin filaments, indicating that they are formed via actin polymerization. However, whereas ruffles from the NCM460 FLAG-$KRAS^{WT}$ cells are smaller and derived exclusively from the cell edges, the NCM460 FLAG-$KRAS^{G12V}$ cells demonstrate a higher and more confusing ruffling which occur in a disseminate manner, suggesting that these changes could be due to the presence of mutated KRAS. Furthermore, the yellow/orange staining exhibited in these microphotography's suggest co-localization between FLAG-KRAS and F-actin.

From the quantitative analysis (Table 4.5.) PCC as well OC values (Figure 4.12.) demonstrated a positive correlation and co-localization possibility since we obtained values above 0.5 and between 0.8 and 0.9, respectively, meaning a high degree of co-localization. M1 and M2 values were, once more, inconclusive.

Our results suggest that KRAS might co-localize with actin clusters in the NCM460 cells and that induction of autophagy lead to changes in actin network polymerization.

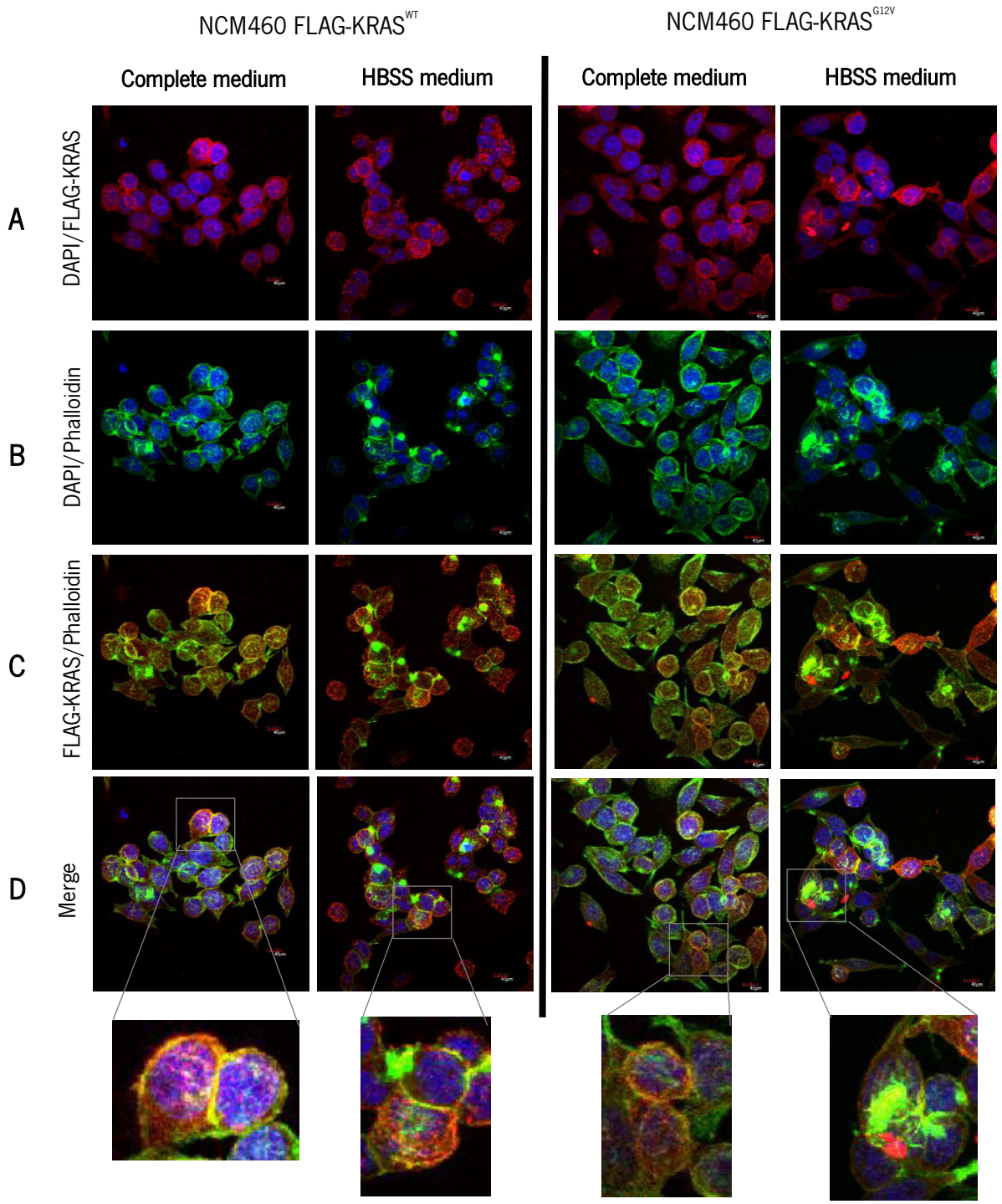


Figure 4.11. Co-immunofluorescence of FLAG-KRAS with Phalloidin 647 in NCM460 cell lines in complete and HBSS medium. A. Confocal images of FLAG-KRAS (red) + DAPI in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12} (right) cells under complete and HBSS medium. **B.** Confocal images of Phalloidin 647 (green) + DAPI in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12} (right) cells under complete and HBSS medium. **C.** Confocal images of FLAG-KRAS (red) + Phalloidin 647 (green) in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12} (right) cells under complete and HBSS medium. **D.** Confocal images of DAPI + FLAG-KRAS + Phalloidin 647 (Merge) in NCM460 FLAG-KRAS^{WT} (left) and NCM460 FLAG-KRAS^{G12} (right) cells under complete and HBSS medium – 100x magnification.

Table 4.5. Values of Actin/FLAG-KRAS co-localization analysis in both NCM460 FLAG-KRAS cell lines, under complete and incomplete nutrient conditions. Pearson's Correlation Coefficient (PCC), Overlap Coefficient (OC) and Mander's coefficients M1 and M2 were obtained performing the co-localization analysis by JACoP. These values were adjusted to the threshold pre-defined for each fluorochrome and applied for all conditions in order to have comparable results.

NCM460 FLAG – $KRAS^{WT}$	PC	OC	M1	M2
Complete medium	0,581	0,825	0,598	0,043
HBSS medium	0,557	0,882	0,416	0,068
NCM460 FLAG – $KRAS^{G12V}$				
Complete medium	0,542	0,897	0,742	0,030
HBSS medium	0,592	0,853	0,692	0,099

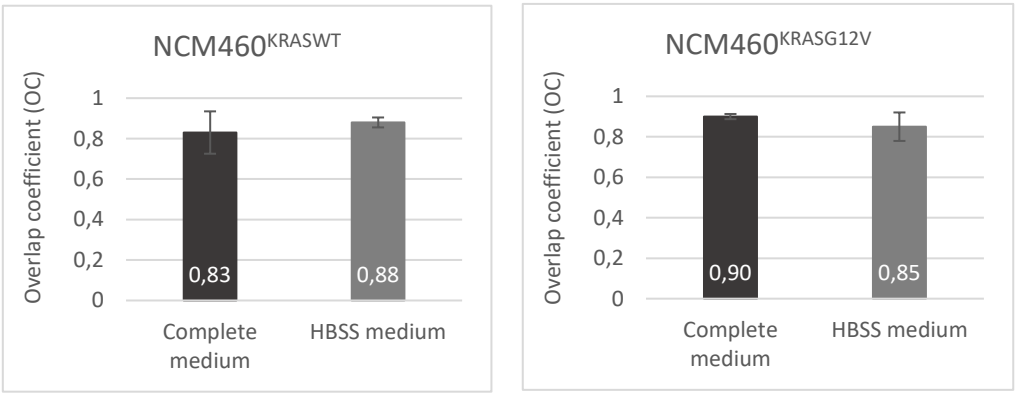


Figure 4.12. Graphical representation of the overlap coefficient between Phalloidin/FLAG-KRAS in NCM460 cell lines, under complete and incomplete nutrient conditions. From the OC values we may infer that both wild-type (left) and mutated KRAS (right) co-localizes with actin, under complete and incomplete medium conditions, once the co-localization values are close to 1.

PCC and OC are suitable for comparative analysis but not for the estimation of the quantity of co-localization, for this reason Mander's overlap coefficient, M1 and M2 (MOC) were also calculated. M1 and M2 not only give us the degree of overlap, but also the estimation of the contribution of each channel in the co-localized areas (Adler & Parmryd, 2010; Comeau, Costantino, & Wiseman, 2006; Zinchuk, Zinchuk, & Okada, 2007). However, M1 and M2 parameters obtained in each immunofluorescence assay performed were unreadable and inconclusive. It is important to remember that confocal images suffer from a high level of background noise, thus analyze and correct the background is essential for properly co-localization measurements. The extent of background depends on factors such as the intensity of immunofluorescence or the models of microscopes used to acquire images and will impact the outcome of the coefficients calculation. Another obstacle in obtaining reliable coefficients results is the high crosstalk between fluorophores which makes confocal images unusable for quantitative co-localization analysis (Zinchuk et al., 2007). Taking this into account, it is suggested that the background noise could be responsible to the low M2 values observed, once it tends to decrease it to zero. Moreover, although we selected fluorophores with well-separated excitation and emission spectra and confirmed the absence of cross-reaction between our antibodies through the negative controls, crosstalk between the fluorophores could also explain these results.

4.2. Effect of MSG-111-cd3 compound in colorectal cancer cells

As referred above, MSG-111-cd3 is a phenoxazine derivative with antiproliferative proprieties which has been tested as an antimicrobial agent. Previous results have shown that MSG-111-cd3 decreased cell survival in the yeast model. Also, in a preliminary assay it was observed that cell death process induced by this compound is dependent of the vacuolar protease Pep4p, whose homologous in human cells is Cathepsin-D (Cat-D).

4.2.1. MSG-111-cd3 decreased cell growth in colorectal cancer cells but not in normal cells

We used colorectal cancer derived cell lines RKO and SW480 and a noncancerous cell line derived from normal colon epithelial cells NCM460, to evaluate the effect on cell growth of different concentrations of the MSG –111–cd3 compound by Sulphorhodamine B (SRB) assay.

We could observe that upon exposure to the compound, CRC and normal cells showed different sensitivities (Figure 4.13.). RKO cell line was the most sensitive cell line exhibiting a significant inhibition of cell growth at low doses of the compound. In SW480 cell line the compound also induced cell growth inhibition but for higher concentrations when compared with RKO cells. Interestingly, normal colon derived cell NCM460 shown high resistance to this compound when compared to CRC cells as for concentrations of compound that showed to have a drastic effect in colorectal cancer cells only a discreet decrease in cell growth was observed.

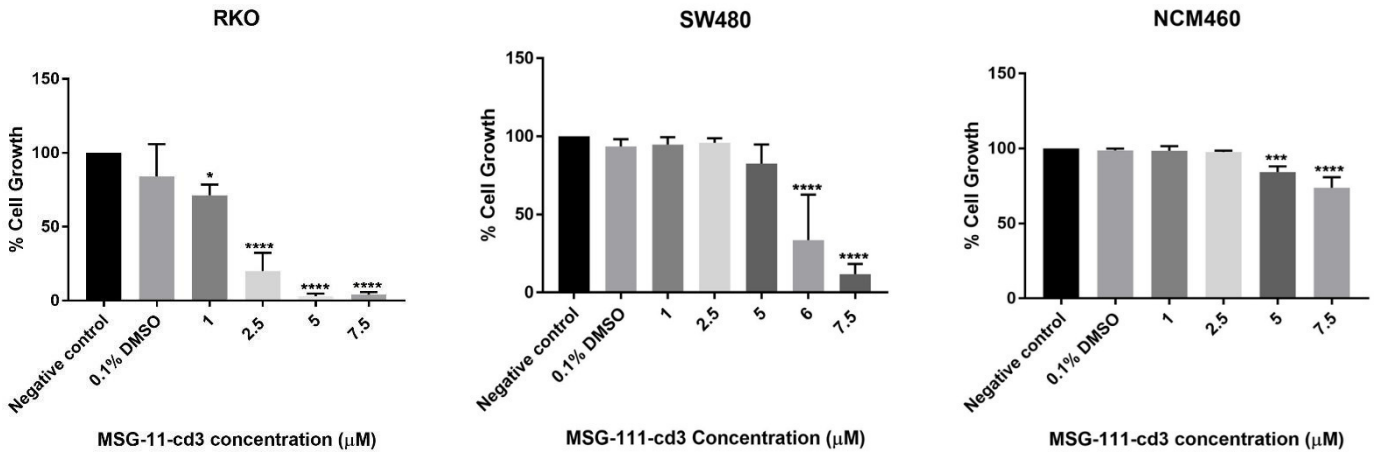
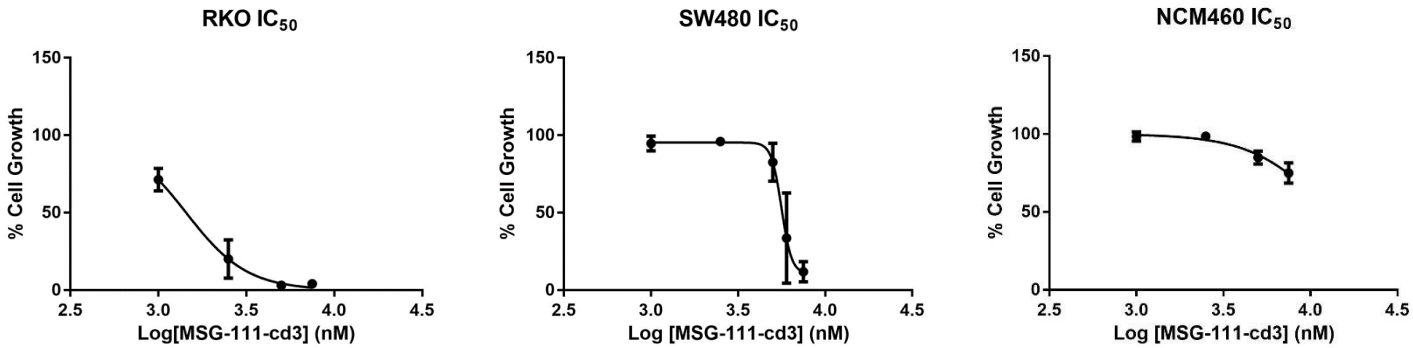


Figure 4.13. Effect of MSG-111-cd3 on cell proliferation of RKO, SW480 and NCM460 cell lines, determined by SRB assay. RKO, SW480 and NCM460 cell lines were incubated for 48 hours with increasing concentrations of MSG-111-cd3. Percentage of cell growth relatively to the negative control in RKO (left), SW480 (middle) and NCM460 cell line (right). These percentages were determined after the period of incubation and expressed as a mean ± SD for each treatment, from at least three independent experiments.

The concentration that inhibit 50% of cell growth (IC_{50}) were determined and confirmed the different sensitivity of the cell lines to the MSG-111-cd3 compound.

The IC_{50} of RKO and SW480 cells were 1.4 μM and 5.6 μM, respectively, whereas the IC_{50} of NCM460 estimated by extrapolation is about 12.8 μM (Figure 4.14), confirming that RKO and SW480 cells were more sensitive to the compound comparing to NCM460 cells.



	IC_{50} (μM)
RKO	1.4 ± 0.1
SW480	5.6 ± 0.2
NCM460	12.8 ± 2.0

Figure 4.14. RKO and SW460 have an IC_{50} value significantly lower than NCM460. IC_{50} values determined using GraphPad Prism 6 software, applying a dose *vs.* response non-linear regression (n=3).

4.2.2. Cathepsin-D might have a protective role in response to MSG-111-cd3 compound

In order to understand if Cat-D (whose homologous is Pep4p vacuolar protease in yeast) is involved in the cell-death mechanism induced by MSG-111-cd3, as demonstrated in yeast, we silenced Cat-D in CRC cells followed by treatment with the IC_{50} of MSG-111-cd3 and analyzed the effect in the cell proliferation.

Our preliminary results (Figure 4.15.) showed in both RKO and SW480 cell lines, that cathepsin-D silenced sensitizes the cells to MSG-111-cd3 compound having a more potent effect than the IC_{50} of the compound alone.

Although preliminary, these results suggest that cathepsin-D might have a protective role in response to the compound and might act as a mechanism of cell defense against the compound.

It is important to say that the experiments must be repeated to decrease the standard deviation and to obtain accurate conclusions. However, it is intriguing, that in both cell lines cathepsin-D silencing sensitizes the cells to MSG-111-cd3 compound having a more potent effect than the IC_{50} of the compound alone.

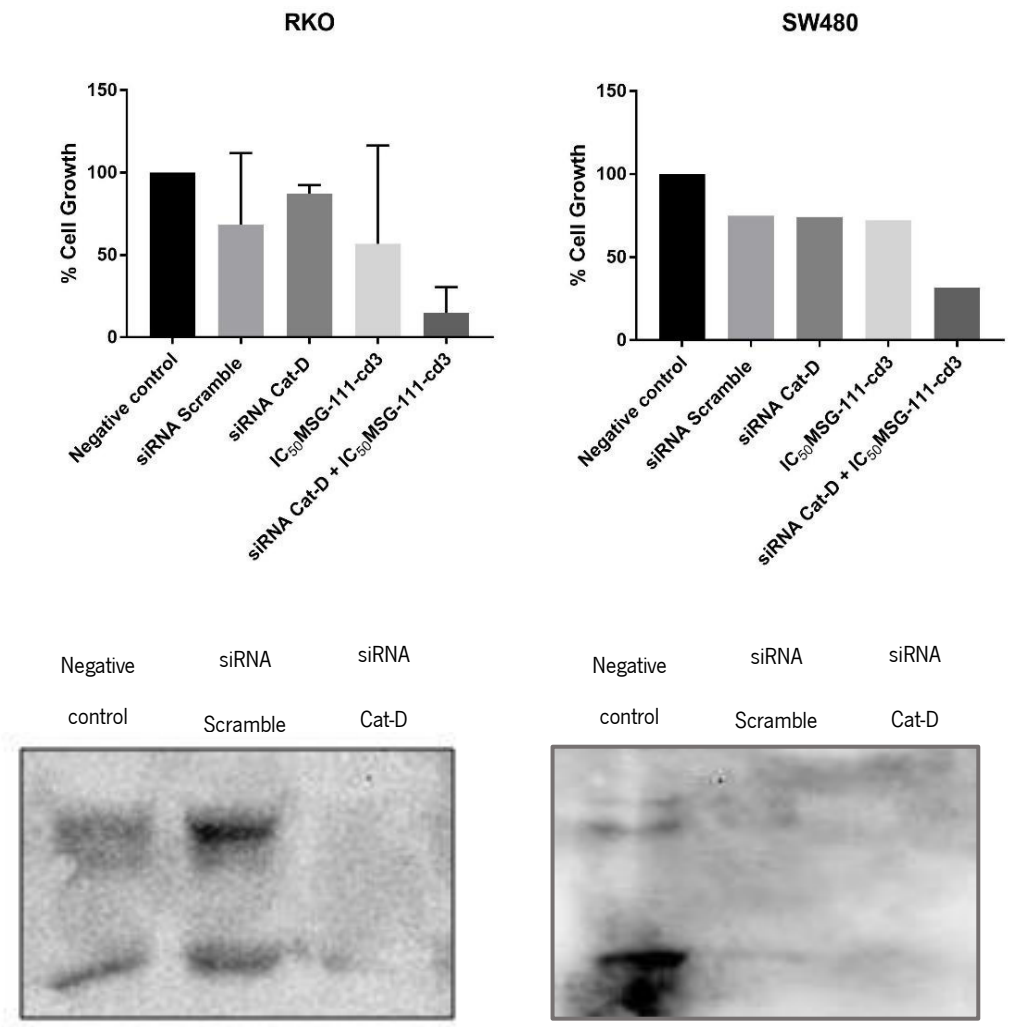


Figure 4.15. Cat-D silencing effects in RKO and SW460 cell lines together with MSG treatment. Cat-D seems to have a protective role against the antiproliferative effects of MSG-111-cd3 compound since when it is silenced cell death is enhanced. These percentages were determined after the period of incubation and, in the case of RKO, expressed as a mean ± SD for each treatment, from two independent experiments. For SW480, only one experiment is represented. Western blot analyses showed that Cat-D (28, 48 and 50 KDa) was inhibited.

5. Discussion

Despite the advances in surgical techniques and chemotherapy, colorectal cancer (CRC) is still the third leading cause of cancer and cancer related death worldwide (Loree et al., 2017). Most colorectal cancers arise from adenomas, in a process described as the adenoma-carcinoma sequence. Like other cancers, initiation and progression of CRC are associated with alterations in key regulatory genes (Ashktorab et al., 2010). Among the regulatory genes affected, KRAS oncogene is one of the most frequently mutated oncogene in CRC being mutated in approximately 35%-45% of CRC cases (Tan & Du, 2012). The most common mutations in this gene are the point substitutions mutations in codons 12 ($KRAS^{G12V}$ or $KRAS^{G12D}$) and 13 ($KRAS^{G13D}$) that are associated with significantly poorer survival after diagnosis (Phipps et al., 2013).

KRAS is a member of the GTPase family capable of changing from an inactive state GDP-bound to an active state GTP-bound thus triggering several signaling cascades in the cell by interaction with its effectors (Filchtinski et al., 2010). When KRAS carries the above-mentioned mutations, conformational changes occur so that KRAS is continuously in its active state, causing persistent stimulation of downstream signaling pathways, such as MAPK and PI3K, that drive many of the hallmarks of CRC (Fernandez-Medarde & Santos, 2011).

KRAS-mediated signaling depends on its level of enrichment on the plasma membrane (PM) (Schmick et al., 2014) as well as its localization within the cell, which is dictated by its hypervariable region (HVR). Indeed, subcellular localization influences the biological outcome of RAS (Casar et al., 2009). KRAS is described to be involved in a wide variety of signaling networks connecting a large set of upstream signals to a vast extent of downstream pathways related to cell cycle progression, growth, cytoskeletal changes, senescence, apoptosis and more recently, autophagy (Alves et al., 2015; Amaravadi, 2015; Fernandez-Medarde & Santos, 2011).

Autophagy is a self-degradative process that is important for balancing sources of energy at critical times in response to nutrient stress (Glick, Barth, & Macleod, 2010). Upon starvation, this mechanism can promote either cell survival or death, having both tumor-promoting as well as tumor suppressing properties (Das, Shravage, & Baehrecke, 2012).

It appears that RAS can upregulate or downregulate autophagy through several signaling pathways, making this crosstalk highly complex (Schmukler et al., 2014). Specifically, in the case of KRAS, our group and others have shown that KRAS mutations are associated with increased

autophagic levels, which seems to enhance cell survival (Alves et al., 2015; J. Y. Guo et al., 2011; J. Y. Guo et al., 2013; S. Yang et al., 2011).

In the last years, our group have been focusing on understanding the role of KRAS mutations in the carcinogenic process of CRC. Indeed, the group had already explored the role of mutant KRAS alleles in autophagy in both non-cancer colon cells derived from normal human colon epithelium and CRC-derived cell lines as well as in a "humanized" *Saccharomyces cerevisiae* model. The major findings of these studies reported that KRAS mutations up-regulated autophagy in both human colon cell lines and yeast models, and accordingly, downregulation of KRAS by siRNA in CRC-derived cell lines lead to impaired autophagic flux (Alves et al., 2015).

Taking into account the results obtained, we questioned if the subcellular localization of mutated KRAS might be important in the control of autophagy mechanism induction in the cell, by interacting with molecules or organelles important for KRAS activation platform. Here we wanted to understand if upon autophagy induction conditions (nutrient limitation), KRAS changed its localization in the cell. Thus, we questioned if KRAS upon starvation-induced autophagy could re-localize to mitochondria and/or lysosomes. Furthermore, we also explored whether Gal-3/KRAS and actin/KRAS known interactions might change upon autophagy induction. For that, we used two NCM460 cell lines, one with wild-type KRAS and other with a mutated KRAS ($KRAS^{G12V}$), and we performed immunofluorescence to determine KRAS co-localization with these subcellular compartments/molecules.

Our immunofluorescence assay using FLAG-KRAS and TOMM20, showed that when starvation was induced mitochondria acquired a more perinuclear distribution and that KRAS suffered a re-localization acquiring a sub-membranous/cytosolic distribution in NCM460 cells. During starvation induction, autophagy has been reported to be an essential event among eukaryotic models, such as in *S. cerevisiae*, for which cells with impaired autophagy die more readily when deprived of carbon and nitrogen (Scott et al., 1996; Tsukada & Ohsumi, 1993). As described in previous chapters, for proper autophagy, autophagosome formation is required, however, the specific membrane origin of autophagosomes remains unclear (Juhasz & Neufeld, 2006) with studies reporting contribution from a wide range of sources such as the endoplasmic reticulum (ER), trans-Golgi network (TGN), and mitochondria (Axe et al., 2008; Reggiori, Shintani, Nair, & Klionsky, 2005; Young et al., 2006), thus suggesting the possibility that the membrane of autophagosomes could be variable. Hailey and co-workers explored the formation of starvation-induced autophagosomes in a mammalian tissue culture cell model and reported that

mitochondrial outer membranes are used in autophagosome biogenesis (Hailey et al., 2010). Moreover, they demonstrate that the mitochondria-lipids delivered to the autophagosome membrane formation came from ER which is the "major lipid factory" within the cell. Indeed they showed that when mitochondria/ER connection was perturbed, the formation of starvation-induced autophagossomes was inhibited (Flis & Daum, 2013; Hailey et al., 2010). Taking this into account we could hypothesize that the reason why we observed that upon autophagy induction mitochondria moved to the perinuclear region, might be due to the fact that in eukaryotic cells the nucleus is surrounded by ER. In this way, mitochondria movement from the cytosol to surrounding zones of the nucleus could allow them to tether with ER, recruit the necessary lipids and enable autophagosome formation, thus supporting autophagy during the nutrient stress period. Moreover, Gomes and colleagues (2011), have demonstrated that during autophagy mitochondria elongates, what could possibly explain why we observed an elongated mitochondrial network upon starvation: it elongates to sustain cellular ATP levels and viability and thus promoting autophagy (Gomes, Di Benedetto, & Scorrano, 2011).

KRAS re-localization from plasma membrane to the cytosol strongly suggests that, when autophagy is stimulated, this isoform can move between compartments by diffusing through the cytosol supporting that KRAS could interact with a large set of organelles. Indeed, exciting new developments have shown that RAS is able to signal from intracellular organelles in addition to the plasma membrane (Hancock, 2003). Great evidence of that was reported by Chiu and colleagues (2002) which have shown that oncogenic HRAS and NRAS engage RAF at the Golgi and that unpalmitoylated HRAS is activated in response to mitogens at the ER with subsequent triggering of ERK pathway (Chiu et al., 2002).

Confocal immunofluorescence images of FLAG-KRAS/TOMM20 analyzed for co-localization levels using the overlap coefficient (OC), a variation of PCC, which considers the differences in intensities between two channels and the background noise (Adler & Parmryd, 2010; Comeau et al., 2006), suggested that $KRAS^{WT}$ seems to co-localize to the mitochondria and that $KRAS^{G12V}$ presence had a vital role in preventing FLAG-KRAS co-localization with TOMM20 since in the cell line with wild-type KRAS co-localization is found either in complete medium and starvation conditions, according to its OC values above 0.7. One possible explanation for these results rely on Bivona and co-workers (2006) reports which demonstrated that protein kinase C (PKC) phosphorylation of wild-type KRAS promote release from the PM and translocation to several internal membranes, including ER and mitochondrial membrane (Bivona et al., 2006). Because it

is described that PKC requires phosphoinositide 3-kinase (PI3K) to become active (Cosentino-Gomes, Rocco-Machado, & Meyer-Fernandes, 2012) and autophagy induced by $KRAS^{MUT}$ is mediated through up-regulation of MEK pathway and down-regulation of PI3K pathway (Alves et al., 2015), we may hypothesize that co-localization between $KRAS^{G12V}$ and mitochondria does not occur since PI3K signaling pathway is downregulated not allowing PKC activation, and subsequently not allowing KRAS phosphorylation by PKC thus preventing its translocation to the mitochondria.

Unexpectedly, this translocation relates to cell death, that was firstly associated with Bcl-xL dependent apoptosis induction (Bivona et al., 2006). However, when Sung and co-workers (2013) investigate the mechanism behind this effect, they demonstrated that KRAS associates with inositol triphosphate (InsP3) receptors (IP3Rs) on the ER, in a Bcl-xL dependent manner, thereby blocking the ability of Bcl-xL to potentiate the InsP3 regulated release of calcium from ER to mitochondria that is required for efficient respiration and inhibition of autophagy (Sung et al., 2013). In contrast to what was previously reported by Bivona and co-workers, Sung and colleagues, found that KRAS phosphorylation by PKC did not activate apoptosis but instead is associated with induction of autophagy by blocking Bcl-xL and its control over InsP3, thus enhancing autophagy (Sung et al., 2013). The fact that wild-type KRAS signaling through IP3Rs at the ER promotes cell death, led us to question why is $KRAS^{WT}$ co-localized with mitochondria both under normal and autophagy-induction conditions? Could $KRAS^{WT}$ localize at the mitochondria to regulate the balance between apoptosis and autophagy?

Immunofluorescence results using FLAG-KRAS and LAMP2 showed that lysosomes have a heterogeneous distribution. Interestingly the cell line with mutated $KRAS^{G12V}$ present a higher number of lysosomes suggesting an increased need of these organelles upon constitutive activation of KRAS. This result is in accordance to our previous report showing that KRAS mutations induce autophagy which depends on lysosomes availability in the cell. Moreover, in both cell lines, when starvation was induced the number of lysosomes appeared to accumulate in the perinuclear region which is where autophagosome-lysosomes fusion primarily takes place associated with the ER (Xu & Ren, 2015), thus being a common feature observed during autophagy.

Concerning the co-localization quantification of FLAG-KRAS/LAMP2 immunofluorescence assay, we obtained inconclusive PCC values below 0.5 whereas the OC values, suggested co-localization of FLAG-KRAS with LAMP2 in both cell lines and independently of the nutrient status. In eukaryotic cells, proteins are transported via endocytic pathways capable to internalize

molecules from the PM and then direct them into sorting endosomes where they can either be recycled back to the PM or travel to late endosomes (Les) and lysosomes for degradation (Elkin, Lakoduk, & Schmid, 2016). However, in contrast what was traditionally thought, endocytic membrane trafficking and cell signaling are now recognized as intimately linked processes in animal cells (Sorkin & von Zastrow, 2009).

It was already reported that from the early endosomes, KRAS but not HRAS nor NRAS, was transported to late endosomes (Les) and eventually targeted to lysosomes (Lu et al., 2009). Moreover, using fluorescent probes, it was shown that KRAS was active on Les since it elicited a signal output through RAF to promote MAPK signaling cascade (Lu et al., 2009). This report suggests that KRAS may resort to endosomal trafficking to activate specific signaling pathways. Accordingly, its counterpart HRAS appear to be endocytosed as part of their normal function in propagating signals from activated cell surface receptors (Jiang & Sorkin, 2002). Furthermore, signaling of HRAS to RAF is also reported to be dependent on endocytosis (Roy, Wyse, & Hancock, 2002). Taking this into account, we suggest that KRAS co-localizes with lysosomes as a need to control downstream effectors or as a mechanism to recycle KRAS back to the PM. In fact, it was already reported that RAS proteins can interact with ESCRT-III, a machinery known to delivery ubiquitin tagged proteins to the endosome acting as a sorting station, to recycle back to PM thus enhancing RAS-induced activities (Zheng et al., 2012). A key question for the future is to unravel how the decision is made for KRAS protein in the endosome to remain internalized and be later degraded or to be sent back to the PM.

From the immunofluorescence assay of FLAG-KRAS together with Gal-3 we could observe that Gal-3 has a heterogeneous cellular distribution being found in the cytoplasm, perinuclear as well as nuclear regions in all the conditions, which is already described by the literature (Dumic, Dabelic, & Flogel, 2006). Looking for the PCC values obtained, we could find a positive correlation between FLAG-KRAS/Gal-3, in both cell lines, and accordingly the OC values also support that KRAS co-colocalizes with Gal-3 in all the conditions, which is in concordance with the literature that describes Gal-3 as an important modulator of KRAS signal output by interfering with its nanoclustering formation in the PM (Elad-Sfadia et al., 2004; R. Levy et al., 2011). Furthermore, Gal-3 seemed to be more expressed in the cell line with $KRAS^{G12V}$ mutation. Gal-3 is a member of the galectin family with ubiquitous expression levels performing a wide range of key cellular roles that are defined by its localization inside the cell as well as the interacting molecules (Hirabayashi & Kasai, 1993; Rabinovich, 1999). For example, cytoplasmic Gal-3 is described to be involved in

regulation of apoptosis (Fukumori et al., 2006) whereas when located in the nucleus is associated with the ribonucleoprotein complexes (Laing & Wang, 1988). Importantly, it is involved in cell proliferation and differentiation by interacting with KRAS (Elad-Sfadia et al., 2004; R. Levy et al., 2011; R. Levy, Grafi-Cohen, Kraiem, & Kloog, 2010; Shalom-Feuerstein et al., 2008). KRAS interacts with Gal-3 mainly in cytosol and PM and only when it is GTP-loaded indicating that this interaction should be higher in the cell line with mutated KRAS once mutation maintains KRAS in its active state (Elad-Sfadia et al., 2004; Shalom-Feuerstein et al., 2008). It has been hypothesized that Gal-3 stabilizes KRAS in its active state thereby enabling the promotion of pro-growth signaling pathways. Increased expression and availability of cytoplasmic Gal-3 is related to cells increased tumorigenic properties which can explain why we noticed a higher level of Gal-3 expression in the cell line with mutated KRAS. Moreover, we might also suppose that cells with mutated KRAS need to recruit more Gal-3 to easily maintain its active status promoted by the mutation itself.

Concerning the results on FLAG-KRAS/Phalloidin we observed a clear rearrangement of actin cytoskeleton under limiting-nutrient conditions suggesting a metabolic adaptation to this stimulus. In fact, Ras-transformed cells are usually characterized by a remodeled actin cytoskeleton which causes poor adhesion, increased motility, invasiveness, and contact-independent growth (Bar-Sagi & Feramisco, 1986; Pawlak & Helfman, 2001). The OC values of the immunofluorescence assay of FLAG-KRAS/Phalloidin (actin) demonstrated a positive correlation with high levels of co-localization in both cell lines and independently of the nutrient status. These results support the active role of actin in KRAS nanoclustering formation (Hancock & Parton, 2005) as well as the role of KRAS in maintaining cytoskeletal changes, motility and adhesion (Pollock et al., 2005).

Looking to our results, under starvation, membrane ruffling was observed with differences between the cell lines. Membrane ruffles, also known as membrane protrusions, are formed via actin polymerization and are essential for cell motility (Mahankali, Peng, Cox, & Gomez-Cambronero, 2011). Whereas NCM460 FLAG-KRAS^{WT} cells present smaller ruffles derived exclusively from the cell edges, the NCM460 FLAG-KRAS^{G12V} cells demonstrate a more confusing and higher ruffling which occur in a disseminate manner. According to reports already made, the molecules possibly involved in this actin cytoskeleton reorganization are the RAS effectors, RAF and PI3K that act accordingly with the cell-type (Joneson, White, Wigler, & Bar-Sagi, 1996; Rodriguez-Viciana et al., 1997). Furthermore, it was recently described that RAS activation, either by growth factor stimulation or through oncogenic mutation, leads to increased membrane ruffling

and subsequent metabolic adaptation to confer cells the ability to sustain cell growth in severe conditions by macropinocytosis, explaining why we observed an increased ruffling in the mutated cell line (Recouvreux & Commisso, 2017). Thus, our results suggest that $KRAS^{G12V}$ presence has a strong influence in actin polymerization with subsequent impact in the membrane ruffles formation and adaptation strategies promoted. In fact, we may infer that oncogenic KRAS maintained a cytoskeletal structure favorable to CRC cells proliferation and survival (Bar-Sagi & Feramisco, 1986; Pawlak & Helfman, 2001; Pollock et al., 2005). In addition, these data strongly suggest an impact of KRAS mutations and actin in autophagy induction since actin polymerization is described as an essential event to autophagosome formation and our results report that KRAS mutations seem to manipulate and enhance actin polymerization in NCM460 cells (Aguilera et al., 2012).

Summing up, our results show that wild type KRAS co-localize with mitochondria and suggest that mutated $KRAS^{G12V}$ seems to prevent KRAS co-localization with mitochondria, independently of autophagy induction. In contrast, KRAS seems to co-localize with lysosomes in all the conditions probably as a need to control downstream effectors or as a mechanism to recycle itself back to the PM to create a positive feedback loop and enhancing KRAS signaling. Moreover, we observed that in both cell lines KRAS and Gal-3 co-localize in all conditions. Finally, our data supports KRAS co-localization with actin in both cell lines which is consistent with the symbiotic relationship suggested, with actin influencing KRAS signal output modulation and KRAS maintaining actin cytoskeleton organization.

Concerning our second aim where we further investigate the effect of a new phenoxazine derivative (MSG-111-cd3) in CRC cells, as a possible new drug for potentially use in CRC therapy, we could observe that after exposure to the compound CRC and normal cells exhibited different sensitivities, which demonstrated a promising effect of this compound in CRC treatment. CRC cells sensitivity was also different, RKO with $BRAF^{V600E}$ displayed higher sensitivity to the compound comparing to SW480 with $KRAS^{G12V}$ suggesting that the mutation harbored by each cell line may influence the effect of MSG-111-cd3 in CRC. In fact, it is already known that CRC patients with KRAS or BRAF mutations demonstrated different resistances to available therapies, such as the anti-EGRF ones, with RAS mutations exhibiting a more pronounced resistance effect comparing to BRAF mutations (Arcila, Lau, Nafa, & Ladanyi, 2011; Bokemeyer et al., 2008; Bokemeyer et al., 2009; Bronte et al., 2015).

Our results on the role of Cathepsin-D in MSG-111-cd3 effect in the RKO and SW480 cell lines, demonstrated different responses comparing to yeast. Previous results in yeast demonstrated that MSG-111-cd3 compound induces cell death through a mechanism dependent on the release of Pep4p from the vacuole to the cytosol, suggesting an effector role of Pep4p. In contrast, our results suggest a protective role of Cat-D, what is according to previously report in CRC cells showing that Cat-D has a protective effect in acetate-induced apoptosis (Marques et al., 2013).

6. Conclusions and future perspectives

6.1 Conclusions

Previous work from the group showed that $KRAS^{MUT}$ upregulate autophagy through MEK/ERK signaling pathway thus promoting cell survival, however little is known about the localization and protein interaction changes induced by KRAS mutations under autophagy conditions.

During this project we had two different purposes: First, we aim to study the influence of starvation-induced autophagy in KRAS localization within the cell and KRAS interaction with proteins, to potentially identify new signaling platforms for KRAS activity. Second, because $KRAS^{MUT}$ are a predictive biomarker of resistance to CRC therapies, we aim to test new promising anti-cancer agents for CRC treatment. For that, we tested for the first time in human colon cell culture models, the effect of a newly phenoxazine derivative named MSG-111-cd3, synthetized by the Chemical group of University of Minho and already tested in yeast by the Microbiology lab. MSG-111-cd3 is described as a promising antitumoral agent and in yeast, exhibited interesting results since it decreased cell survival through a mechanism dependent of the vacuolar Pep4p, whose homologous in human cells is Cathepsin-D.

We were able to answer the questions raised in the beginning of the project and the results obtained in this project could be summarized as follow:

- Answering to our first question: "Does autophagy induction by starvation lead to co-localization of KRAS protein to mitochondria or lysosomes?". Our data suggested that **$KRAS^{WT}$ localize at the mitochondria** which is lost when KRAS is mutated. Our results also suggested that **KRAS co-localize to the lysosome.** Moreover, we can infer that in both mitochondria and lysosomes KRAS co-localization occur independently of autophagy induction and interestingly KRAS mutations influence the extent of co-localization with mitochondria.

- Concerning the question: "Are the complexes KRAS-Gal-3 and KRAS-actin affected under starvation-induced autophagy?". Our data suggest that **Gal-3 and KRAS co-localize in NCM460 cells** and that **KRAS might co-localize with actin cytoskeleton** with possible

influence in **polymerization.** Autophagy induction does not seem to influence the KRAS-Gal-3 complex localization, however, KRAS mutations appear to influence the number of existing complexes. Concerning KRAS-actin interaction, starvation-induced autophagy promoted actin cytoskeleton remodeling possibly in a KRAS-dependent manner since co-localization levels were high and the cell line with mutated KRAS exhibited a different actin rearrangement.

- Regarding the question: "Is MSG-111-cd3 a promising anticancer agent in CRC?". Our data showed that **MSG-111-cd3 decreased cell growth in colorectal cancer cells but not in normal cells** suggesting that could be a potential candidate for CRC treatment. Cathepsin-D also seems to have a role in the cell death induced by MSG-111-cd3 in CRC cells, however, the results suggest that its mechanism of action may be different from the one described in yeast.

6.2 Future perspectives

Concerning the **first aim**, although this work gave some new clues on the extent to which KRAS influences autophagy through the study of its localization, optimizations are needed and further techniques could be performed to better support our results and explore several other aspects:

- In order to **improve our co-localization quantifications**, that exhibited some inconclusive values, an accurate correction of background could be performed, by using optimized emission filters, and a strict control of background levels using deconvolution (Zinchuk et al., 2007) as well as by avoiding acquire too bright and too contrast images, as it may result in image saturation (Zinchuk & Zinchuk, 2008).

- To better support results obtained from immunofluorescence assays and confocal microscopy about KRAS co-localization with mitochondria, lysosomes, Gal-3 and F-actin, **complementary biochemical assays** such as proximity ligation (PLA) and/or fluorescence resonance energy transfer (FRET) assays should be performed. PLA extends the

capabilities of traditional immunoassays to include direct detection, visualization and quantification of individual endogenous proteins and its interactions with high sensitivity/specificity and repeatability (Debaize, Jakobczyk, Rio, Gandemer, & Troadec, 2017) whereas FRET measures the energy transferred between two fluorophores determining spatial and temporal distribution of proteins associations (Sekar & Periasamy, 2003). Furthermore, **bioinformatic analysis** could be a great tool to enhance credibility of the immunofluorescence results since there are several computational methods for protein-protein interaction prediction available (Zahiri, Bozorgmehr, & Masoudi-Nejad, 2013).

- It would be interesting to perform these same immunofluorescence assays in **other KRAS-transfected cell lines** such as NCM460 Flag-KRAS^{G12D} and Flag-KRAS^{G13D}, to infer if different KRAS mutations alter KRAS co-localization with mitochondria and lysosomes as well as KRAS/Gal-3 and KRAS/actin complexes, as described here in NCM460 Flag-KRAS^{WT} and NCM460 Flag-KRAS^{G12V}. Moreover, explore **other possible localizations** of KRAS, under limiting-nutrient conditions, such as the endoplasmic reticulum (ER), which is known as a platform where KRAS suffers key biochemical modifications that dictate its localization within the cell (Hancock, 2003), may give us additional valuable insights about autophagy influence in KRAS functional properties and understand KRAS mutations role.

- **Deeper exploitation of autophagy** in these immunofluorescence assays is also suggested as a future perspective. Some authors reported that a key point in starvation-induced autophagy occurs at the end of the process, where lysosomes are regenerated from autolysosomes through a pathway termed autophagic lysosome reformation (ALR), leading to the activation of new pathways (Munson & Ganley, 2015; Yu et al., 2010). Thus, this arise the question "Inducing autophagy during less or more time, will lead to differential KRAS localization within the cell?".

- Also, as **yeast is a useful organism** to address the function of KRAS in autophagy, as proved by Alves *et al.* (2015), this model can be further explored to address other specific cellular functions of KRAS or KRAS localization within cellular compartments.

- Recently, KRAS has been supposed to be involved in the **interplay between autophagy and apoptosis**, which are mechanisms described as having increased activity in CRC tumors bearing activating mutations in KRAS (Liu, Jakubowski, & Hunt, 2011; S. Yang et al., 2011). Understanding how KRAS mutations orchestrate the balanced regulation of apoptosis and autophagy in the colon model and the role of Beclin-1 and Bcl-2 protein family members in this control, could be a contribute to new perspectives for therapeutic approaches in resistant CRC harboring KRAS mutations. In fact, some studies exploring this issue were already made and it was found that in HCT116 colorectal cancer cells, anti - apoptotic proteins Bcl-2 and Bcl-xL stimulated pro-survival autophagy, with a differential dependence on Beclin-1 (Priault et al., 2010).

Concerning the **second aim**, taking into account that we have shown here for the first time results of the effects of the new compound MSG-111-cd3 in colon model human cells, many findings are still to come and for that further exploitation is necessary:

- Concerning the assays where we inhibited Cat-D and treated with MSG-111-cd3 IC_{50}, **the assays must be repeated** in order to support the preliminary conclusions that we made. Moreover, it would be interesting to understand better the role of the mutations in MSG-111-cd3 treatment resistance by silencing KRAS and BRAF genes by RNAi and studying if resistance levels still the same. Also, yeast transformed with the different KRAS isoforms could unravel if this oncogene, ant its mutation may influence the resistance to the compound.

- Since the outcome of Cat-D involvement in MSG-111-cd3 seems to be different in yeast and human cells, the next step that we suggest is trying to **understand the cell-death mechanism involved**. For that, the expression levels of molecular biomarkers of autophagy and apoptosis should be study under the effect of the compound.

7. References

Aagaard, L., & Rossi, J. J. (2007). RNAi therapeutics: principles, prospects and challenges. *Adv Drug Deliv Rev, 59*(2-3), 75-86. doi: 10.1016/j.addr.2007.03.005

Abankwa, D., Gorfe, A. A., & Hancock, J. F. (2007). Ras nanoclusters: molecular structure and assembly. *Semin Cell Dev Biol, 18*(5), 599-607. doi: 10.1016/j.semcdb.2007.08.003

Abe, A., Yamane, M., & Tomoda, A. (2001). Prevention of growth of human lung carcinoma cells and induction of apoptosis by a novel phenoxazinone, 2-amino-4,4alpha-dihydro-4alpha,7-dimethyl-3H-phenoxazine-3-one. *Anticancer Drugs, 12*(4), 377-382.

Adler, J., & Parmryd, I. (2010). Quantifying colocalization by correlation: the Pearson correlation coefficient is superior to the Mander's overlap coefficient. *Cytometry A, 77*(8), 733-742. doi: 10.1002/cyto.a.20896

Agudo-Ibanez, L., Herrero, A., Barbacid, M., & Crespo, P. (2015). H-ras distribution and signaling in plasma membrane microdomains are regulated by acylation and deacylation events. *Mol Cell Biol, 35*(11), 1898-1914. doi: 10.1128/MCB.01398-14

Aguilera, M. O., Beron, W., & Colombo, M. I. (2012). The actin cytoskeleton participates in the early events of autophagosome formation upon starvation induced autophagy. *Autophagy, 8*(11), 1590-1603. doi: 10.4161/auto.21459

Ahearn, I. M., Haigis, K., Bar-Sagi, D., & Philips, M. R. (2011). Regulating the regulator: post-translational modification of RAS. *Nat Rev Mol Cell Biol, 13*(1), 39-51. doi: 10.1038/nrm3255

Ahmed, D., Eide, P. W., Eilertsen, I. A., Danielsen, S. A., Eknaes, M., Hektoen, M., . . . Lothe, R. A. (2013). Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis, 2*, e71. doi: 10.1038/oncsis.2013.35

Al-Sohaily, S., Biankin, A., Leong, R., Kohonen-Corish, M., & Warusavitarne, J. (2012). Molecular pathways in colorectal cancer. *J Gastroenterol Hepatol, 27*(9), 1423-1431. doi: 10.1111/j.1440-1746.2012.07200.x

Allegra, C. J., Jessup, J. M., Somerfield, M. R., Hamilton, S. R., Hammond, E. H., Hayes, D. F., . . . Schilsky, R. L. (2009). American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol, 27*(12), 2091-2096. doi: 10.1200/JCO.2009.21.9170

Alves, S., Castro, L., Fernandes, M. S., Francisco, R., Castro, P., Priault, M., . . . Preto, A. (2015). Colorectal cancer-related mutant KRAS alleles function as positive regulators of autophagy. *Oncotarget, 6*(31), 30787-30802. doi: 10.18632/oncotarget.5021

Amaravadi, R. K. (2015). Transcriptional regulation of autophagy in RAS-driven cancers. *J Clin Invest, 125*(4), 1393-1395. doi: 10.1172/JCI81504

Amaravadi, R. K., Yu, D., Lum, J. J., Bui, T., Christophorou, M. A., Evan, G. I., . . . Thompson, C. B. (2007). Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. *J Clin Invest, 117*(2), 326-336. doi: 10.1172/JCI28833

Arcila, M., Lau, C., Nafa, K., & Ladanyi, M. (2011). Detection of KRAS and BRAF mutations in colorectal carcinoma roles for high-sensitivity locked nucleic acid-PCR sequencing and broad-spectrum mass spectrometry genotyping. *J Mol Diagn, 13*(1), 64-73. doi: 10.1016/j.jmoldx.2010.11.005

Arends, M. J. (2013). Pathways of colorectal carcinogenesis. *Appl Immunohistochem Mol Morphol, 21*(2), 97-102. doi: 10.1097/PAI.0b013e31827ea79e

Armaghany, T., Wilson, J. D., Chu, Q., & Mills, G. (2012). Genetic alterations in colorectal cancer. *Gastrointest Cancer Res, 5*(1), 19-27.

Ashktorab, H., Schaffer, A. A., Daremipouran, M., Smoot, D. T., Lee, E., & Brim, H. (2010). Distinct genetic alterations in colorectal cancer. *PLOS ONE, 5*(1), e8879. doi: 10.1371/journal.pone.0008879

Axe, E. L., Walker, S. A., Manifava, M., Chandra, P., Roderick, H. L., Habermann, A., . . . Ktistakis, N. T. (2008). Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol, 182*(4), 685-701. doi: 10.1083/jcb.200803137

Badadani, M. (2012). Autophagy Mechanism, Regulation, Functions, and Disorders. *ISRN Cell Biology, 2012*, 11. doi: 10.5402/2012/927064

Bar-Sagi, D., & Feramisco, J. R. (1986). Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. *Science, 233*(4768), 1061-1068.

Barras, D. (2015). BRAF Mutation in Colorectal Cancer: An Update. *Biomark Cancer, 7*(Suppl 1), 9-12. doi: 10.4137/BIC.S25248

Bertram, J. S. (2000). The molecular biology of cancer. *Mol Aspects Med, 21*(6), 167-223.

Bivona, T. G., Quatela, S. E., Bodemann, B. O., Ahearn, I. M., Soskis, M. J., Mor, A., . . . Philips, M. R. (2006). PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its

association with Bcl-XL on mitochondria and induces apoptosis. *Mol Cell, 21*(4), 481-493. doi: 10.1016/j.molcel.2006.01.012

Bokemeyer, C., Bondarenko, I., Hartmann, J. T., de Braud, F., Schuch, G., Zubel, A., . . . Koralewski, P. (2011). Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study. *Ann Oncol, 22*(7), 1535-1546. doi: 10.1093/annonc/mdq632

Bokemeyer, C., Bondarenko, I., Hartmann, J. T., De Braud, F. G., Volovat, C., Nippgen, J., . . . Koralewski, P. (2008). KRAS status and efficacy of first-line treatment of patients with metastatic colorectal cancer (mCRC) with FOLFOX with or without cetuximab: The OPUS experience. *Journal of Clinical Oncology, 26*(15_suppl), 4000-4000. doi: 10.1200/jco.2008.26.15_suppl.4000

Bokemeyer, C., Bondarenko, I., Makhson, A., Hartmann, J. T., Aparicio, J., de Braud, F., . . . Koralewski, P. (2009). Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J Clin Oncol, 27*(5), 663-671. doi: 10.1200/JCO.2008.20.8397

Boland, C. R., Sinicrope, F. A., Brenner, D. E., & Carethers, J. M. (2000). Colorectal cancer prevention and treatment. *Gastroenterology, 118*(2 Suppl 1), S115-128.

Bolognese, A., Correale, G., Manfra, M., Lavecchia, A., Mazzoni, O., Novellino, E., . . . Loddo, R. (2002). Antitumor Agents. 2. Synthesis, Structure−Activity Relationships, and Biological Evaluation of Substituted 5H-Pyridophenoxazin-5-ones with Potent Antiproliferative Activity. *J Med Chem, 45*(24), 5217-5223. doi: 10.1021/jm020918w

Bolognese, A., Correale, G., Manfra, M., Lavecchia, A., Novellino, E., & Pepe, S. (2006). Antitumor Agents. 5. Synthesis, Structure−Activity Relationships, and Biological Evaluation of Dimethyl-5H-pyridophenoxazin-5-ones, Tetrahydro-5H-benzopyridophenoxazin-5-ones, and 5H-Benzopyridophenoxazin-5-ones with Potent Antiproliferative Activity. *J Med Chem, 49*(17), 5110-5118. doi: 10.1021/jm050745l

Bos, J. L., Rehmann, H., & Wittinghofer, A. (2007). GEFs and GAPs: critical elements in the control of small G proteins. *Cell, 129*(5), 865-877. doi: 10.1016/j.cell.2007.05.018

Bouchahda, M., Karaboue, A., Saffroy, R., Innominato, P., Gorden, L., Guettier, C., . . . Levi, F. (2010). Acquired KRAS mutations during progression of colorectal cancer metastases: possible implications for therapy and prognosis. *Cancer Chemother Pharmacol, 66*(3), 605-609. doi: 10.1007/s00280-010-1298-9

Boya, P., Gonzalez-Polo, R. A., Casares, N., Perfettini, J. L., Dessen, P., Larochette, N., . . . Kroemer, G. (2005). Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol, 25*(3), 1025-1040. doi: 10.1128/MCB.25.3.1025-1040.2005

Bronte, G., Silvestris, N., Castiglia, M., Galvano, A., Passiglia, F., Sortino, G., . . . Russo, A. (2015). New findings on primary and acquired resistance to anti-EGFR therapy in metastatic colorectal cancer: do all roads lead to RAS? *Oncotarget, 6*(28), 24780-24796. doi: 10.18632/oncotarget.4959

Burada, F., Nicoli, E. R., Ciurea, M. E., Uscatu, D. C., Ioana, M., & Gheonea, D. I. (2015). Autophagy in colorectal cancer: An important switch from physiology to pathology. *World J Gastrointest Oncol, 7*(11), 271-284. doi: 10.4251/wjgo.v7.i11.271

Cabrera-Mendoza, F., Gainza-Lagunes, S., Castaneda-Andrade, I., & Castro-Zarate, A. (2014). [Clinical relevance of the K-ras oncogene in colorectal cancer: experience in a Mexican population]. *Rev Gastroenterol Mex, 79*(3), 166-170. doi: 10.1016/j.rgmx.2014.07.002

Carvalho, R. E. S. (2011). Staining pattern and potential targets of a benzo[a]phenoxazinium chloride with antiproliferative activity.

Casar, B., Arozarena, I., Sanz-Moreno, V., Pinto, A., Agudo-Ibanez, L., Marais, R., . . . Crespo, P. (2009). Ras subcellular localization defines extracellular signal-regulated kinase 1 and 2 substrate specificity through distinct utilization of scaffold proteins. *Mol Cell Biol, 29*(5), 1338-1353. doi: 10.1128/MCB.01359-08

Castellano, E., & Santos, E. (2011). Functional specificity of ras isoforms: so similar but so different. *Genes Cancer, 2*(3), 216-231. doi: 10.1177/1947601911408081

Cazzanelli, G., Pereira, F., Alves, S., Francisco, R., Azevedo, L., Dias Carvalho, P., . . . Preto, A. (2018). The Yeast Saccharomyces cerevisiae as a Model for Understanding RAS Proteins and their Role in Human Tumorigenesis. *Cells, 7*(2). doi: 10.3390/cells7020014

Centelles, J. J. (2012). General aspects of colorectal cancer. *ISRN Oncol, 2012*, 139268. doi: 10.5402/2012/139268

Chen, N., & Karantza-Wadsworth, V. (2009). Role and regulation of autophagy in cancer. *Biochim Biophys Acta, 1793*(9), 1516-1523. doi: 10.1016/j.bbamcr.2008.12.013

Chichili, G. R., & Rodgers, W. (2009). Cytoskeleton-membrane interactions in membrane raft structure. *Cell Mol Life Sci, 66*(14), 2319-2328. doi: 10.1007/s00018-009-0022-6

Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., . . . Philips, M. R. (2002). Ras signalling on the endoplasmic reticulum and the Golgi. *Nat Cell Biol, 4*(5), 343-350. doi: 10.1038/ncb783

Choi, K. S. (2012). Autophagy and cancer. *Exp Mol Med, 44*(2), 109-120. doi: 10.3858/emm.2012.44.2.033

Choy, E., Chiu, V. K., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., . . . Philips, M. R. (1999). Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. *Cell, 98*(1), 69-80. doi: 10.1016/S0092-8674(00)80607-8

Comeau, J. W., Costantino, S., & Wiseman, P. W. (2006). A guide to accurate fluorescence microscopy colocalization measurements. *Biophys J, 91*(12), 4611-4622. doi: 10.1529/biophysj.106.089441

Cosentino-Gomes, D., Rocco-Machado, N., & Meyer-Fernandes, J. R. (2012). Cell signaling through protein kinase C oxidation and activation. *Int J Mol Sci, 13*(9), 10697-10721. doi: 10.3390/ijms130910697

Coutts, A. S., & La Thangue, N. B. (2016). Regulation of actin nucleation and autophagosome formation. *Cell Mol Life Sci, 73*(17), 3249-3263. doi: 10.1007/s00018-016-2224-z

Cox, A. D., Fesik, S. W., Kimmelman, A. C., Luo, J., & Der, C. J. (2014). Drugging the undruggable RAS: Mission possible? *Nat Rev Drug Discov, 13*(11), 828-851. doi: 10.1038/nrd4389

Das, G., Shravage, B. V., & Baehrecke, E. H. (2012). Regulation and function of autophagy during cell survival and cell death. *Cold Spring Harb Perspect Biol, 4*(6). doi: 10.1101/cshperspect.a008813

De Roock, W., Jonker, D. J., Di Nicolantonio, F., Sartore-Bianchi, A., Tu, D., Siena, S., . . . Tejpar, S. (2010). Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA, 304*(16), 1812-1820. doi: 10.1001/jama.2010.1535

De Stefano, A., & Carlomagno, C. (2014). Beyond KRAS: Predictive factors of the efficacy of anti-EGFR monoclonal antibodies in the treatment of metastatic colorectal cancer. *World J Gastroenterol, 20*(29), 9732-9743. doi: 10.3748/wjg.v20.i29.9732

Debaize, L., Jakobczyk, H., Rio, A. G., Gandemer, V., & Troadec, M. B. (2017). Optimization of proximity ligation assay (PLA) for detection of protein interactions and fusion proteins in non-adherent cells: application to pre-B lymphocytes. *Mol Cytogenet, 10*, 27. doi: 10.1186/s13039-017-0328-2

Degenhardt, K., Mathew, R., Beaudoin, B., Bray, K., Anderson, D., Chen, G., . . . White, E. (2006). Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell, 10*(1), 51-64. doi: 10.1016/j.ccr.2006.06.001

Desouza, M., Gunning, P. W., & Stehn, J. R. (2012). The actin cytoskeleton as a sensor and mediator of apoptosis. *Bioarchitecture, 2*(3), 75-87. doi: 10.4161/bioa.20975

Di Nicolantonio, F., Martini, M., Molinari, F., Sartore-Bianchi, A., Arena, S., Saletti, P., . . . Bardelli, A. (2008). Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol, 26*(35), 5705-5712. doi: 10.1200/JCO.2008.18.0786

Dinu, D., Dobre, M., Panaitescu, E., Birla, R., Iosif, C., Hoara, P., . . . Ardeleanu, C. (2014). Prognostic significance of KRAS gene mutations in colorectal cancer–preliminary study. *J Med Life, 7*(4), 581-587.

Dumic, J., Dabelic, S., & Flogel, M. (2006). Galectin-3: an open-ended story. *Biochim Biophys Acta, 1760*(4), 616-635. doi: 10.1016/j.bbagen.2005.12.020

Elad-Sfadia, G., Haklai, R., Balan, E., & Kloog, Y. (2004). Galectin-3 augments K-Ras activation and triggers a Ras signal that attenuates ERK but not phosphoinositide 3-kinase activity. *J Biol Chem, 279*(33), 34922-34930. doi: 10.1074/jbc.M312697200

Elkin, S. R., Lakoduk, A. M., & Schmid, S. L. (2016). Endocytic pathways and endosomal trafficking: a primer. *Wien Med Wochenschr, 166*(7-8), 196-204. doi: 10.1007/s10354-016-0432-7

Ellis, C. N. (2005). Inherited Colorectal Cancer Syndromes. *Clinics in Colon and Rectal Surgery, 18*(3), 150-162. doi: 10.1055/s-2005-916276

Fearon, E. R., & Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell, 61*(5), 759-767.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., . . . Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer, 136*(5), E359-386. doi: 10.1002/ijc.29210

Fernandez-Medarde, A., & Santos, E. (2011). Ras in cancer and developmental diseases. *Genes Cancer, 2*(3), 344-358. doi: 10.1177/1947601911411084

Ferreira, J. C. C. (2014). Envolvimento da proteína HMGB1 na morte induzida pela Benzofenoxazina MSG-111-cd3.

Ferreira, J. C. C. (2017). Characterization of vacuole permeabilization and HMGB1 nuclear release in the yeast cell death induced by a benzo[a]phenoxazine derivative.

Fey, D., Matallanas, D., Rauch, J., Rukhlenko, O. S., & Kholodenko, B. N. (2016). The complexities and versatility of the RAS-to-ERK signalling system in normal and cancer cells. *Semin Cell Dev Biol, 58*, 96-107. doi: 10.1016/j.semcdb.2016.06.011

Filchtinski, D., Sharabi, O., Ruppel, A., Vetter, I. R., Herrmann, C., & Shifman, J. M. (2010). What makes Ras an efficient molecular switch: a computational, biophysical, and structural study of Ras-GDP interactions with mutants of Raf. *J Mol Biol, 399*(3), 422-435. doi: 10.1016/j.jmb.2010.03.046

Flis, V. V., & Daum, G. (2013). Lipid transport between the endoplasmic reticulum and mitochondria. *Cold Spring Harb Perspect Biol, 5*(6). doi: 10.1101/cshperspect.a013235

Frade, V., Sousa, M.J., Moura, J., and Gonçalves, M.S. (2007). Synthesis, characterization and antimicrobial activity of new benzo[a]phenoxazine based fluorophores. *Tetrahedron Letters, 8347-8352*.

Frade, V. H. J., Sousa, M. J., Moura, J. C. V. P., and Gonçalves, M. S. T. (2008). Synthesis of naphtho[2,3-a]phenoxazinium chlorides: Structure-activity relationships of these heterocycles and benzo[a]phenoxazinium chlorides as new antimicrobials. *Bioorganic Med. Chem, 16*, 3274–3282.

Fredericks, E. (2015). *Fredericks E, Dealtry G, Roux S (2015) Molecular aspects of Colorectal Carcinogenesis: A Review. J Cancer Biol Res 3(1): 1057* (Vol. 3(1)).

Fukumori, T., Oka, N., Takenaka, Y., Nangia-Makker, P., Elsamman, E., Kasai, T., . . . Raz, A. (2006). Galectin-3 regulates mitochondrial stability and antiapoptotic function in response to anticancer drug in prostate cancer. *Cancer Res, 66*(6), 3114-3119. doi: 10.1158/0008-5472.CAN-05-3750

Gajate, P., Sastre, J., Bando, I., Alonso, T., Cillero, L., Sanz, J., . . . Diaz-Rubio, E. (2012). Influence of KRAS p.G13D mutation in patients with metastatic colorectal cancer treated with cetuximab. *Clin Colorectal Cancer, 11*(4), 291-296. doi: 10.1016/j.clcc.2012.02.003

Galluzzi, L., Pietrocola, F., Bravo-San Pedro, J. M., Amaravadi, R. K., Baehrecke, E. H., Cecconi, F., . . . Kroemer, G. (2015). Autophagy in malignant transformation and cancer progression. *EMBO J, 34*(7), 856-880. doi: 10.15252/embj.201490784

Glick, D., Barth, S., & Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *J Pathol, 221*(1), 3-12. doi: 10.1002/path.2697

Gomes, L. C., Di Benedetto, G., & Scorrano, L. (2011). During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. *Nat Cell Biol, 13*(5), 589-598. doi: 10.1038/ncb2220

Gomez-Llobregat, J., Buceta, J., & Reigada, R. (2013). Interplay of cytoskeletal activity and lipid phase stability in dynamic protein recruitment and clustering. *Sci Rep, 3*, 2608. doi: 10.1038/srep02608

Grande Pulido, E. (2011). Molecular biology of colorectal cancer. In E. Cidón (Ed.), *The Challenge of Colorectal Cancer: A Review Book* (pp. 31-51). Kerala: Research Signpost.

Greenfield, L. K., & Jones, N. L. (2013). Modulation of autophagy by Helicobacter pylori and its role in gastric carcinogenesis. *Trends Microbiol, 21*(11), 602-612. doi: 10.1016/j.tim.2013.09.004

Guo, J. Y., Chen, H. Y., Mathew, R., Fan, J., Strohecker, A. M., Karsli-Uzunbas, G., . . . White, E. (2011). Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev, 25*(5), 460-470. doi: 10.1101/gad.2016311

Guo, J. Y., Karsli-Uzunbas, G., Mathew, R., Aisner, S. C., Kamphorst, J. J., Strohecker, A. M., . . . White, E. (2013). Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. *Genes Dev, 27*(13), 1447-1461. doi: 10.1101/gad.219642.113

Guo, Y., Bao, Y., & Yang, W. (2017). Regulatory miRNAs in Colorectal Carcinogenesis and Metastasis. *Int J Mol Sci, 18*(4). doi: 10.3390/ijms18040890

Gysin, S., Salt, M., Young, A., & McCormick, F. (2011). Therapeutic strategies for targeting ras proteins. *Genes Cancer, 2*(3), 359-372. doi: 10.1177/1947601911412376

Hailey, D. W., Rambold, A. S., Satpute-Krishnan, P., Mitra, K., Sougrat, R., Kim, P. K., & Lippincott-Schwartz, J. (2010). Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell, 141*(4), 656-667. doi: 10.1016/j.cell.2010.04.009

Hale, A. N., Ledbetter, D. J., Gawriluk, T. R., & Rucker, E. B., 3rd. (2013). Autophagy: regulation and role in development. *Autophagy, 9*(7), 951-972. doi: 10.4161/auto.24273

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi: 10.1016/j.cell.2011.02.013

Hancock, J. F. (2003). Ras proteins: different signals from different locations. *Nat Rev Mol Cell Biol, 4*(5), 373-384. doi: 10.1038/nrm1105

Hancock, J. F., & Parton, R. G. (2005). Ras plasma membrane signalling platforms. *Biochem J, 389*(Pt 1), 1-11. doi: 10.1042/BJ20050231

He, C., & Klionsky, D. J. (2009). Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet, 43*, 67-93. doi: 10.1146/annurev-genet-102808-114910

Heinemann, V., Douillard, J. Y., Ducreux, M., & Peeters, M. Targeted therapy in metastatic colorectal cancer – An example of personalised medicine in action. *Cancer Treatment Reviews, 39*(6), 592-601. doi: 10.1016/j.ctrv.2012.12.011

Heinemann, V., von Weikersthal, L. F., Decker, T., Kiani, A., Vehling-Kaiser, U., Al-Batran, S.-E., . . . Stintzing, S. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. *The Lancet Oncology, 15*(10), 1065-1075. doi: 10.1016/S1470-2045(14)70330-4

Heo, W. D., Inoue, T., Park, W. S., Kim, M. L., Park, B. O., Wandless, T. J., & Meyer, T. (2006). PI(3,4,5)P3 and PI(4,5)P2 lipids target proteins with polybasic clusters to the plasma membrane. *Science, 314*(5804), 1458-1461. doi: 10.1126/science.1134389

Hernandez-Valladares, M., & Prior, I. A. (2015). Comparative proteomic analysis of compartmentalised Ras signalling. *Sci Rep, 5*, 17307. doi: 10.1038/srep17307

Hirabayashi, J., & Kasai, K. (1993). The family of metazoan metal-independent beta-galactoside-binding lectins: structure, function and molecular evolution. *Glycobiology, 3*(4), 297-304.

Hisamuddin, I. M., & Yang, V. W. (2006). Molecular Genetics of Colorectal Cancer: An Overview. *Curr Colorectal Cancer Rep, 2*(2), 53-59.

Hobbs, G. A., Der, C. J., & Rossman, K. L. (2016). RAS isoforms and mutations in cancer at a glance. *J Cell Sci, 129*(7), 1287-1292. doi: 10.1242/jcs.182873

Hohla, F., Winder, T., Greil, R., Rick, F. G., Block, N. L., & Schally, A. V. (2014). Targeted therapy in advanced metastatic colorectal cancer: current concepts and perspectives. *World J Gastroenterol, 20*(20), 6102-6112. doi: 10.3748/wjg.v20.i20.6102

Hunter, J. C., Manandhar, A., Carrasco, M. A., Gurbani, D., Gondi, S., & Westover, K. D. (2015). Biochemical and Structural Analysis of Common Cancer-Associated KRAS Mutations. *Mol Cancer Res, 13*(9), 1325-1335. doi: 10.1158/1541-7786.MCR-15-0203

Imai, K., & Yamamoto, H. (2008). Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. *Carcinogenesis, 29*(4), 673-680. doi: 10.1093/carcin/bgm228

Jancik, S., Drabek, J., Radzioch, D., & Hajduch, M. (2010). Clinical relevance of KRAS in human cancers. *J Biomed Biotechnol, 2010*, 150960. doi: 10.1155/2010/150960

Janku, F., McConkey, D. J., Hong, D. S., & Kurzrock, R. (2011). Autophagy as a target for anticancer therapy. *Nat Rev Clin Oncol, 8*(9), 528-539. doi: 10.1038/nrclinonc.2011.71

Jasperson, K. W., Tuohy, T. M., Neklason, D. W., & Burt, R. W. (2010). Hereditary and familial colon cancer. *Gastroenterology, 138*(6), 2044-2058. doi: 10.1053/j.gastro.2010.01.054

Jia, S., Zhang, R., Li, Z., & Li, J. (2017). Clinical and biological significance of circulating tumor cells, circulating tumor DNA, and exosomes as biomarkers in colorectal cancer. *Oncotarget*. doi: 10.18632/oncotarget.17184

Jiang, X., & Sorkin, A. (2002). Coordinated traffic of Grb2 and Ras during epidermal growth factor receptor endocytosis visualized in living cells. *Mol Biol Cell, 13*(5), 1522-1535. doi: 10.1091/mbc.01-11-0552

Johnson, D. S., & Chen, Y. H. (2012). Ras family of small GTPases in immunity and inflammation. *Curr Opin Pharmacol, 12*(4), 458-463. doi: 10.1016/j.coph.2012.02.003

Joneson, T., White, M. A., Wigler, M. H., & Bar-Sagi, D. (1996). Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. *Science, 271*(5250), 810-812.

Jose, J., and Burgess, K. (2006). Syntheses and properties of water-soluble Nile Red derivatives. *J. Org. Chem, 71*.

Juhasz, G., & Neufeld, T. P. (2006). Autophagy: a forty-year search for a missing membrane source. *PLoS Biol, 4*(2), e36. doi: 10.1371/journal.pbio.0040036

Kang, R., Zeh, H. J., Lotze, M. T., & Tang, D. (2011). The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ, 18*(4), 571-580. doi: 10.1038/cdd.2010.191

Kast, D. J., Zajac, A. L., Holzbaur, E. L., Ostap, E. M., & Dominguez, R. (2015). WHAMM Directs the Arp2/3 Complex to the ER for Autophagosome Biogenesis through an Actin Comet Tail Mechanism. *Curr Biol, 25*(13), 1791-1797. doi: 10.1016/j.cub.2015.05.042

Kaur, J., & Debnath, J. (2015). Autophagy at the crossroads of catabolism and anabolism. *Nat Rev Mol Cell Biol, 16*(8), 461-472. doi: 10.1038/nrm4024

Kenworthy, A. K. (2007). Nanoclusters digitize Ras signalling. *Nat Cell Biol, 9*(8), 875-877. doi: 10.1038/ncb0807-875

Kim, D., & Rossi, J. (2008). RNAi mechanisms and applications. *Biotechniques, 44*(5), 613-616. doi: 10.2144/000112792

Kim, H. S., Heo, J. S., Lee, J., Lee, J. Y., Lee, M. Y., Lim, S. H., . . . Park, Y. S. (2016). The impact of KRAS mutations on prognosis in surgically resected colorectal cancer patients with liver and lung metastases: a retrospective analysis. *BMC Cancer, 16*, 120. doi: 10.1186/s12885-016-2141-4

Ko, A., Kanehisa, A., Martins, I., Senovilla, L., Chargari, C., Dugue, D., . . . Deutsch, E. (2014). Autophagy inhibition radiosensitizes in vitro, yet reduces radioresponses in vivo due to deficient immunogenic signalling. *Cell Death Differ, 21*(1), 92-99. doi: 10.1038/cdd.2013.124

Korphaisarn, K., & Kopetz, S. (2016). BRAF-Directed Therapy in Metastatic Colorectal Cancer. *Cancer J, 22*(3), 175-178. doi: 10.1097/PPO.0000000000000189

Kroemer, G., Marino, G., & Levine, B. (2010). Autophagy and the integrated stress response. *Mol Cell, 40*(2), 280-293. doi: 10.1016/j.molcel.2010.09.023

Kruppa, A. J., Kendrick-Jones, J., & Buss, F. (2016). Myosins, Actin and Autophagy. *Traffic, 17*(8), 878-890. doi: 10.1111/tra.12410

Kucukkilinc, T., and Ozer, I. . (2007). ulti-site inhibition of human plasma cholinesterase by cationic phenoxazine and phenothiazine dyes. *Archives of biochemistry and biophysics, 461*, 294-298.

Laing, J. G., & Wang, J. L. (1988). Identification of carbohydrate binding protein 35 in heterogeneous nuclear ribonucleoprotein complex. *Biochemistry, 27*(14), 5329-5334.

Langlois, M.-J., Bergeron, S., Bernatchez, G., Boudreau, F., Saucier, C., Perreault, N., . . . Rivard, N. (2010). The PTEN Phosphatase Controls Intestinal Epithelial Cell Polarity and Barrier Function: Role in Colorectal Cancer Progression. *PLOS ONE, 5*(12), e15742. doi: 10.1371/journal.pone.0015742

Lee, J. Y., Koga, H., Kawaguchi, Y., Tang, W., Wong, E., Gao, Y. S., . . . Yao, T. P. (2010). HDAC6 controls autophagosome maturation essential for ubiquitin-selective quality-control autophagy. *EMBO J, 29*(5), 969-980. doi: 10.1038/emboj.2009.405

Lee, Y. K., & Lee, J. A. (2016). Role of the mammalian ATG8/LC3 family in autophagy: differential and compensatory roles in the spatiotemporal regulation of autophagy. *BMB Rep, 49*(8), 424-430.

Leslie, A., Carey, F. A., Pratt, N. R., & Steele, R. J. (2002). The colorectal adenoma-carcinoma sequence. *Br J Surg, 89*(7), 845-860. doi: 10.1046/j.1365-2168.2002.02120.x

Levy, J. M., Thompson, J. C., Griesinger, A. M., Amani, V., Donson, A. M., Birks, D. K., . . . Thorburn, A. (2014). Autophagy inhibition improves chemosensitivity in BRAF(V600E) brain tumors. *Cancer Discov, 4*(7), 773-780. doi: 10.1158/2159-8290.CD-14-0049

Levy, J. M. M., Towers, C. G., & Thorburn, A. (2017). Targeting autophagy in cancer. *Nat Rev Cancer, 17*(9), 528-542. doi: 10.1038/nrc.2017.53

Levy, R., Biran, A., Poirier, F., Raz, A., & Kloog, Y. (2011). Galectin-3 mediates cross-talk between K-Ras and Let-7c tumor suppressor microRNA. *PLOS ONE, 6*(11), e27490. doi: 10.1371/journal.pone.0027490

Levy, R., Grafi-Cohen, M., Kraiem, Z., & Kloog, Y. (2010). Galectin-3 promotes chronic activation of K-Ras and differentiation block in malignant thyroid carcinomas. *Mol Cancer Ther, 9*(8), 2208-2219. doi: 10.1158/1535-7163.MCT-10-0262

Lewis, M. R. G., P. P.; Sloviter, H. A. (1949). The action of oxazine dyes on tumors in mice. *Cancer Res., 9*, 736-740.

Liu, X., Jakubowski, M., & Hunt, J. L. (2011). KRAS gene mutation in colorectal cancer is correlated with increased proliferation and spontaneous apoptosis. *Am J Clin Pathol, 135*(2), 245-252. doi: 10.1309/AJCP7FO2VAXIVSTP

Lopes, C. (2015). Molecular pathways involved in the yeast cell death induced by a benzo[a]phenoxazine derivative.

Loree, J. M., Pereira, A. A. L., Lam, M., Willauer, A. N., Raghav, K., Dasari, A., . . . Kopetz, S. (2017). Classifying Colorectal Cancer by Tumor Location Rather than Sidedness Highlights a Continuum in Mutation Profiles and Consensus Molecular Subtypes. *Clin Cancer Res*. doi: 10.1158/1078-0432.CCR-17-2484

Lu, A., Tebar, F., Alvarez-Moya, B., Lopez-Alcala, C., Calvo, M., Enrich, C., . . . Bachs, O. (2009). A clathrin-dependent pathway leads to KRas signaling on late endosomes en route to lysosomes. *J Cell Biol, 184*(6), 863-879. doi: 10.1083/jcb.200807186

Mah, L. Y., & Ryan, K. M. (2012). Autophagy and cancer. *Cold Spring Harb Perspect Biol, 4*(1), a008821. doi: 10.1101/cshperspect.a008821

Mahankali, M., Peng, H. J., Cox, D., & Gomez-Cambronero, J. (2011). The mechanism of cell membrane ruffling relies on a phospholipase D2 (PLD2), Grb2 and Rac2 association. *Cell Signal, 23*(8), 1291-1298. doi: 10.1016/j.cellsig.2011.03.010

Maiuri, M. C., Tasdemir, E., Criollo, A., Morselli, E., Vicencio, J. M., Carnuccio, R., & Kroemer, G. (2009). Control of autophagy by oncogenes and tumor suppressor genes. *Cell Death Differ, 16*(1), 87-93. doi: 10.1038/cdd.2008.131

Marisa, L., de Reynies, A., Duval, A., Selves, J., Gaub, M. P., Vescovo, L., . . . Boige, V. (2013). Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. *PLoS Med, 10*(5), e1001453. doi: 10.1371/journal.pmed.1001453

Markman, B., Javier Ramos, F., Capdevila, J., & Tabernero, J. (2010). EGFR and KRAS in colorectal cancer. *Adv Clin Chem, 51*, 71-119.

Marques, C., Oliveira, C. S., Alves, S., Chaves, S. R., Coutinho, O. P., Corte-Real, M., & Preto, A. (2013). Acetate-induced apoptosis in colorectal carcinoma cells involves lysosomal membrane permeabilization and cathepsin D release. *Cell Death Dis, 4*, e507. doi: 10.1038/cddis.2013.29

Martelli, A. M., Tazzari, P. L., Evangelisti, C., Chiarini, F., Blalock, W. L., Billi, A. M., . . . Cocco, L. (2007). Targeting the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin module for acute myelogenous leukemia therapy: from bench to bedside. *Curr Med Chem, 14*(19), 2009-2023.

Matallanas, D., Arozarena, I., Berciano, M. T., Aaronson, D. S., Pellicer, A., Lafarga, M., & Crespo, P. (2003). Differences on the inhibitory specificities of H-Ras, K-Ras, and N-Ras (N17) dominant negative mutants are related to their membrane microlocalization. *J Biol Chem, 278*(7), 4572-4581. doi: 10.1074/jbc.M209807200

Matallanas, D., Romano, D., Al-Mulla, F., O'Neill, E., Al-Ali, W., Crespo, P., . . . Kolch, W. (2011). Mutant K-Ras activation of the proapoptotic MST2 pathway is antagonized by wild-type K-Ras. *Mol Cell, 44*(6), 893-906. doi: 10.1016/j.molcel.2011.10.016

Mathew, R., Karantza-Wadsworth, V., & White, E. (2007). Role of autophagy in cancer. *Nat Rev Cancer, 7*(12), 961-967. doi: 10.1038/nrc2254

Meijer, A. J., & Codogno, P. (2009). Autophagy: regulation and role in disease. *Crit Rev Clin Lab Sci, 46*(4), 210-240. doi: 10.1080/10408360903044068

Metzger, B., Chambeau, L., Begon, D. Y., Faber, C., Kayser, J., Berchem, G., . . . Wenner, T. (2011). The human epidermal growth factor receptor (EGFR) gene in European patients with advanced colorectal cancer harbors infrequent mutations in its tyrosine kinase domain. *BMC Med Genet, 12*, 144. doi: 10.1186/1471-2350-12-144

Mizushima, N. (2007). Autophagy: process and function. *Genes Dev, 21*(22), 2861-2873. doi: 10.1101/gad.1599207

Mizushima, N., & Klionsky, D. J. (2007). Protein turnover via autophagy: implications for metabolism. *Annu Rev Nutr, 27*, 19-40. doi: 10.1146/annurev.nutr.27.061406.093749

Mokarram, P., Albokashy, M., Zarghooni, M., Moosavi, M. A., Sepehri, Z., Chen, Q. M., . . . Ghavami, S. (2017). New frontiers in the treatment of colorectal cancer: Autophagy and the unfolded protein response as promising targets. *Autophagy, 13*(5), 781-819. doi: 10.1080/15548627.2017.1290751

Morselli, E., Galluzzi, L., Kepp, O., Vicencio, J. M., Criollo, A., Maiuri, M. C., & Kroemer, G. (2009). Anti- and pro-tumor functions of autophagy. *Biochim Biophys Acta, 1793*(9), 1524-1532. doi: 10.1016/j.bbamcr.2009.01.006

Munson, M. J., & Ganley, I. G. (2015). MTOR, PIK3C3, and autophagy: Signaling the beginning from the end. *Autophagy, 11*(12), 2375-2376. doi: 10.1080/15548627.2015.1106668

Nagahara, H., Mimori, K., Ohta, M., Utsunomiya, T., Inoue, H., Barnard, G. F., . . . Mori, M. (2005). Somatic mutations of epidermal growth factor receptor in colorectal carcinoma. *Clin Cancer Res, 11*(4), 1368-1371. doi: 10.1158/1078-0432.CCR-04-1894

Nazemalhosseini Mojarad, E., Kuppen, P. J., Aghdaei, H. A., & Zali, M. R. (2013). The CpG island methylator phenotype (CIMP) in colorectal cancer. *Gastroenterol Hepatol Bed Bench, 6*(3), 120-128.

Niv, H., Gutman, O., Kloog, Y., & Henis, Y. I. (2002). Activated K-Ras and H-Ras display different interactions with saturable nonraft sites at the surface of live cells. *J Cell Biol, 157*(5), 865-872. doi: 10.1083/jcb.200202009

Oh, B. Y., Lee, R. A., Chung, S. S., & Kim, K. H. (2011). Epidermal growth factor receptor mutations in colorectal cancer patients. *J Korean Soc Coloproctol, 27*(3), 127-132. doi: 10.3393/jksc.2011.27.3.127

Overmeyer, J. H., & Maltese, W. A. (2011). Death pathways triggered by activated Ras in cancer cells. *Front Biosci (Landmark Ed), 16*, 1693-1713.

Pawlak, G., & Helfman, D. M. (2001). Cytoskeletal changes in cell transformation and tumorigenesis. *Curr Opin Genet Dev, 11*(1), 41-47.

Phipps, A. I., Buchanan, D. D., Makar, K. W., Win, A. K., Baron, J. A., Lindor, N. M., . . . Newcomb, P. A. (2013). KRAS-mutation status in relation to colorectal cancer survival: the joint impact

of correlated tumour markers. *Br J Cancer, 108*(8), 1757-1764. doi: 10.1038/bjc.2013.118

Pollock, C. B., Shirasawa, S., Sasazuki, T., Kolch, W., & Dhillon, A. S. (2005). Oncogenic K-RAS is required to maintain changes in cytoskeletal organization, adhesion, and motility in colon cancer cells. *Cancer Res, 65*(4), 1244-1250. doi: 10.1158/0008-5472.CAN-04-1911

Porta, C., Paglino, C., & Mosca, A. (2014). Targeting PI3K/Akt/mTOR Signaling in Cancer. *Front Oncol, 4*, 64. doi: 10.3389/fonc.2014.00064

Priault, M., Hue, E., Marhuenda, F., Pilet, P., Oliver, L., & Vallette, F. M. (2010). Differential dependence on Beclin 1 for the regulation of pro-survival autophagy by Bcl-2 and Bcl-xL in HCT116 colorectal cancer cells. *PLOS ONE, 5*(1), e8755. doi: 10.1371/journal.pone.0008755

Prior, I. A., & Hancock, J. F. (2012). Ras trafficking, localization and compartmentalized signalling. *Semin Cell Dev Biol, 23*(2), 145-153. doi: 10.1016/j.semcdb.2011.09.002

Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Troxel, A., . . . Levine, B. (2003). Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest, 112*(12), 1809-1820. doi: 10.1172/JCI20039

Rabinovich, G. A. (1999). Galectins: an evolutionarily conserved family of animal lectins with multifunctional properties; a trip from the gene to clinical therapy. *Cell Death Differ, 6*(8), 711-721. doi: 10.1038/sj.cdd.4400535

Rajalingam, K., Schreck, R., Rapp, U. R., & Albert, S. (2007). Ras oncogenes and their downstream targets. *Biochim Biophys Acta, 1773*(8), 1177-1195. doi: 10.1016/j.bbamcr.2007.01.012

Rajasekharan, S. K., & Raman, T. (2013). Ras and Ras mutations in cancer. *Central European Journal of Biology, 8*(7), 609-624. doi: 10.2478/s11535-013-0158-5

Recouvreux, M. V., & Commisso, C. (2017). Macropinocytosis: A Metabolic Adaptation to Nutrient Stress in Cancer. *Front Endocrinol (Lausanne), 8*, 261. doi: 10.3389/fendo.2017.00261

Reggiori, F., Shintani, T., Nair, U., & Klionsky, D. J. (2005). Atg9 cycles between mitochondria and the pre-autophagosomal structure in yeasts. *Autophagy, 1*(2), 101-109.

Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., . . . Downward, J. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell, 89*(3), 457-467.

Roper, J., & Hung, K. E. (2013). Molecular Mechanisms of Colorectal Carcinogenesis. In P. D. K. M. Haigis (Ed.), *Molecular Pathogenesis of Colorectal Cancer* (pp. 25-65). New York, NY: Springer New York.

Roy, S., Plowman, S., Rotblat, B., Prior, I. A., Muncke, C., Grainger, S., . . . Hancock, J. F. (2005). Individual palmitoyl residues serve distinct roles in H-ras trafficking, microlocalization, and signaling. *Mol Cell Biol, 25*(15), 6722-6733. doi: 10.1128/MCB.25.15.6722-6733.2005

Roy, S., Wyse, B., & Hancock, J. F. (2002). H-Ras signaling and K-Ras signaling are differentially dependent on endocytosis. *Mol Cell Biol, 22*(14), 5128-5140.

Rustgi, A. K. (2007). The genetics of hereditary colon cancer. *Genes Dev, 21*(20), 2525-2538. doi: 10.1101/gad.1593107

Sakitani, K., Hirata, Y., Hikiba, Y., Hayakawa, Y., Ihara, S., Suzuki, H., . . . Koike, K. (2015). Inhibition of autophagy exerts anti-colon cancer effects via apoptosis induced by p53 activation and ER stress. *BMC Cancer, 15*, 795. doi: 10.1186/s12885-015-1789-5

Sameer, A. S. (2013). Colorectal cancer: molecular mutations and polymorphisms. *Front Oncol, 3*, 114. doi: 10.3389/fonc.2013.00114

Schmick, M., Vartak, N., Papke, B., Kovacevic, M., Truxius, D. C., Rossmannek, L., & Bastiaens, P. I. H. (2014). KRas localizes to the plasma membrane by spatial cycles of solubilization, trapping and vesicular transport. *Cell, 157*(2), 459-471. doi: 10.1016/j.cell.2014.02.051

Schmidt, C. (2007). Book Review of "The Molecular Biology of Cancer" by Stella Pelengaris, Michael Khan (Editors). *Molecular Cancer, 6*, 72-72. doi: 10.1186/1476-4598-6-72

Schmukler, E., Kloog, Y., & Pinkas-Kramarski, R. (2014). Ras and autophagy in cancer development and therapy. *Oncotarget, 5*(3), 577-586. doi: 10.18632/oncotarget.1775

Scott, S. V., Hefner-Gravink, A., Morano, K. A., Noda, T., Ohsumi, Y., & Klionsky, D. J. (1996). Cytoplasm-to-vacuole targeting and autophagy employ the same machinery to deliver proteins to the yeast vacuole. *Proc Natl Acad Sci U S A, 93*(22), 12304-12308.

Sekar, R. B., & Periasamy, A. (2003). Fluorescence resonance energy transfer (FRET) microscopy imaging of live cell protein localizations. *J Cell Biol, 160*(5), 629-633. doi: 10.1083/jcb.200210140

Shalom-Feuerstein, R., Plowman, S. J., Rotblat, B., Ariotti, N., Tian, T., Hancock, J. F., & Kloog, Y. (2008). K-ras nanoclustering is subverted by overexpression of the scaffold protein galectin-3. *Cancer Res, 68*(16), 6608-6616. doi: 10.1158/0008-5472.CAN-08-1117

Sorkin, A., & von Zastrow, M. (2009). Endocytosis and signalling: intertwining molecular networks. *Nat Rev Mol Cell Biol, 10*(9), 609-622. doi: 10.1038/nrm2748

Souglakos, J. (2007). Genetic alterations in sporadic and hereditary colorectal cancer: implementations for screening and follow-up. *Dig Dis, 25*(1), 9-19. doi: 10.1159/000099166

Stigliano, V., Sanchez-Mete, L., Martayan, A., & Anti, M. (2014). Early-onset colorectal cancer: a sporadic or inherited disease? *World J Gastroenterol, 20*(35), 12420-12430. doi: 10.3748/wjg.v20.i35.12420

Sui, G., & Shi, Y. (2005). Gene silencing by a DNA vector-based RNAi technology. *Methods Mol Biol, 309*, 205-218. doi: 10.1385/1-59259-935-4:205

Sung, P. J., Tsai, F. D., Vais, H., Court, H., Yang, J., Fehrenbacher, N., . . . Philips, M. R. (2013). Phosphorylated K-Ras limits cell survival by blocking Bcl-xL sensitization of inositol trisphosphate receptors. *Proc Natl Acad Sci U S A, 110*(51), 20593-20598. doi: 10.1073/pnas.1306431110

Suzuki, F., Hashimoto, K., Ishihara, M., Westman, G., Samuelsson, K., Kawase, M., Motohashi, N., and Sakagami, H. . (2007). Tumor-specificity and type of cell death induced by phenoxazines. *Anticancer Res., 27*, 4233–4238.

Szpon, L., Stal, A., Zawadzki, M., Lis-Nawara, A., Kielan, W., & Grzebieniak, Z. (2016). K-ras gene mutation as an early prognostic marker of colon cancer. *Pol Przegl Chir, 88*(1), 15-19. doi: 10.1515/pjs-2016-0021

Tan, C., & Du, X. (2012). KRAS mutation testing in metastatic colorectal cancer. *World J Gastroenterol, 18*(37), 5171-5180. doi: 10.3748/wjg.v18.i37.5171

Tariq, K., & Ghias, K. (2016). Colorectal cancer carcinogenesis: a review of mechanisms. *Cancer Biol Med, 13*(1), 120-135. doi: 10.28092/j.issn.2095-3941.2015.0103

Tian, T., Plowman, S. J., Parton, R. G., Kloog, Y., & Hancock, J. F. (2010). Mathematical modeling of K-Ras nanocluster formation on the plasma membrane. *Biophys J, 99*(2), 534-543. doi: 10.1016/j.bpj.2010.04.055

Tsuchida, N., Murugan, A. K., & Grieco, M. (2016). Kirsten Ras* oncogene: significance of its discovery in human cancer research. *Oncotarget, 7*(29), 46717-46733. doi: 10.18632/oncotarget.8773

Tsukada, M., & Ohsumi, Y. (1993). Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS Lett, 333*(1-2), 169-174.

Van Cutsem, E., Kohne, C. H., Lang, I., Folprecht, G., Nowacki, M. P., Cascinu, S., . . . Ciardiello, F. (2011). Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol, 29*(15), 2011-2019. doi: 10.1200/JCO.2010.33.5091

Vigil, D., Cherfils, J., Rossman, K. L., & Der, C. J. (2010). Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? *Nat Rev Cancer, 10*(12), 842-857. doi: 10.1038/nrc2960

Walther, A., Johnstone, E., Swanton, C., Midgley, R., Tomlinson, I., & Kerr, D. (2009). Genetic prognostic and predictive markers in colorectal cancer. *Nat Rev Cancer, 9*(7), 489-499. doi: 10.1038/nrc2645

Wennerberg, K., Rossman, K. L., & Der, C. J. (2005). The Ras superfamily at a glance. *J Cell Sci, 118*(Pt 5), 843-846. doi: 10.1242/jcs.01660

White, E., & DiPaola, R. S. (2009). The double-edged sword of autophagy modulation in cancer. *Clin Cancer Res, 15*(17), 5308-5316. doi: 10.1158/1078-0432.CCR-07-5023

Wong, A. S., Cheung, Z. H., & Ip, N. Y. (2011). Molecular machinery of macroautophagy and its deregulation in diseases. *Biochim Biophys Acta, 1812*(11), 1490-1497. doi: 10.1016/j.bbadis.2011.07.005

Worthley, D. L., & Leggett, B. A. (2010). Colorectal Cancer: Molecular Features and Clinical Opportunities. *The Clinical Biochemist Reviews, 31*(2), 31-38.

Xu, H., & Ren, D. (2015). Lysosomal physiology. *Annu Rev Physiol, 77*, 57-80. doi: 10.1146/annurev-physiol-021014-071649

Yamagishi, H., Kuroda, H., Imai, Y., & Hiraishi, H. (2016). Molecular pathogenesis of sporadic colorectal cancers. *Chinese Journal of Cancer, 35*, 4. doi: 10.1186/s40880-015-0066-y

Yang, S., Wang, X., Contino, G., Liesa, M., Sahin, E., Ying, H., . . . Kimmelman, A. C. (2011). Pancreatic cancers require autophagy for tumor growth. *Genes Dev, 25*(7), 717-729. doi: 10.1101/gad.2016111

Yang, Z. J., Chee, C. E., Huang, S., & Sinicrope, F. A. (2011). The role of autophagy in cancer: therapeutic implications. *Mol Cancer Ther, 10*(9), 1533-1541. doi: 10.1158/1535-7163.MCT-11-0047

Yoo, B. H., Wu, X., Li, Y., Haniff, M., Sasazuki, T., Shirasawa, S., . . . Rosen, K. V. (2010). Oncogenic ras-induced down-regulation of autophagy mediator Beclin-1 is required for malignant

transformation of intestinal epithelial cells. *J Biol Chem, 285*(8), 5438-5449. doi: 10.1074/jbc.M109.046789

Young, A. R., Chan, E. Y., Hu, X. W., Kochl, R., Crawshaw, S. G., High, S., . . . Tooze, S. A. (2006). Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. *J Cell Sci, 119*(Pt 18), 3888-3900. doi: 10.1242/jcs.03172

Yu, L., McPhee, C. K., Zheng, L., Mardones, G. A., Rong, Y., Peng, J., . . . Lenardo, M. J. (2010). Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature, 465*(7300), 942-946. doi: 10.1038/nature09076

Yue, Z., Jin, S., Yang, C., Levine, A. J., & Heintz, N. (2003). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A, 100*(25), 15077-15082. doi: 10.1073/pnas.2436255100

Zahiri, J., Bozorgmehr, J. H., & Masoudi-Nejad, A. (2013). Computational Prediction of Protein-Protein Interaction Networks: Algo-rithms and Resources. *Curr Genomics, 14*(6), 397-414. doi: 10.2174/1389202911314060004

Zeitouni, D., Pylayeva-Gupta, Y., Der, C. J., & Bryant, K. L. (2016). KRAS Mutant Pancreatic Cancer: No Lone Path to an Effective Treatment. *Cancers (Basel), 8*(4). doi: 10.3390/cancers8040045

Zekri, J. (2016). Frequency and Clinical Impact of KRAS Mutations in Patients with Colorectal Cancer from the Middle East. *J Anal Oncol, 5*, 67-74.

Zhai, H., Song, B., Xu, X., Zhu, W., & Ju, J. (2013). Inhibition of autophagy and tumor growth in colon cancer by miR-502. *Oncogene, 32*(12), 1570-1579. doi: 10.1038/onc.2012.167

Zhang, J., Zheng, J., Yang, Y., Lu, J., Gao, J., Lu, T., . . . Liu, T. (2015). Molecular spectrum of KRAS, NRAS, BRAF and PIK3CA mutations in Chinese colorectal cancer patients: analysis of 1,110 cases. *Sci Rep, 5*, 18678. doi: 10.1038/srep18678

Zhang, Y. J., Dai, Q., Sun, D. F., Xiong, H., Tian, X. Q., Gao, F. H., . . . Fang, J. Y. (2009). mTOR signaling pathway is a target for the treatment of colorectal cancer. *Ann Surg Oncol, 16*(9), 2617-2628. doi: 10.1245/s10434-009-0555-9

Zhao, B., Wang, L., Qiu, H., Zhang, M., Sun, L., Peng, P., . . . Yuan, X. (2017). Mechanisms of resistance to anti-EGFR therapy in colorectal cancer. *Oncotarget, 8*(3), 3980-4000. doi: 10.18632/oncotarget.14012

Zheng, Z. Y., Cheng, C. M., Fu, X. R., Chen, L. Y., Xu, L., Terrillon, S., . . . Chang, E. C. (2012). CHMP6 and VPS4A mediate the recycling of Ras to the plasma membrane to promote growth factor signaling. *Oncogene, 31*(43), 4630-4638. doi: 10.1038/onc.2011.607

Zientara-Rytter, K., & Subramani, S. (2016). Role of actin in shaping autophagosomes. *Autophagy, 12*(12), 2512-2515. doi: 10.1080/15548627.2016.1236877

Zinchuk, V., & Zinchuk, O. (2008). Quantitative colocalization analysis of confocal fluorescence microscopy images. *Curr Protoc Cell Biol, Chapter 4*, Unit 4 19. doi: 10.1002/0471143030.cb0419s39

Zinchuk, V., Zinchuk, O., & Okada, T. (2007). Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena. *Acta Histochem Cytochem, 40*(4), 101-111. doi: 10.1267/ahc.07002