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MESTRADO EM ONCOLOGIA

ESPECIALIZAÇÃO EM ONCOLOGIA LABORATORIAL

Deciphering the functional role of KRAS-induced SUSD2 downregulation in colorectal cancer

Diogo Rafael Coelho

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Dissertação de Candidatura ao grau de **Mestre em Oncologia** – **Especialização em Oncologia Laboratorial** submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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“The woods are lovely, dark, and deep, but I have promises to keep, and miles to go before I sleep, and miles to go before I sleep.”

Robert Frost's

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Resumo

INTRODUÇÃO: O Cancro colorretal é um dos cancros mais incidentes e mortais em todo o mundo. O envelhecimento, fator de risco não modificável, aumenta o risco de desenvolvimento desta neoplasia. Para além disso, muitos outros fatores, como a obesidade, história familiar e atividade física demonstram influenciar o risco de cancro colorretal. Molecularmente, esta neoplasia encontra-se associada a três vias moleculares: a via da instabilidade cromossómica, a via da instabilidade microssatélite e a via da polipose serreada. O KRAS é um importante biomarcador nesta doença. Pacientes com mutações KRAS, não beneficiam de uma abordagem terapêutica anti-EGFR. Para além disso, o KRAS, frequentemente mutado em cancro colorretal, é um regulador de vários processos oncogénicos, como a proliferação celular, sobrevivência e regulação do microambiente tumoral, promovendo o início e progressão carcinogénica neste tipo de cancro. Estudos prévios do grupo sugerem que o SUSD2 é uma proteína regulada pelo KRAS mutante. Em cancro colorretal, o SUSD2 demonstra possuir um papel supressor tumoral, regulando a proliferação, no entanto, o seu papel nos restantes processos tumorais continua por ser esclarecido. Assim, o nosso objetivo foi avaliar o papel do aumento de expressão do SUSD2 como mediador dos efeitos supressores do silenciamento do *KRAS*.

MATERIAL E MÉTODOS: Inicialmente, para validar o aumento de expressão do SUSD2 após silenciamento do *KRAS*, realizou-se qRT-PCR e citometria de fluxo, recorrendo-se das linhas celulares HCT-15, HCT-116, SW480, SW620 e LS174T. Posteriormente, para avaliar o papel do SUSD2 em CRC, avaliou-se a sua capacidade de regular vários marcadores relacionados com diferentes processos oncogénicos. Para além disso, avaliou-se o papel do SUSD2 no crescimento celular, apoptose e na agregação tumoral.

RESULTADOS: O aumento de expressão do SUSD2 a nível proteico após o silenciamento do *KRAS* foi observado na linha celular HCT-116. Com isto, recorreu-se a esta linha celular para avaliar o papel do SUSD2 em CRC, e o seu potencial como mediador dos efeitos supressores do silenciamento do *KRAS*. Os resultados demonstram que em cancro colorretal, o SUSD2 desempenhava um papel no crescimento celular e na capacidade de as células tumorais agregarem, enquanto que na apoptose não foram observadas diferenças. Para além disso, os resultados sugerem que o SUSD2 possui um papel duplo em cancro colorretal, atuando tanto como oncogene, através regulação exercida no LGR5, TWIST1, CD24 e CD44v6, como supressor tumoral, regulando o ZEB1, CD44 e CD133. Para além disso, foi observado que a diminuição de expressão do SUSD2

em cancro colorretal com KRAS mutante correlaciona-se com estabilidade microssatélite e estágio I.

CONCLUSÃO E PERSPECTIVAS FUTURAS: Os nossos resultados demonstram que o SUSD2 é uma proteína regulada pelo KRAS mutante, e que diversos efeitos observados com o silenciamento do *KRAS* são regulados pelo consequente aumento de expressão do SUSD2.

Abstract

INTRODUCTION: Colorectal cancer is one of the most incident and deadly cancers worldwide. Aging, a non-modifiable risk factor, increases the risk of developing colorectal cancer. Besides, several other risk factors such as obesity, family history, and physical activity have been shown to influence the risk of colorectal cancer. Molecularly, colorectal cancer is associated with three molecular pathways: the chromosomal instability pathway, the microsatellite instability pathway, and the serrated pathway. KRAS is an important biomarker in this disease. Patients harboring KRAS mutations do not benefit from an anti-EGFR therapeutic approach. Furthermore, KRAS is frequently mutated in colorectal cancer and a regulator of several oncogenic traits, such as proliferation, survival, and regulation of the tumor microenvironment, promoting the initiation and progression of this cancer. Previous studies by our group suggest that SUSD2 is regulated by the mutant KRAS. In colorectal cancer, SUSD2 has a tumor suppressor role, regulating proliferation, however, its role in other tumor-related processes remains to be elucidated. Thus, we aimed to assess the role of SUSD2 upregulation as a mediator of the suppressive effects of *KRAS* silencing.

MATERIALS AND METHODS: Firstly, to validate SUSD2 upregulation upon *KRAS* silencing, qRT-PCR and flow cytometry were performed, using a panel of colorectal cancer cell lines: HCT-15, HCT-116, SW480, SW620, and LS174T. Subsequently, to assess the role of SUSD2 in CRC, its ability to regulate several markers related to different oncogenic processes was evaluated. Furthermore, the role of SUSD2 in cell growth, apoptosis, and tumor aggregation was also evaluated.

RESULTS: Increased expression of SUSD2 at the protein level after *KRAS* silencing was observed only in the HCT-116 cell line. Therefore, this cell line was used to assess the role of SUSD2 in CRC, and its potential as a mediator of the suppressive effects of *KRAS* silencing. The results demonstrate that in colorectal cancer, SUSD2 plays a role in cell growth and regulates tumor cells ability to aggregate, whereas in apoptosis no differences were observed. Furthermore, the results suggest that SUSD2 has a dual role in colorectal cancer, acting both as an oncogene, through regulation exerted on LGR5, TWIST1, CD24, and CD44v6, as a tumor suppressor, regulating ZEB1, CD44, and CD133. Furthermore, it was observed that decreased SUSD2 expression in colorectal cancer with mutant KRAS correlates with microsatellite stability and stage I patients.

CONCLUSION AND FUTURE PERSPECTIVES: Our results demonstrate that SUSD2 is a mutant KRAS-regulated protein and that several effects observed upon *KRAS* silencing are regulated by the consequent increase in SUSD2 expression.

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Abbreviations and Acronyms

AMOP - Adhesion-associated Domain in MUC4 and other proteins

APC - Adenomatous Polyposis Coli

CAC - Colitis-associated Cancer

CDH1 - Cadherin 1

CDKN2A - Cyclin-dependent Kinase Inhibitor 2A

cDNA - Complementary Deoxyribonucleic Acid

CIMP - CpG Island Methylator phenotype

CIN - Chromosomal Instability Pathway

CMS - Consensus Molecular Subtype

CRC - Colorectal Cancer

CSBF - Colon-derived SUSD2 Binding Factor

CSC - Cancer Stem Cells

CTLA4 - Cytotoxic T Lymphocyte-associated Antigen 4

dMMR - Defective Mismatch Repair System

EGFR - Epidermal Growth Factor Receptor

EMT - Epithelial-mesenchymal Transition

ERK5 - Extracellular Signal-regulated Kinase 5

FACS - Fluorescence-activated Cell Sorting

FBS - Fetal Bovine Serum

FDRs - First-degree Relatives

FRCR - Family Relative Colorectal Cancer Risk

Gal-1 - Galectin-1

GAPDH - Glyceraldehyde 3-phosphate Dehydrogenase

GAPs - GTPase-activating Proteins

GDPH - Glycine-aspartic acid-proline-histidine

GEF - Guanine Nucleotide Exchange Factor

GLUT-1 - Glucose Transporter 1

GPCRs - G-protein-coupled Receptors

HCC - Hepatocellular Carcinoma

HGSOC - High-grade Serous Ovarian Cancer

HIF-1 - Hypoxia-inducible Factor 1

Hsc70 - Heat Shock Protein 70

HVR - Hypervariable Region

IGFs - Insulin-like Growth Factors

JNK - c-Jun N-terminal Kinase

KRAS - Kirsten Rat Sarcoma Viral Oncogene Homolog

LGR5 - Leucine Rich Repeat Containing G Protein-Coupled Receptor 5

MAPK - Mitogen-activated Protein Kinase

MCP-1 - Monocyte Chemoattractant Protein-1

mCRC - Metastatic Colorectal Cancer

MDSCs - Myeloid-derived Suppressor Cells

MFI - Median Fluorescence Intensity

MHCI - Major Histocompatibility Complex I

MMR - Mismatch Repair System

MSI - Microsatellite Instability

MSS - Microsatellite Stability

OS - Overall Survival

PBS - Phosphate-buffered Saline

PD-1 - Programmed Cell Death Protein 1

PD-L1 - Programmed Death-ligand 1

RCF - Relative Centrifugal Force

RFS - Relapse Free-survival

ROS - Reactive Oxygen Species

RPIA - Ribose 5-phosphate Isomerase A

RT - Room Temperature

RTKs - Receptor Tyrosine Kinases

RT-qPCR - Quantitative Real-Time PCR

siRNA - small interfering RNA

SUSD2 - Sushi Domain Containing 2

TAMs - Tumor-associated Macrophages

TGF-β - Transforming Growth Factor β

TME - Tumor Microenvironment

TP53 - Tumor Protein p53

TPM4 - Tropomyosin 4

Treg cells - Regulatory T cells

TSG - Tumor Suppressor Gene

VEGF - Vascular endothelial growth factor

vWFD - von Willebrand Factor type D

ZEB1 - Zinc Finger E-Box Binding Homeobox 1

I. INTRODUCTION

1. Colorectal Cancer: Epidemiology

Cancer has become one of the major public health problems. Aging, consequent of scientific and medical progression, has increased the prevalence of particular diseases, such as cancer (1). According to GLOBOCAN estimates, in 2018 there were 17 million new cancer cases (excluding non-melanomatous skin cancers) and 9.5 million cancer-related deaths (1). Both incidence and mortality display an increasing trend. In 2040, new cancer cases are expected to reach 26 million, an increase of 53% compared to 2018 (2).

Colorectal cancer (CRC) is the most frequently diagnosed gastrointestinal neoplasia, affecting the colon and rectum (3). CRC is the fourth most incident and the third more deadly neoplasia worldwide (1). In 2020, according to GLOBOCAN, were an estimated 1.931.590 new CRC cases and 935.173 deaths, nearly 10% of all new cancer cases and deaths reported annually. Despite being the second most commonly identified cancer in females, CRC has a higher incidence and mortality in males (4). CRC incidence is higher in developed countries (55% of cases) (4, 5). However, the majority of CRC-related deaths (52% of deaths) were reported in developing countries (6).

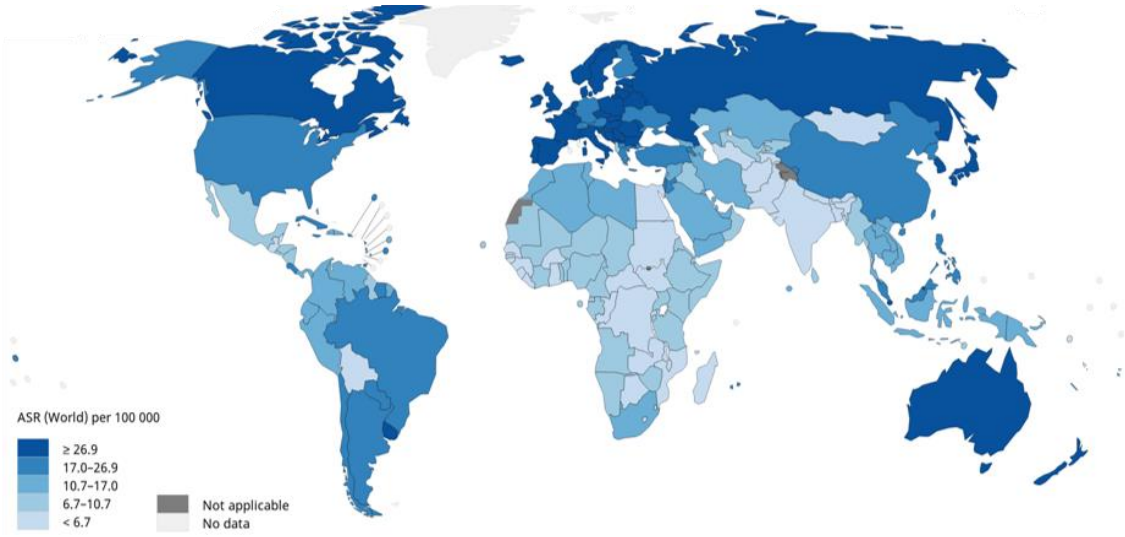


Figure 1. Worldwide incidence of colorectal cancer in 2020 (From Globocan 2020).

In Portugal, CRC is the third most frequent and the second most lethal cancer. According to GLOBOCAN estimates, in 2020, 10.501 new cancer cases were diagnosed in Portugal. Of these, 6.418 were detected in males. Furthermore, 4.320 CRC-related deaths were reported, 59% of whom were males.

Industrialization and economic growth in developing countries will stimulate CRC incidence increase. In 2035, it is expected CRC incidence rise to more than 2.5 million new CRC cases, an increase of 30% compared to CRC cases in 2020 (4).

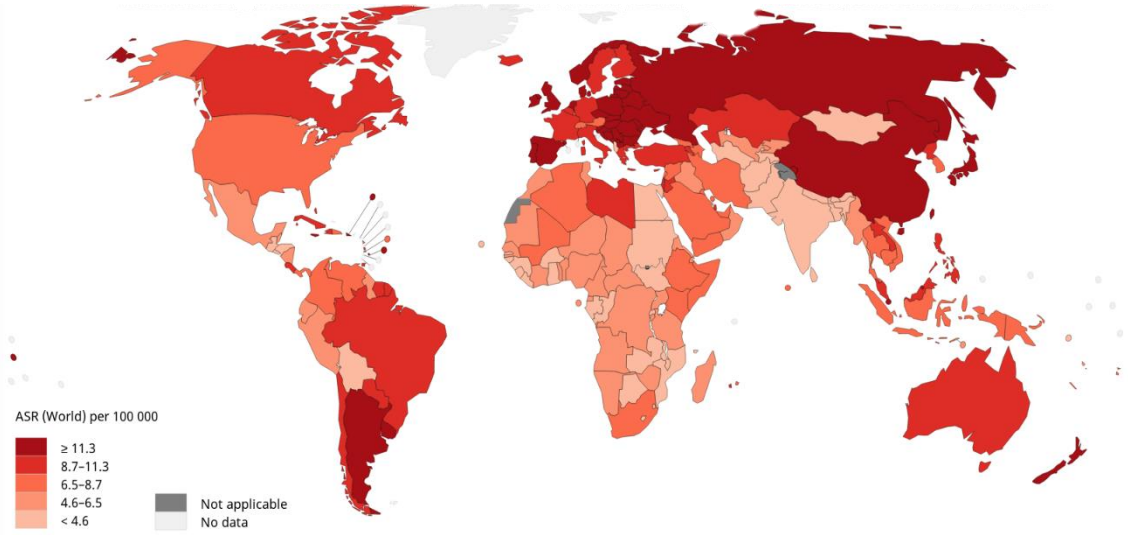


Figure 2. Worldwide mortality of colorectal cancer in 2020 (From Globocan 2020).

2. Colorectal Cancer: Risk Factors

Lifetime risk of developing CRC is about 4-5% (7). Concomitantly, genetic, and environmental factors have been shown to play an important role in CRC development (4, 8, 9). Aging, a non-modifiable risk factor, is described as the main risk factor for CRC (4, 7). The half-century of age marks a critical moment for increased risk of this type of cancer (9). However, several other risk factors can be mentioned:

Gender: Previously described, males evidence a higher CRC risk compared to females (8, 10). Both incidence and mortality are 25% higher in males (4, 11). Behaviors associated with an increased CRC risk, such as alcohol intake and smoking, and lower adherence to CRC screening based on faecal samples explain the higher CRC risk observed in males (8, 12, 13).

Physical Activity: Low physical activity promoted by western lifestyles has been shown to enhance CRC risk, particularly colon cancer (14-16). Elevated insulin levels or insulin resistance, inflammation, and high adiposity are possible mechanisms by how low physical activity rises CRC risk (14, 16).

Obesity: High-level adiposity contributes to CRC, namely colon cancer (17). CRC risk enhances about 5% per 5 kg/m^2 increase in body mass index (BMI) (18). In Europe, overweight and obesity (IBM ≥ 30 kg/m^2) are responsible for 11% of CRC cases (19). It is believed that the pro-inflammatory and tumoral microenvironment supported by adipocytes, and high lipid peroxidation rates observed in obese patients, are mechanisms by which obesity promotes CRC (20).

Family History: A quarter of all sporadic CRC tumors affect patients with a positive family history of the disease (21). The degree, the number of first-degree relatives (FDRs) with CRC, and the age at diagnosis are factors that impact family relative colorectal cancer risk (FRCR) (21-23). Having FDRs with CRC promotes a 2-fold increase in FRCR (22). The number of FDRs and the FRCR exhibit a positive correlation (23). Besides that, FDRs diagnosed with CRC at an age younger than 50 increase 3.31 times FRCR (23).

Diseases: Ulcerative colitis, a disease marked for the permanent immunological aggression on colon and rectum mucosa, is associated with a 2.4 times CRC risk increase (24). Colitis-associated cancer (CAC), a CRC subtype, is responsible for 1-2% of all CRC cases (25). Moreover, 20% of inflammatory bowel disease patients will develop CAC, with a mortality rate of about 50% (26). On the other hand, type 2 diabetes mellitus, a metabolic disorder characterized by insulin resistance and insufficient insulin production, is associated with a 1.27-folder higher CRC risk (27). Hyperinsulinemia resulting from hyperglycemia in these patients is accompanying by an environment abundant in insulin-like growth factors (IGFs) and rare in IGFs-binding proteins (27). In turn, these physiological alterations are associated with cell growth and apoptosis evasion, promoting CRC (27).

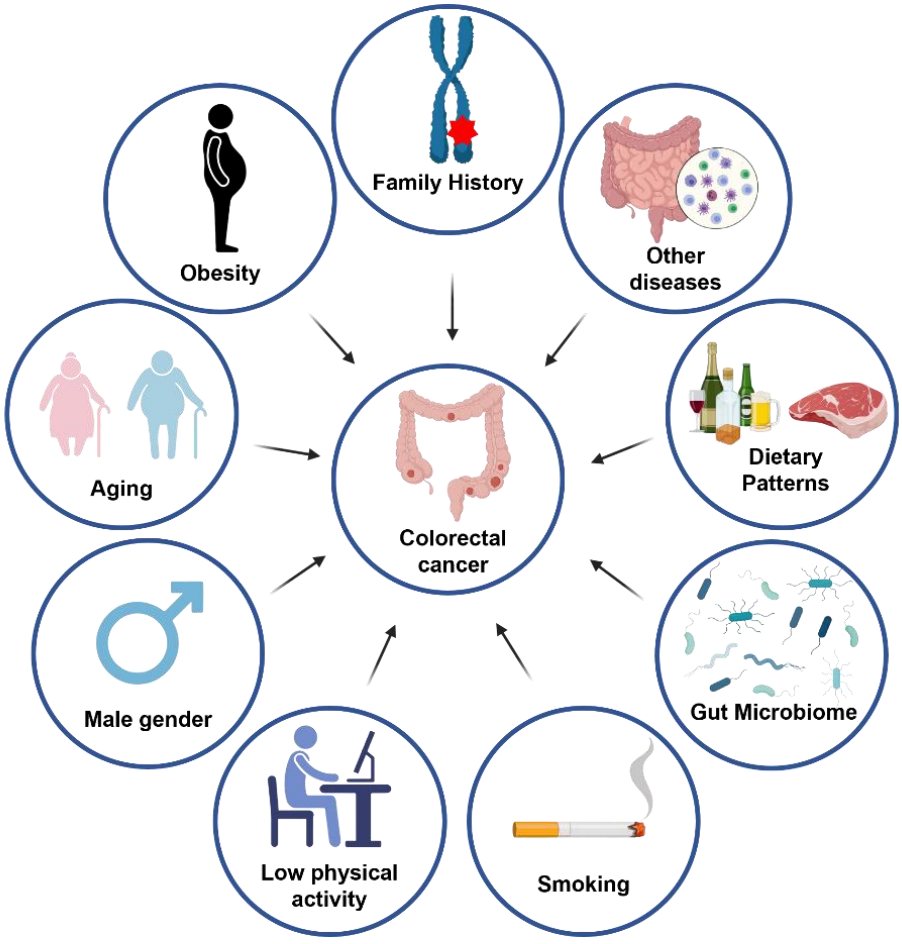


Figure 3. Colorectal cancer risk factors. Created with BioRender.com.

Dietary Patterns: It is believed that moderate alcohol consumption (>1-4 drinks/day, or 12.6-49.9 g/day of ethanol) increases the CRC risk by 21% (13). Carcinogenic behavior performed by acetaldehyde, a metabolite from alcohol metabolism, is the main mechanism by which alcohol intake increases CRC risk (28, 29). Additionally, a diet rich in red and processed meat (>160 g/day) is shown to increase CRC risk by 35%, compared to low intake (<20 g/day) (30).

Gut Microbiome: Dysbiosis (Imbalance of microbiota homeostasis) is considered a CRC promoter (31). *Fusobacterium nucleatum*, a gram-negative bacterium, colonizes more frequently the intestine of CRC patients, especially in the adenoma stage (32). FadA release by this bacterium is related to cell growth, through FadA binding to E-cadherin and consequent internalization and cytoplasmic accumulation (32, 33). Furthermore, *Escherichia coli*, a commensal bacteria, was demonstrated to promote CRC, interacting with mismatch repair system (34).

Smoking: Current smokers have a 14% higher risk than non-smokers, while former smokers possess a 17% higher risk (12). Besides that, smoking one cigarette pack a day for 50 years or two packs for 25 years increases CRC risk by 1.24 times, compared to someone who never smoked (35). CpG islands methylation and the ability to induce high prostaglandin E2 levels are potential pathways by which smoking raises CRC risk (12, 36-38).

3. Colorectal Cancer: Pathogenesis

The progressive accumulation of genetic and epigenetic alterations promotes CRC. Stem cells, located in colon and rectum crypts, enable the rapid self-renew observed in these organs (39). Consequently, in these cells, the risk of mutations improves. The adenoma-carcinoma sequence has emerged to explain the natural history of CRC (40). Tumor suppressor genes (TSG) silencing and oncogenes activation, through genetic and epigenetic alterations, promote the transition from stem cells or mature somatic cells to cancer stem cells (CSC) transition (41). It is believed that CSC play an important role in the initiation of CRC, which will progress to a dysplastic epithelial premalignant lesion, known as a polyp (40-42). Consequently, 15% of these polyps, with new mutations will evolve to CRC (7).

Colorectal carcinogenesis is related to three distinct molecular pathways: chromosomal instability pathway, microsatellite instability pathway, and serrated pathway (43).

Chromosomal instability pathway (CIN) accounts for 65-70% of sporadic CRC and is recognized by the presence of structural and numerical chromosomal abnormalities, namely

aneuploidy (6, 41). Inactivating mutations in *adenomatous polyposis coli* (*APC*) gene at early stages is an ordinary event in this molecular pathway (44, 45). Multi-protein complex APC-GSK3β-Axin binds β-catenin and promotes its degradation. APC loss induces cytosolic β-catenin accumulation and Wnt signaling activation (45-47). Aberrant proliferation and differentiation caused by Wnt signaling deregulation leads to dysplastic crypts, which will result in adenomas (adenomatous polyps). Afterward, Kirsten rat sarcoma viral oncogene homolog (KRAS) activating and tumor protein p53 (TP53) inactivating mutations promote adenoma growth and adenoma-carcinoma transition, respectively (43, 47).

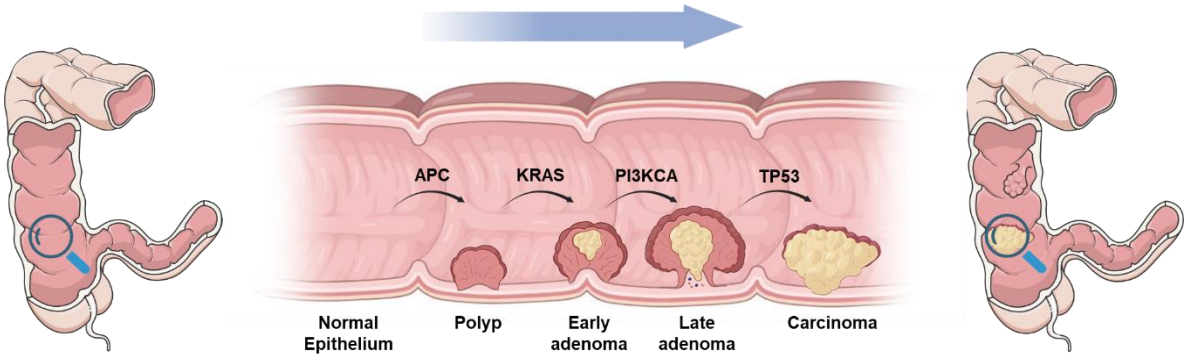


Figure 4. The adenoma-carcinoma sequence. Created with BioRender.com.

The microsatellite instability (MSI) pathway, responsible for 15-20% of sporadic CRC, is characterized by a defective mismatch repair system (MMR) (44, 47). Biallelic inactivation of *MLH1*, through promoter hypermethylation, is the primary cause of the defective MMR (dMMR) (45, 48). Short nucleotide tandem repeats in DNA sequences, also known as microsatellite sequences, are frequent across the human genome. However, these sequences are hotspots for mutations. Traditionally, these mutations would be recognized and repaired by the MMR (7). Nevertheless, in the absence of MMR, these mutations will be replicated, promoting CRC.

Serrated pathway, also known as the CpG island methylator phenotype (CIMP), is reported as an alternative carcinogenic pathway to the traditional adenoma-carcinoma sequence (48-50). Accounting for 10-20% of sporadic CRC cases, the high frequency of methylation in CpG islands (Cluster of CpG dinucleotides at the promoter regions or first exons) is a major trait of this pathway (45). Methylation is an epigenetic mechanism capable of regulating gene expression. Cytosine methylation from CpG islands induces TSG silencing, such as *MLH1* and *cyclin-dependent kinase inhibitor 2A* (*CDKN2A*) (6, 50). Furthermore, mutations in *BRAF* oncogene at early stages are described as a characteristic event of this pathway (45, 49). It has been reported that BRAF promotes CpG islands methylation (51). Besides, BRAF activation stimulates the Mitogen-activated protein kinase

(MAPK) signaling, inducing uncontrolled proliferation and, consequently, serrated adenomas capable to progress to CRC (45).

4. Colorectal Cancer: Consensus Molecular Subtype

CRCs' ability to develop through several carcinogenic pathways contributes to the massive clinical and biological heterogeneity observed in this disease. In order to obtain a CRC classification system based on tumor biology, the Consensus Molecular Subtype (CMS) classification was created (52). As a result, four subtypes have been established, allowing the categorization of most CRCs. CMS1 tumors (14% of CRCs) are predominantly hypermutated MSI, also known as MSI immune (52-54). *MLH1* silencing succeeded by dMMR, high hypermethylation levels, and BRAF mutations (mostly $BRAF^{V600E}$) are common features in this subtype (45, 54). For that reason, these tumors have a high tumor burden that supports a strong immune infiltration in the tumor microenvironment (52, 54). Moreover, CMS1 tumors are associated with the worst relapse free-survival (RFS) among CMS (45, 52). CMS2 (37% of CRCs), or canonical subtype, is characterized by tumors with microsatellite stability (MSS) and low hypermethylation levels (54). These tumors carry a marked upregulation of WNT and MYC downstream targets (52, 54). Besides, TSGs loss and the oncogenes overexpression are commonly noted in CMS2 tumors (52). Regarding prognosis, CMS2 tumors are related to better overall survival (OS) and RFS (52, 53). Referred to as metabolic subtype, CMS3 tumors (13% of CRC) are characterized by metabolic reprogramming (52-54). Moreover, KRAS-activating mutations are more prevalent in this class of tumors (53, 54). Lastly, CMS4 tumors (23% of CRCs), also known as mesenchymal tumors, exhibit similarities to CMS2 tumors, such as MSS and low methylation levels (54). Nevertheless, overexpression of genes related to epithelial-mesenchymal transition (EMT), stemness, extracellular matrix remodeling, and angiogenesis are distinctive traits (52, 54). CMS4 tumors are usually diagnosed at more advanced stages (III and IV) and are related to poor OS and RFS (53, 55). The remaining CRCs (13% of CRCs) did not classify into any CMS due to the absence or presence of mixed molecular alterations (45).

5. Colorectal Cancer: Therapeutic Approaches

Screening programs and increasing therapeutic options favor CRC diagnosis at early-stages, improving its prognosis. Surgery is the basis of curative treatment in CRC non-metastasized (9). In the polyp stage, polypectomy allows complete recession, without it developing into carcinoma (56). Resection by laparoscopy with resection of adjacent lymph vessels, succeeded by adjuvant chemotherapy in patients with a high risk of local recurrence is the standard surgical procedure for colon cancer in many countries (4, 9).

Nevertheless, in rectal cancer, total mesorectal excision is the main surgical approach, following by neoadjuvant therapy in patients at high risk of relapse (9).

Chemotherapy is mainly applied as adjuvant therapy after curative surgery (57). 5-Fluorouracil or capecitabine, both fluoropyrimidines, along with oxaliplatin (FOLFOX4), or irinotecan (FOLFOXIRI) as an alternative strategy, and folic acid is the standard chemotherapy for CRC (4, 58). In turn, chemoradiotherapy with fluoropyrimidine as a radiation sensitizer provides tumor downsizing before being surgically removed (4, 57).

Concerning metastatic CRC (mCRC), most countries have not yet implemented CRC screening, meaning that mCRC remains frequently diagnosed. In first-world countries, up to 30% of all CRCs are mCRCs at diagnosis (55). The 5-year relative survival of mCRC patients is poor, about 11.7% (57). Nonetheless, the development and identification of new treatments have improved the prognosis of these patients. Targeted therapy plays a major role in mCRC management. Bevacizumab, an anti-VEGF (Vascular endothelial growth factor) monoclonal antibody, was the first target therapy for mCRC, improving the prognosis of these patients, even when used with chemotherapy (4, 59). Most recently, new therapeutic biomarkers, such as KRAS, BRAF, and MSI status have proven to play a crucial role in which therapeutic approach should be implemented in these patients (60). Anti-EGFR (Epidermal growth factor receptor) therapy (Cetuximab and Panitumumab) enhances mCRC patients' survival and their response to standard chemotherapy (58). However, in Ras-mutated patients, anti-EGFR therapy promotes worse OS and RFS (58, 60). Therefore, at mCRC diagnosis, RAS testing should be assessed (60). In addition, mCRC with BRAF mutations are more aggressive and do not respond to anti-EGFR therapy (60, 61). MSI status should be evaluated, as mCRC patients with dMMR and MSI-H exhibit great responses to immunotherapy. Programmed cell death protein 1 (PD-1) inhibitors (Pembrolizumab and Nivolumab) improve the prognosis in this mCRC subtype, handled as monotherapy, or with an anti-CTLA4 (Cytotoxic T lymphocyte-associated antigen 4) (Ipilimumab) (60, 62).

6. Colorectal Cancer: The role of the MAPK pathway

MAPK cascades are important regulators of signal transduction. ERK/MAPK, c-Jun N-terminal kinase (JNK), p38MAPK, and extracellular signal-regulated kinase 5 (ERK5) are the better-known MAPK pathways (63, 64). Traditionally, these cascades are composed of 3 consecutively activated kinases (63, 65). Scaffold proteins promote a multi-protein complex formation, composed of all kinases, increasing pathway efficiency (66).

ERK/MAPK pathway is the well-documented MAPK cascade. Commonly associated with the Ras-Raf-MEK-ERK sequence, this signaling pathway is often triggered by growth factors binding to their receptor tyrosine kinases (RTKs), typically the EGRF (67). In the cytoplasmic membrane, activated RTKs promote Grb2-SOS complex recruitment, which through the SOS protein, a guanine nucleotide exchange factor (GEF), induces GTP/GDP exchange on Ras, activating it (64, 67). Sequentially, Ras binding to the regulatory N-terminal domain of Raf proteins (A-Raf, B-Raf, and C-Raf) favors its activation, through conformational changes that disfavor its self-inhibited state (64). Afterward, Raf will induce phosphorylation of two serines, activating MEK1/2. Consequently, ERK1/2 will be activated through threonine-X-tyrosine activation loop phosphorylation impelled by MEK1/2 (64, 68). Finally, activated ERK1/2 are translocated to the nucleus, stimulating transcription factors associated with several cancer traits, such as cell proliferation, differentiation, apoptosis, angiogenesis, and metastasis, or activate cytoplasmic protein (63-66).

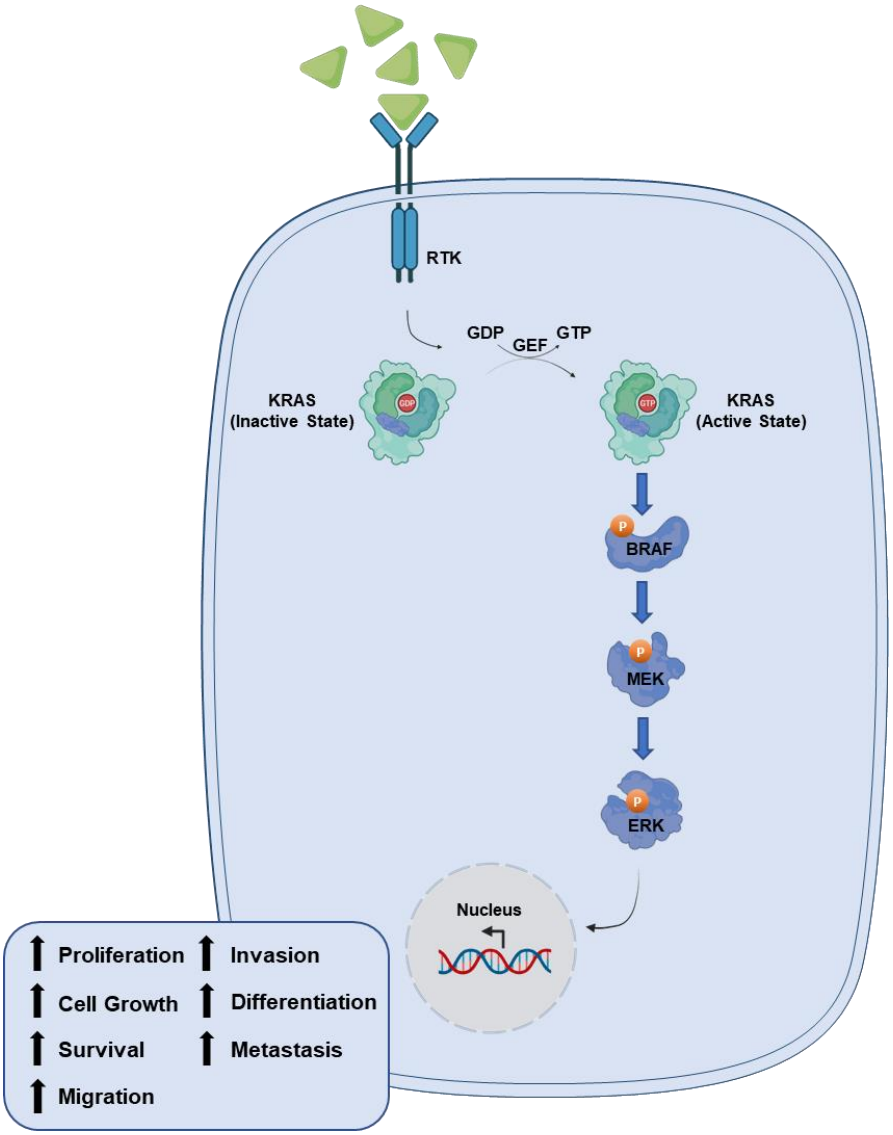


Figure 5. The ERK/MAPK pathway. Created with BioRender.com.

Several reports support the idea that the ERK/MAPK pathway is overactivated in CRC, playing an important role in CRC carcinogenesis initiation and progression. Dysregulations in this cascade are recurrently founded in CRC, being associated with cell proliferation, survival, migration and invasion, and metastasis (69). KRAS and BRAF are proto-oncogenes frequently mutated in CRC. Activating mutations in these genes are related to tumor aggressiveness, being present in about 40% and 10-15% of CRC tumors, respectively (70, 71). Moreover, EGFR, the major mediator of this signaling pathway activation, is overexpressed in 80% of CRCs (9). Due to the large portion of tumors harboring mutations in components of the ERK/MAPK pathway, producing new targeted therapies has become of utmost importance. In order, BRAF inhibitor combination with an anti-EGFR and a MEK inhibitor has been shown to reduce mortality in mCRC patients with $BRAF^{V600E}$ mutation (72). Furthermore, $KRAS^{G12C}$ inhibitors, such as sotorasib and adagrasib, responsible for hold $KRAS^{G12C}$ mutant in its inactivated state, have been connected with sustained inhibition of phospho-MEK and phospho-ERK when used simultaneously with an EGFR inhibitor (73). Nonetheless, more efforts are required to discover new targeted therapies that allow achieving better clinical benefits.

7. Ras Family

Identified in the 60s, the Ras family encodes three small monomeric GTPases, HRAS, KRAS, and NRAS, with a predictive molecular weight of 21 kDa (74, 75). Described as switcher proteins that cycle between an active GTP-bound state and an inactive GDP-bound state, this group of GTPases plays a central role in extracellular signals transduction, being initiated by RTKs, as EGFR and PDGFR-β, G-protein-coupled receptors (GPCRs), and integrins (74, 76). GTPase-activating proteins (GAPs) are major regulators of their signaling, potentiating GTP hydrolysis to GDP. Known as GTPases with low hydrolytic activity, GAPs regulation is vital, preventing their persistent activation (76, 77). In contrast, GEFs are proteins that make Ras activation a more efficient process. GEF/GTPase complex helps GDP dissociation, supporting natural GTP binding, whose cytosolic concentration is ten times higher than GDP concentration (76, 78). The GDP-GTP exchange will translate into conformational changes in Ras proteins, enabling their cellular activity (76).

In cancer, Ras proteins act as oncogenes and are recognized by their regulator role in several oncogenic traits, such as proliferation, cell growth, differentiation, angiogenesis, migration, and metastasis (74, 79). Gain-of-function missense mutations in Ras are found in about 30% of all cancers, awarding RAS the title of the most frequent mutant oncogene (74, 80). KRAS is the most mutated Ras GTPase in cancer, accounting for 85% of all mutations, therefore being the most mutated oncogene in this disease (76).

8. KRAS

KRAS is located on chromosome 12p12.1 and encodes two different monomeric GTPases by alternative splicing: KRAS4A and KRAS4B, being the last one the predominant splice variant (81, 82). Despite being expressed in most tissues, this GTPase display in skeletal muscle, myocardium, uterus, adrenal cortex, and bone marrow stem cells higher expression (81).

Functionally, KRAS modulates several cell signaling events, such as proliferation, differentiation, survival, and cell metabolism (83). KRAS signaling depends on its association with the cytoplasmic membrane. Here, proteins belonging to GEF family are recruited by activated RTKs, GRPCs, and integrins, supporting the activation of this small GTPase (76). Thus, both variants undergo post-translational modifications that promote their membrane localization. Regarding KRAS4B, this variant is farnesylated, cleaved, and methylated at cysteine 185 (C185), located in the C-terminal hypervariable region (HVR) region (84). Unlike all Ras family variants, KRAS4B does not require palmitoylation to translocate into the cytoplasmic membrane (75, 76). Furthermore, a poly-lysine sequence in the HVR region benefits membrane association. Contrarily, KRAS4A does not possess this poly-lysine region undergoing an additional palmitoylation at the cysteine residue (C180) (75, 84).

9. KRAS Mutation

KRAS is the most mutated oncogene in cancer. KRAS imbalances are detected in 55% of cancers (81). The wild-type KRAS allele displays an unexpected tumor suppressor feedback under the mutated KRAS allele. To overcome this, wild-type allele loss, or gain in the number of copies of the mutated allele, are mechanisms frequently adopted by tumor cells to undergo tumorigenesis induced by mutated allele, producing KRAS imbalances (81, 83).

Missense mutations (Change of a single nucleotide leading to substitution of an amino acid in the protein) are the most frequently observed mutations in KRAS (82). In cancer, KRAS is not mutated and overexpressed in tissues where normally have greater expression, but in other tissues, such as pancreatic ductal adenocarcinoma (96%), CRC (40%), and lung adenocarcinoma (32%) (81, 85, 86). Besides, KRAS mutations are responsible for nearly 1 million annual cancer-related deaths (76). Constituted by six exons, mutations in this oncogene often affect exons 2, 3, and 4 (81). In 90% of cases, mutations occur in codon 12, located in exon 2 (83). The G12D (substitute glycine for aspartate) and G12V (substitute glycine for valine) mutations are the most frequently found in this protein. However, mutations in codons 13, 61, and 146 are also detected with some relevance in

particular types of cancer (81, 83). Interestingly, each type of KRAS mutation modifies specifically some activating mechanism resulting in its permanent activation, promoting the constitutive activation of its downstream effectors. Activating mutations in codon 12 decrease the intrinsic GTPase activity and the binding to GAPs, while codon 13 mutations, in addition to that, increases the GTP-KRAS binding affinity by 10-fold (83). Additionally, Q61 mutations abolish both intrinsic GTPase activity and that promoted by GAPs (76). While A146 mutations favor GDP-KRAS dissociation, promoting the active GTP-bound state of KRAS (76, 87). Furthermore, as a result of its central role in cancer, KRAS mutations have been evaluated for their potential role as biomarkers for survival and response to therapy, having been reported that each mutation has a specific role concerning that in each type of cancer (83).

10. Oncogenic Signaling of KRAS in Cancer

KRAS role as a cancer-associated molecule was uncovered in the 80s (88). Since then, several efforts have been made to fully understood its role in cancer. In addition to its intracellular role, where KRAS regulates several growth factor signaling pathways, this oncogene plays a central role in tumor microenvironment (TME) regulation (44, 89). Its ability to induce uncontrolled proliferation and cell growth is a well-recognized process. The ability to promote such effects by distinct pathways is of great importance for its role in tumor progression since it has been reported that excessive ERK signaling induces cell senescence (71, 90). Therefore, beyond inducing uncontrolled proliferation and cell growth through this pathway, mutated KRAS, through PI3K type I activation, induces PI3K/AKT/mTORC1 signaling, promoting such oncogenic events. Furthermore, through PI3K/AKT signaling, mutated KRAS allows tumor cells to evade apoptosis, promoting cell survival (74, 91).

High proliferation rates demand large amounts of components necessary for new tumor cells division and formation. Adoption of aerobic glycolytic metabolism by cancer cells (Warburg effect) though metabolic remodeling is described as a hallmark of cancer (92, 93). In the past years, KRAS has demonstrated a central role in metabolic rewiring in cancer (74, 94). Mutated KRAS has been reported to promote glycolysis by increasing glucose uptake (94). Through hypoxia-inducible factor (HIF)-1 upregulation, as a result of KRAS modulation on PI3K/AKT/mTORC1 signaling, oncogenic KRAS promotes glycolytic enzymes upregulation and amino acids uptake (95). Besides that, gain in the number of copies of the mutated KRAS supports glutathione biosynthesis, important for reactive oxygen species (ROS) management, whereas KRAS signaling induces ribose 5-phosphate isomerase A (RPIA) upregulation, an enzyme involved in amino acid synthesis (96, 97). Additionally, lipid metabolism regulation by KRAS is another highlight of its mutation. Lipids

are important elements of cell membranes and play a crucial role in energy storage. Oncogenic KRAS signaling controls β-oxidation and lipogenesis (96, 98). Lastly, mutated KRAS tumor cells constrained to adverse conditions can carry out mechanisms such as macropinocytosis, essential to sequester albumin, and autophagy, obtaining nutrients from stromal cells (96, 99).

KRAS ability to regulate processes that enhance sustains tumor metastasis makes this oncogene one of the most important promoters of tumor progression. Synergistic with transforming growth factor (TGF)-β signaling, mutated KRAS increases cellular expression of mesenchymal markers, such vimentin, and downregulates epithelial markers, such E-cadherin (100). Furthermore, through ERK/MAPK, PI3K/AKT, and TIAM1 signaling, KRAS promotes cell motility and EMT-associated genes upregulation (95, 101). Tumor ability to evade the immune system control is one of the most important traits in cancer. KRAS mutations support a pro-tumorigenic TME (74, 89, 102, 103). IL-17-secreting Th17 cells recruitment is one of the mechanisms by which KRAS fosters this microenvironment (89, 102). Besides, KRAS ability to induce alterations in immune-related membrane receptors expression, such as the decrease of Major Histocompatibility Complex I (MHCI) proteins and increase of Programmed Death-ligand 1 (PD-L1) expression, is crucial for evading cytotoxic responses by $CD8^+$ T cells (102, 103). Moreover, KRAS promotes immunosuppressive cells recruitment to the TME, such as Myeloid-derived suppressor cells (MDSCs), through IL-6 and CXCL3 release (74, 89, 103). Through CCL9 and IL-23 release, mutated KRAS tumor cells recruit M2 macrophages and exclude B, T and, NK cells from TME (74, 102). Finally, KRAS mutations are related to STK11/LKN1 loss and consequent STING inhibition, promoting type I interferon genes and chemokines downregulation, crucial for T cells recruitment, proving KRAS powerful role in several oncogenic-related events to tumor maintenance and progression (102).

11. KRAS Role in Colorectal Cancer Development

KRAS is the most mutated Ras isoform in CRC. About 40% of CRCs display mutations in this oncogene, corresponding to 90% of all Ras mutations in CRC (86). The G12D mutation is the most detected in this type of cancer, attending to 13.4% of CRC cases, followed by G13D (substitute glycine for aspartate) (7.8% of CRCs) and G12V mutations (7.5% of CRCs) (104).

Concerning colorectal carcinogenesis, KRAS is a major regulator of proliferation and cell growth. In the adenoma-carcinoma sequence, KRAS mutations frequently occur after APC loss, at early adenoma stages, promoting uncontrolled proliferation by ERK/MAPK and PI3K/AKT signaling (105, 106). Moreover, it has been reported that APC loss and KRAS

activating mutations synergy is a colorectal tumorigenesis initiation key event, promoting cancer stem cells activation (107). Previously described, mutated KRAS induces class I PI3K activation through interactions with the catalytic subunit of PI3K, encouraging PIP2 phosphorylation to PIP3, and consequently, AKT activation. Besides that, AKT regulation may also be through KRAS ability to promote PIP2 hydrolysis to PIP3 by activating phospholipase Cε (108). Therefore, through AKT regulation, mutated KRAS induces mTORC1 activation by TSC1/TSC2 complex phosphorylation and inhibition, sustaining uncontrolled cell proliferation (95). Furthermore, it is well-known that AKT inhibits GSK3 by phosphorylation, a member of the multi-protein complex responsible for regulating cytoplasmic β-catenin. Thus, mutated KRAS increases cytosolic β-catenin, promoting cell proliferation by cell-cycle progression genes upregulation, such c-MYC and cyclin D1 (95, 109). Besides that, AKT regulation by KRAS extends to other oncogenic hallmarks, such as cell survival. Regulation exerted by AKT on mTORC1 results in S6K1 activation, promoting anti-apoptotic protein BAD expression (110). Lastly, mutated KRAS fosters anoikis resistance via BCL-XL stabilization, a crucial event for anchorage-independent growth (86).

Interestingly, KRAS mutations are commonly found in CMS3 CRCs, where tumors undergo extensive metabolic reprogramming (44, 54). As described above, oncogenic KRAS plays a central role in this rewiring. In CRC, mutated KRAS increases surface expression of Glucose transporter 1 (GLUT-1) and glucose uptake, supporting glycolysis (94, 111). Through this, pentose phosphate pathway intermediates and amino acid biosynthesis is favored (95, 96, 111). Furthermore, KRAS controls glutamine metabolism and phosphoserine synthesis in CRC, enhancing the central role played by KRAS (94, 96, 111).

The oncogenic signaling of KRAS on the orchestration of the crosstalk between cancer cells and the TME extends to CRC. The oncogenic KRAS enables CRC cells to evade immune surveillance via MHC1 downregulation, making these cells resistant to the cytotoxic response of $CD8^+$ T cells (89, 103). Besides that, through MEK/ERK/AP-1 signaling, KRAS induces IL-10 and TGF-β1 secretion, promoting $CD4^+$ T cells differentiation into regulatory T cells (Treg cells), supporting a tolerogenic TME (102, 112). In addition to these pro-tumorigenic effects, mutated KRAS promotes immune cells recruitment, favoring tumor maintenance and progression. IL-17-secreting Th17 cells recruitment, and upregulation of IL-17A receptor in CRC cells, stimulate pro-inflammatory cytokines, such as IL-6, CXCL2, MCP-1 and ARG1, and metalloproteases upregulation, such MMP-7 and MMP-12, and MDSCs recruitment (89). MDSCs display an immunosuppressive and pro-tumoral role and can also be recruited through IRF2 downregulation and GM-CSF upregulation, backed by mutated KRAS in CRC (103, 113). Angiogenesis (new blood vessels development through

pre-existing vessels) is another oncogenic trait regulated by KRAS (102, 106). Angiogenesis is a vital event for oxygen and nutrients supply to cells, and tumor spread. In CRC, mutated KRAS cells release IL-8, recruiting endothelial cells to the TME, encouraging angiogenesis (89, 102).

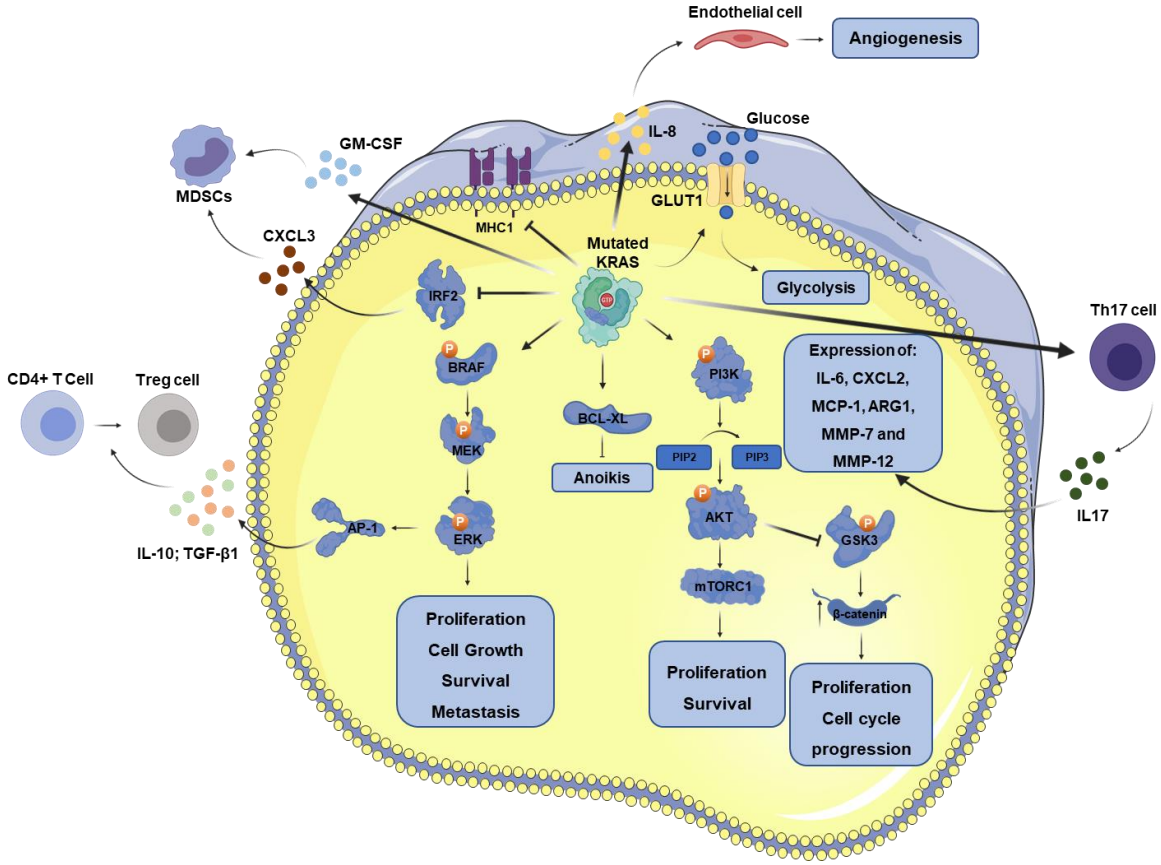


Figure 6. KRAS role in colorectal cancer. Created with BioRender.com.

More recently, it has been reported that KRAS is an indispensable molecule in metastasis and metastasis maintenance (114). CRC frequently metastasizes to liver and lung (115). A recent report shows the important role of ERK2 signaling activation in mutated KRAS cells ability to metastasize the liver (116). Besides that, KRAS mutation association with hepatic-pulmonary metastasis is well-documented in CRC (116, 117). Therefore, in addition to the well-documented role in colorectal tumorigenesis initiation, KRAS mutations orchestrate several other oncogenic traits, supporting CRC maintenance and progression.

12. KRAS Impact in Colorectal Cancer Clinical Management

KRAS plays a crucial role in CRC. Thus, KRAS mutations have been extensively evaluated for their potential role as survival and therapeutic response biomarkers. The predictive role has been showing to be KRAS mutation type dependent. Codon 12 mutations are associated with a worse prognosis in CRC, while codon 13 mutations possess a better prognosis against KRAS wild-type tumors. Moreover, codon 12 mutations are related to

advanced-stage and lymph node metastasis (83). Concerning KRAS predictive role in therapy response, advanced-stage patients with KRAS mutations show worse FOLFOX chemotherapy response (118). Besides, as previously described, KRAS mutations are well-known biomarkers for anti-EGFR therapy in mCRC. About 52,6% of mCRC owning KRAS mutations, excluding anti-EGFR therapy for this group of patients (119). However, KRAS G13D patients have been associated with response to anti-EGFR (120, 121). Besides that, KRAS engages in several events throughout colorectal tumorigenesis, and CRC mutated KRAS cells are normally dependent on the KRAS mutation, making KRAS an ideal therapeutic target (122). Nonetheless, despite several efforts made by the scientific community to target this oncogene, KRAS remains almost undruggable.

13. SUSD2

Sushi Domain Containing 2 (*SUSD2*), also known by its mouse homolog SVS-1 or mSVS-1, is located on chromosome 22q11.23 and encodes a type I transmembrane protein of 822-amino acid (123). Constituted by fifteen exons, SUSD2 has a predicted molecular weight of 90.4 kDa, with the ability to increase to 100-110 kDa, since SUSD2 has nine predicted N-glycosylation asparagine residues (124, 125). SUSD2 is highly expressed in the lung and kidney, whereas low expression levels were detected in other tissues such as the adrenal gland, breast, colon, thyroid, uterus, and adipose tissue (124, 126, 127).

SUSD2 is constituted of three major functional domains: a large extracellular domain composed of somatomedin B, adhesion-associated domain in MUC4 and other proteins (AMOP), von Willebrand factor type D (vWFD), and sushi/CCP/SCR domains, a transmembrane domain, and a small cytosolic domain (123). All extracellular subdomains display a crucial role in their cellular function and location, in the cytoplasmic membrane (127). Besides, post-translational cleavage is an important event in their cell localization, since, in its absence, SUSD2 ability to translocate from the endoplasmic reticulum to the plasma membrane is abolished (125). Post-translational cleavage occurs in the central region of the protein, in the glycine-aspartic acid-proline-histidine (GDPH) amino acid sequence (vWFD domain), between an aspartic acid residue and a proline, producing two fragments (N- and C-terminal) linked by one or more disulfide bonds suggesting SUSD2 mature form as a heterodimer (125, 128). Two distinct mechanisms of SUSD2 cleavage are described, one of them through pH autocatalytic cleavage, while the other happens through serine proteases activity (125). Besides, unlike the C-terminal, the N-terminal region of SUSD2 can be cleaved and released (123). Lastly, it has been reported that SUSD2 can undergo exonization, a process defined by intronic sequences inclusion in mRNA. This alternative splicing mechanism has been demonstrated in lung tumor samples, and occurs between introns 11 and 14, promoting SUSD2 isoforms (129).

14. SUSD2 Role in Cancer: Foe or Ally

SUSD2 role as a cancer-associated molecule has been emerging in the last years. Described for the first time in 2007, SUSD2 was described as a regulator of clonogenicity, anchorage-independent growth, cell migration, and invasion, illustrating a tumor suppressor role in oncogene-v-K-ras transformed NIH3T3 and Ki3T3 cell lines (123). Since then, SUSD2 potential role in other cellular events has been investigated, such as cell proliferation, invasion, migration, metastasis, and TME regulation (127, 130, 131). Intriguingly, SUSD2 exhibits a dual role in cancer. In breast, gastric, and endometrial cancer SUSD2 acts as an oncogene, whereas in CRC, lung cancer, renal cell carcinoma, hepatocellular carcinoma (HCC), and high-grade serous ovarian cancer (HGSOC), it has a tumor-suppressive role (126, 127, 130, 132-134).

Regarding SUSD2 role within the tumor cell, higher levels of SUSD2 have been reported to support uncontrolled proliferation in endometrial and gastric cancer, while in CRC, lung cancer, and HCC, SUSD2 overexpression is associated with a tumor-suppressive role against proliferation (126, 127, 132, 133, 135). In HCC, SUSD2 has been described as a downstream target of Tropomyosin 4 (TPM4), exhibiting a negative correlation between them (136). TPM4 acts as an oncogene, promoting proliferation, invasion, and metastasis (137, 138). SUSD2 upregulation show to reverse all oncogenic roles of TPM4 in HCC. Thus, TPM4 oncogenic role is due to its ability to regulate SUSD2 expression (136). Regarding SUSD2 role as a regulator of cell migration and invasion, SUSD2 overexpression promotes invasion but does not affect cell migration in breast cancer (124). In turn, in gastric cancer, SUSD2 upregulation fosters cell migration and invasion, whereas, in HGSOC, SUSD2 upregulation promotes an opposing effect, inhibiting cell migration and invasion (132, 134, 139). Besides, SUSD2 regulates metastasis in several types of cancer. In HGSOC, the peritoneal cavity is a frequent metastasis location. Unlike most cancer, HGSOC cells need to aggregate and form spheroids to metastasize (140, 141). Moreover, the mesothelial clearance capacity of the primary HGSOC cells is crucial to eliminate mesothelial cells present in the peritoneal cavity, enabling metastasis (134, 142). HGSOC $SUSD2^{+}$ spheroids display a lower mesothelial clearance capacity than $SUSD2^{KD}$ spheroids (134). Furthermore, in vivo, $SUSD2^{KD}$ mouse models are more likely to develop pancreatic metastases than $SUSD2^{+}$ mouse models, thus, demonstrating the antagonistic role played by SUSD2 in HGSOC metastasis (131). Contradictorily, Xu et al. reported that higher SUSD2 levels in HGOSC are related to an increased metastasis ability of tumor cells, through EpCAM upregulation exerted by SUSD2, promoting EMT by downregulation of E-cadherin (143). Besides that, in vivo, using a mouse model of breast cancer with SUSD2

overexpression, the tendency to form lung metastasis was higher, whereas, in lung adenocarcinoma, SUSD2 downregulation is correlated with metastasis (143, 144).

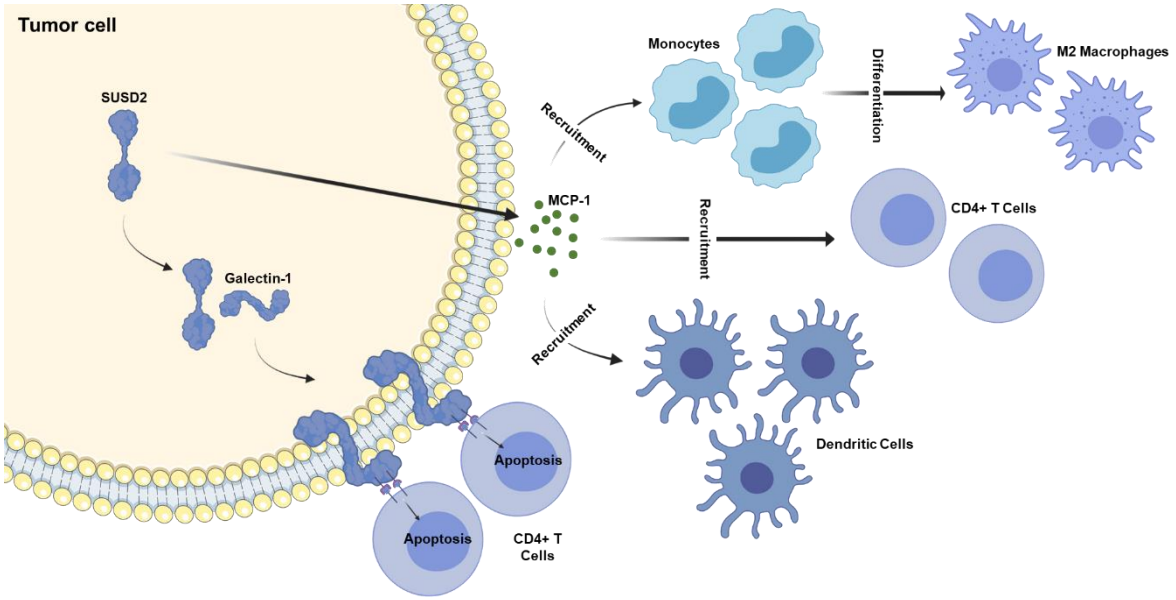


Figure 7. SUSD2 regulation on tumor microenvironment. Created with BioRender.com.

Concerning SUSD2 ability as an EMT regulator, its upregulation has been associated with EMT-related genes upregulation, such as FZD7, TGF-β1, SPARC, and ITGA5, in gastric cancer (132). Contrarily to Xu et al. report, it was described by other authors, *SUSD2* silencing, in HGSOC, promotes upregulation of genes related to a mesenchymal cell phenotype, such as the STEAP1, AHNAK, Snail-1 and -3, and COL5A2 (134). Furthermore, SUSD2 downregulation increases the N-cadherin/E-cadherin ratio, showing a negative correlation between SUSD2 and N-cadherin, a mesenchymal marker related to EMT (134).

In addition to the SUSD2 pro or anti-tumorigenic activities in tumor cells, SUSD2 orchestrates several TME-related events. In breast cancer, *SUSD2* silencing inhibits cell surface Galectin-1 (Gal-1) expression (124). Gal-1 is the most produced galectin in breast cancer, having the ability to regulate TME, supporting a pro-tolerogenic microenvironment (145). Surface expression of Gal-1 by tumor cells raises T cell apoptosis by cell-cell interaction (146). Moreover, in breast cancer, SUSD2 overexpression is associated with T helper cell apoptosis increase (124). Thus, SUSD2 ability to regulate the T helper cell population may be due to the Gal-1 ability to promotes T cell apoptosis. Besides, SUSD2 promotes pro-tumoral immune cells recruitment. In breast cancer, SUSD2 overexpression promotes tumor-associated macrophages (TAMs) recruitment, namely M2 macrophages, an anti-inflammatory and pro-angiogenic phenotype (130). SUSD2 ability to promote a pro-tumorigenic microenvironment is owing to Monocyte Chemoattractant Protein-1 (MCP-1) increased expression and release by SUSD2 overexpressed tumors (130). MCP-1 recruits monocytes, $CD4^+$ T cells, dendritic cells, and promotes macrophages polarization into M2

phenotype (147, 148). Furthermore, in ovarian cancer, *SUSD2* silencing increases tumor cells binding ability to platelets (149). The ability to bind platelets is an escape mechanism from immune surveillance and resistance to chemotherapeutic agents. In addition, *SUSD2* silencing induces a pro-angiogenic microenvironment through GPIIB/IIIA receptor upregulation, essential for platelet aggregation, and consequent VEGF release (149). Therefore, despite being a protein barely described, SUSD2 proves to be a potential regulator of several oncogenic traits.

15. SUSD2 and Colorectal Cancer

SUSD2 was reported as a new potential tumor suppressor gene in CRC (127). SUSD2 is downregulated in CRC, compared to adjacent tissues. Furthermore, C10orf99, also known as colon-derived SUSD2 binding factor (CSBF), is described as a ligand of SUSD2, and is downregulated in CRC. While SUSD2 downregulation occurs by promoter methylation or through epigenetic regulation of transcription factors, C10orf99 expression seems to be regulated by SUSD2, since SUSD2 restoration induces C10orf99 upregulation. Moreover, SUSD2/C10orf99 interaction inhibits proliferation in colon cancer cell lines, through cyclin D1, cyclin D3, and CDK6 downregulation, promoting G1 cell cycle arrest (127). Notably, SUSD2/C10orf99 anti-proliferative effect only occurs through the interaction between both, while alone none of them inhibit proliferation (127). Furthermore, C10orf99 exhibits a potent chemotactic role in lymphocyte recruitment to epithelium through interaction with GPR15, highlighting the potential role of SUSD2 in orchestrating CRC TME (150, 151). Nonetheless, further studies addressing SUSD2 impact in other cancer-related traits, such as migration, invasion, and metastasis are requested to support the role of SUSD2 in CRC.

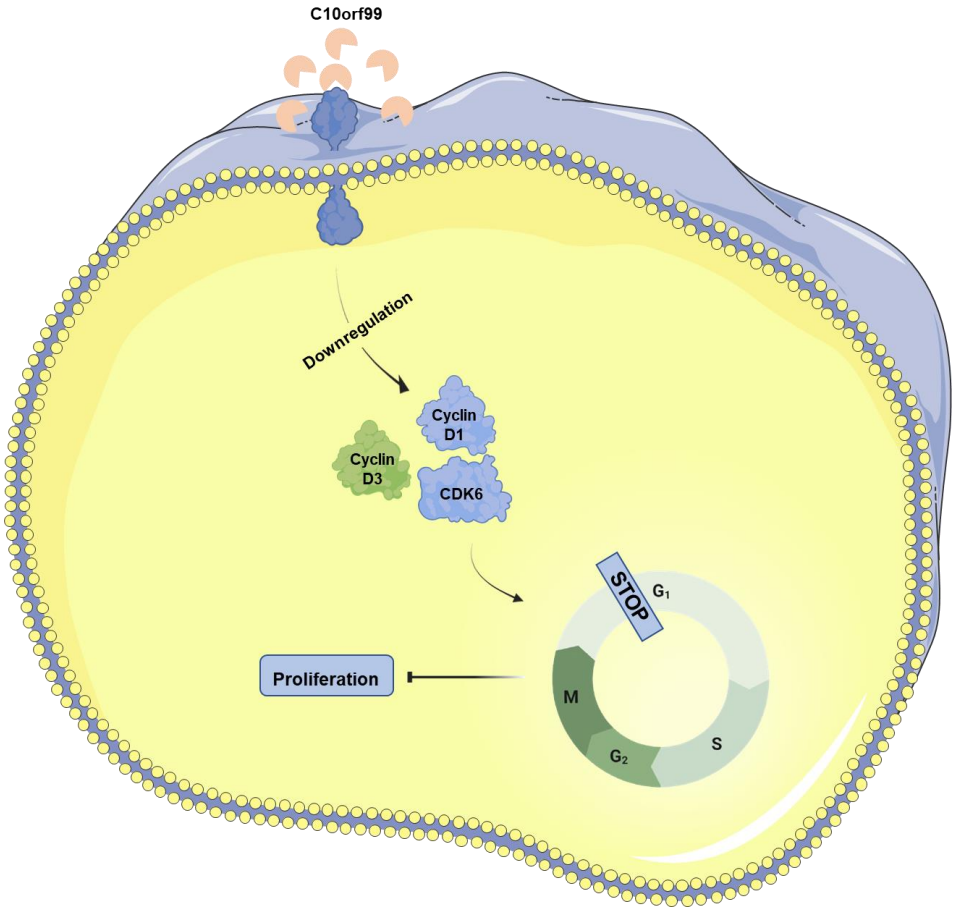


Figure 8. SUSD2 role in colorectal cancer. Created with BioRender.com.

II. AIMS

It is well established that mutated KRAS role in colorectal carcinogenesis goes beyond promoting high rates of proliferation that support the tumorigenesis initiation. Currently, KRAS role in colorectal metastasis and regulation of surrounding tumor microenvironment components is well-documented. Furthermore, mutated KRAS is a well-known CRC biomarker, restricting the use of anti-EGFR therapy in patients harboring mutations in this gene. Therefore, it is of utmost importance to understand the role of this oncogene and the mechanisms underlying their actions in CRC.

Previously, in our group, with the objective of identifying proteins involved in the KRAS signaling pathway, a proteomics analysis was performed on KRAS mutant and silenced KRAS mutant colorectal cancer cell lines. The results demonstrated that SUSD2 expression was upregulated upon *KRAS* silencing, suggesting that SUSD2 may have a tumor suppressor role in colorectal cancer. As such, the main objective of this dissertation is to evaluate the involvement of SUSD2 upregulation in mediating the tumor suppressive effects of *KRAS* silencing.

For that, we intend to address two specific aims:

- Specific aim 1: To validate SUSD2 upregulation at both mRNA and protein levels upon *KRAS* silencing in an extended panel of KRAS mutant CRC cell lines.
- Specific aim 2: To evaluate the functional effects of increasing SUSD2 expression upon silencing the mutated *KRAS* in CRC.

III. MATERIAL AND METHODS

1. Cell Lines and Cell Culture

Human colorectal cancer cell lines HCT-15, HCT-116, SW480, SW620, and LS-174T include in this study were available at i3S. HCT-15, HCT-116, and SW480 were cultured in RPMI medium, whereas SW620 and LS174T were cultured in DMEM medium. All culture media was supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin [100 units/mL of penicillin and 100 µg/mL of streptomycin]. Cells were kept at 37°C in a humidified incubator with 5% carbon dioxide.

For maintenance, cell lines were passaged when 80% confluence, maximum, was reached. For that, Phosphate-buffered saline (PBS) was used to remove FBS (trypsin inhibitor), dead cells, and culture medium. Trypsin was added for long enough to detach cells from the culture flask. Afterward, to inactivate trypsin and provide growth factors and hormones, cell culture medium with FBS was added. 125µl from this flask was transferred to a new flask with a new culture medium.

Table 1. Characterization by the molecular pathways MSI, CIMP, CIN and mutation status of KRAS and BRAF, associated disease and origin of the used CRC cell line in this study. Adapted from Ahmed et al. (152)

Cell Line	MSI Status	CIMP	CIN	KRAS	BRAF	Disease	Derived Tumor
HCT-116	MSI	+	-	G13D	WT	Colon Carcinoma	Primary Tumor
HCT-15	MSI	+	-	G13D	WT	Colon Adenocarcinoma	HCT-15/DLD-1 misclassified
SW480	MSS	-	+	G12V	WT	Colon Adenocarcinoma	Primary Tumor
SW620	MSS	+	+	G12V	WT	Colon Adenocarcinoma	Lymph node metastasis
LS-174T	MSI	-	-	G12D	WT	Colon Adenocarcinoma	Subcultured LS 180

2. *KRAS* and *SUSD2* Gene Silencing

Gene silencing was achieved using two small interfering RNA (siRNA), specific for each gene: KRAS (L-005069-00-0010) and SUSD2 (cat. nº 229610916, Integrated DNA Technologies). A sequence with no homology to any gene, referred to as non-targeting (NT) siRNA, was used as a negative control at the same concentration as the siRNA targeting the genes of interest. *KRAS* silencing was performed in HCT-116, HCT-15, SW480, SW620, and LS-174T cell lines, while *SUSD2* silencing was performed in HCT-116 cell line. An appropriate cell concentration (HCT-116 cells 1.50 x 105, HCT-15 cells 2.00 x 105, SW620 cells 4.00 x 105, SW480 cells 2.50 x 105, LS-174T cells 2.00 x 105) was plated in 6-well plates with appropriate completed culture medium. To enable siRNAs entry within the cells, lipofectamine RNAiMAX was used according to the manufacturer's guidelines. Opti-MEM (1,5 ml) was used to replace the culture medium. 10 nM of each siRNA was added to 250 µl Opti-MEM, and 3 µl of lipofectamine RNAiMAX to another 250 µl of Opti-MEM, then stood for 5 minutes at RT. Individually, both siRNA and lipofectamine were mixed and incubated for 20 minutes at RT. Afterward, both mixes were placed in contact with the cells, followed by 6 hours of incubation at 37°C. After this time, the medium was replaced with a fresh

complete culture medium, followed by incubation at 37° C continued until 48h post-transfection. *KRAS* silencing was assessed by Western blotting and *SUSD2* silencing by flow cytometry.

3. Western Blot Analysis

Western blotting was performed to evaluate *KRAS* silencing and heat shock protein 70 (Hsc70) expression. Hsc70 was used as housekeeping to control the amount of protein lysate loaded into each well.

3.1. Sample Preparation (Protein Extraction and Quantification)

Cells were placed on ice and washed with ice-cold PBS 1X. Cold RIPA buffer (50 mM Tris HCl; 150 mM NaCl; 2 mM EDTA; 1% IGEPAL CA-630; pH=7.5), supplemented with 1:7 proteases inhibitors cocktail (Roche, Basel, Switzerland) and 1:100 phosphatases inhibitors cocktail (Sigma-Aldrich) was used to induce cell lysis. After being scrapped, cells were centrifugated at 14000 rpm at 4°C, for 10 minutes. Supernatants were collected and protein quantification was performed using the Bradford assay with DC Protein assay kit (BioRad, Hercules, CA), and read at an absorbance of 655 nm. Then, 6 µl of Laemmli sample buffer (0.5 M Tris-HCl pH 6.8, 9.2g sodium dodecyl sulfate (SDS), 40mL Glycerol, 5% 2-mercaptoethanol, 5% bromophenol blue) was added to 20 µg of protein, and the mix boiled for 5 minutes at 95°C for protein denaturation.

Table 2. Primary and secondary antibody for Western Blot.

Target molecule	Serum	Molecular Weight	Dilution	Manufacter	Catalog nº	Blockade
Primary Antibodies						
KRAS	Mouse	21	1:4000	LSBio	LS-C175665-100	5% Milk
Hsc70	Mouse	70	1:10000	Santa Cruz Biotechnology	Sc-7298	5% Milk
Secundary Antibodies						
KRAS			1:8000	GE Healthcare	NA931V	
Hsc70			1:20000	GE Healthcare	NA931V	

3.2. Western Blotting

Each sample and a molecular weight ladder (Bio-Rad, Precision Plus protein standard) was loaded on a 7,5% SDS-polyacrylamide gel and run at 80 V for approximately 30 minutes and then at 120 V, until the end. Following electrophoresis, the protein was transferred to Amersham Protran Premium 0,45 µm nitrocellulose blotting membranes (GE Healthcare) for 90 min at 100 V. After gel transfer, the membranes were incubated with Ponceau S solution, allowing fast and reversible staining for protein bands. Then, membranes were blocked with 5% non-fat powder milk

in PBS-T for 1 hour. Subsequently, the membranes were incubated overnight with the primary antibody at 45 rpm, and 4°C. Before being incubated with the HRP-conjugated anti-mouse secondary antibody, the membranes were washed 5 times, each one of 5 minutes, with PBS-T and then incubated with the secondary antibody for 1 hour. After 6 new washes, each one of 6 minutes, the membranes were incubated with the Kit Clarity Western ECL for signal detection. Densitometric analysis of bands was performed with ImageJ (National Institute of Health, USA).

4. SUSD2 Expression Quantification

4.1. Total RNA Extraction/Quantification

Cell culture was removed, and the cells were washed twice with PBS 1X. Cells were lysed by adding 500 µl per well of TripleXtractor, and scrapping. The solution was transferred to an RNase-free Eppendorf and centrifugated at 4°C. In order to isolate RNA, 100 µl of chloroform was added (ratio 1:5 TripleXtractor) to the solution, shaked, and incubated at room temperature (RT) for 3 minutes. After, the samples were submitted to 15 minutes of centrifugation at 15000 relative centrifugal force (rcf) and 4°C. The aqueous phase, where RNA is contained, was extracted into a new DNA/RNAse-free Eppendorf. Isopropanol (500 µl) was added to precipitate the RNA (ratio 1:1 TripleXtractor). After 10 minutes of incubation at RT, the solution was centrifugated at 15000 rcf for 10 minutes at 4°C. The supernatant was discarded, and the pellet was washed two times, both with 1 ml of 70% ethanol (Prepared with DNA/RNAse-free water), followed by centrifugation at 15000 rcf for 5 minutes at 4°C. The ethanol was discarded, and to remove traces of ethanol the samples were air-dried. Afterward, the RNA pellet was dissolved in 20 µl of DNA/RNAse-free water. RNA quantification and purity were obtained using a Nanodrop Spectrophotometer ND-100. Samples were then stored at -80°C.

4.2. cDNA Synthesis

Complementary deoxyribonucleic acid (cDNA) synthesis from 1000 ng of RNA was accomplished with qScriptTM cDNA SuperMix kit, according to manufacturer instructions. The reverse transcriptase reaction was carried out in the thermocycler using the following conditions: 5 minutes at 25°C, 60 minutes at 42°C for reverse transcription, and 5 minutes at 85°C to stop the reaction.

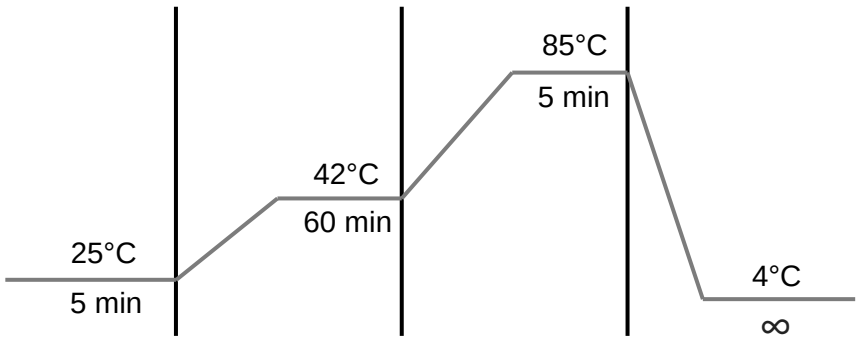


Figure 9. Thermocycler programme.

4.3. Quantitative Real-Time PCR

Quantitative real-time PCR (RT-qPCR) was performed in order to validate SUSD2 upregulation upon *KRAS* silencing, at mRNA level, and to evaluate a possible regulation of SUSD2 on several markers: Leucine Rich Repeat Containing G Protein-Coupled Receptor 5 (LGR5), TWIST, Vimentin, Cadherin 1 (CDH1), Zinc Finger E-Box Binding Homeobox 1 (ZEB1), and NANOG. GAPDH was used as an internal control for normalization. The TaqMan assay was used as a detection method for quantification. The relative quantity of all target genes was determined by subtraction of the internal control gene CT (Threshold cycle) from the target gene CT. The relative expression was calculated using the 2−ΔΔCT method, through subtraction of the non-targeting group ΔCT from the silenced condition ΔCt. Expression differences between each group were achieved by the following formula:

$$Fold\ change = \frac{2^{-\Delta\Delta CT}\ (SiCondition\ group)}{2^{-\Delta\Delta CT}\ (NT\ group)}$$

The mix consists of DNA/RNAse-free water (4 µL), cDNA sample (0.5 µl), interest probe (0,5 µl), and TaqMan Universal PCR Master Mix (5 µl). Triplicates were performed for all samples. To ensure DNA/RNAse-free water and Master Mix were free of contaminants, a negative control for each gene composed of probe mix (5.5 µl) and DNA/RNAse-free water (4.5 µl) was used. The samples were analyzed by the 7500 Fast Real-Time PCR System (Applied Biosystems).

Table 3. Taqman Gene Expression Assays used to assess the mRNA levels for the selected genes.

Gene	TaqMan assay reference	Manufacturer
SUSD2	Hs.PT.58.22484230.gs	Integrated DNA Technologies
KRAS	Hs00270666_m1	Thermo Fischer
CDH1	Hs.PT.53.2388193	Integrated DNA Technologies
NANOG	Hs.PT.58.21480849	Integrated DNA Technologies
TWIST1	Hs.PT.56.18940950	Integrated DNA Technologies
ZEB1	Hs.PT.58.39178574	Integrated DNA Technologies
VIMENTIN	Hs.PT.47.14705389	Integrated DNA Technologies
LGR5	Hs00969422_m1	Thermo Fischer
GAPDH	Hs.PT.39a.22214836	Integrated DNA Technologies

5. Flow Cytometry

Flow cytometry was performed to validate SUSD2 upregulation upon *KRAS* silencing, at protein level, evaluate SUSD2 localization, validate *SUSD2* silencing upon 48 hours of transfection, and measure the expression of cell surface molecules: CD24, CD44, CD44v6, CD47, CD133, CD166, HLA-ABC, and PD-L1. For that, membrane and intracytoplasmic staining were performed. Independently of the type of staining, all samples were acquired in BD Accuri C6, and the data was analyzed in the Accuri CFlow Sampler software (CFlow).

5.1. Membrane Staining

Membrane staining was used to validate SUSD2 upregulation upon *KRAS* silencing, evaluate SUSD2 localization, and measure the expression of cell surface molecules. Cultured cells were isolated from their culture medium, washed with PBS, and trypsinized. Trypsin was neutralized with FBS, and the cells were then left to stand for 30-40 minutes at 37°C to recover the cell surface molecules. Then, cells were washed with flow cytometry buffer (PBS and 1%FBS) or Fluorescence-activated cell sorting (FACS) buffer and centrifugated at 1200 rpm for 5 minutes at 4°C. The primary antibodies (PE-conjugated CD24, APC-conjugated CD44, APC-conjugated CD44v6, FITC-conjugated CD47, APC-conjugated CD133, APC-conjugated CD166, FITC-conjugated PD-L1, PE-conjugated HLA-ABC, Purified anti-human SUSD2) was added, followed by 10 or 15 minutes of incubation at RT. Then, cells were washed with flow cytometry buffer or FACS buffer, followed by centrifugation at 300g for 5 minutes at 4ºC. Since the primary antibody of SUSD2 has no fluorochrome, a secondary antibody (Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, #A11029, Invitrogen) was used, followed by an additional wash step. Lastly, formaldehyde (2%) was used to fix the cells.

5.2. Intracytoplasmic Staining

Intracytoplasmic staining was performed to validate SUSD2 upregulation upon *KRAS* silencing, evaluate SUSD2 localization, and validate *SUSD2* silencing. Before adding the primary antibody (Purified anti-human SUSD2 antibody, #327401, BioLegend), the cells were exposed to formaldehyde (2%) to fix them. After washing the cells with FACS buffer, saponin (0.5%) was added to permeabilize the cells. The remaining steps are the same described above for membrane staining, except that in the end, it is not necessary to fix the cells, as they have already been fixed.

5.3. Annexin V/Propidium Iodide Apoptosis Assay

Annexin V/Propidium Iodide Apoptosis Assay was performed to evaluate the role of SUSD2 in apoptosis. Cells were separated from their culture medium, washed with PBS, and trypsinized. Then we proceed with the washing of cells with PBS 1X, followed by centrifugation for 10 minutes at 2000 rpm and RT. After this step, positive control was created with 50% saponin, to induce cell death. Cells undergo a new wash for 10 minutes at 300g and RT. 200 µl of 1x Annexin-binding buffer and 1 µl of FITC-conjugated Annexin V antibody were added, followed by 15 minutes of incubation at RT, in the dark. After the incubation period, 100 µl of Annexin-binding buffer was added to the cells. Immediately before acquiring the samples in BD Accuri C6, 1 µl of propidium iodide was added to all samples.

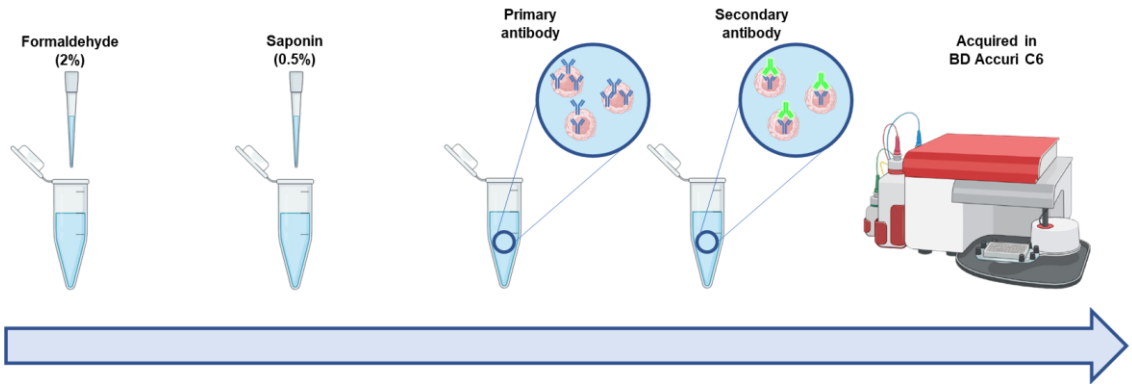


Figure 10. SUSD2 staining for flow cytometry analysis. Created with BioRender.com.

Table 4. Flow cytometry antibodies.

Antibody	Clone	Conjugated fluorophore	Manufacter	Catalog nº	µl/10^5 cells
SUSD2	W5C5		Biolegend	327401	1
2^{ND}		Alexa Fluor 488	Invitrogen	A-11029	0.5
Annexin		FITC	BD Biosciences	556547	1
CD24	32D12	PE	Miltenyi Biotec	130-098-210	0.5
CD44	Discontinued	APC	Miltenyi Biotec	130-098-210	0.5
CD44v6	REA706	APC	Miltenyi Biotec	130-111-425	0.5
CD47	MEM-122	FITC	Immunotools	21270473	0.5
CD133	AC133	APC	Miltenyi Biotec	130-098-826	0.5
CD166	REA442	APC	Miltenyi Biotec	130-106-619	0.5
HLA-ABC	W6/32	PE	Immunotools	21159034	0.5
PD-L1	MIH1	FITC	BD Pharmingen	555748	1

6. Aggregation Assay

200 µl of a cell suspension with a concentration of 1 x 105 cells/ml were plated in a 6-wells plates previously prepared with a solution composed of 100mg of Bacto Agar in 15 ml of PBS 1X. Then cells were incubated at 37ºC for 24 hours, and for 48 hours. Every 24 hours of incubation, images from the wells were collected.

7. *In Silico* Assay

To evaluate SUSD2 expression in CRC, and validate KRAS regulation on SUSD2 expression, an *in silico* analysis of TCGA database was conducted. In this analysis, the association between this regulation and the MSI status and CRC patients stage was also evaluated.

8. Statistical Analysis

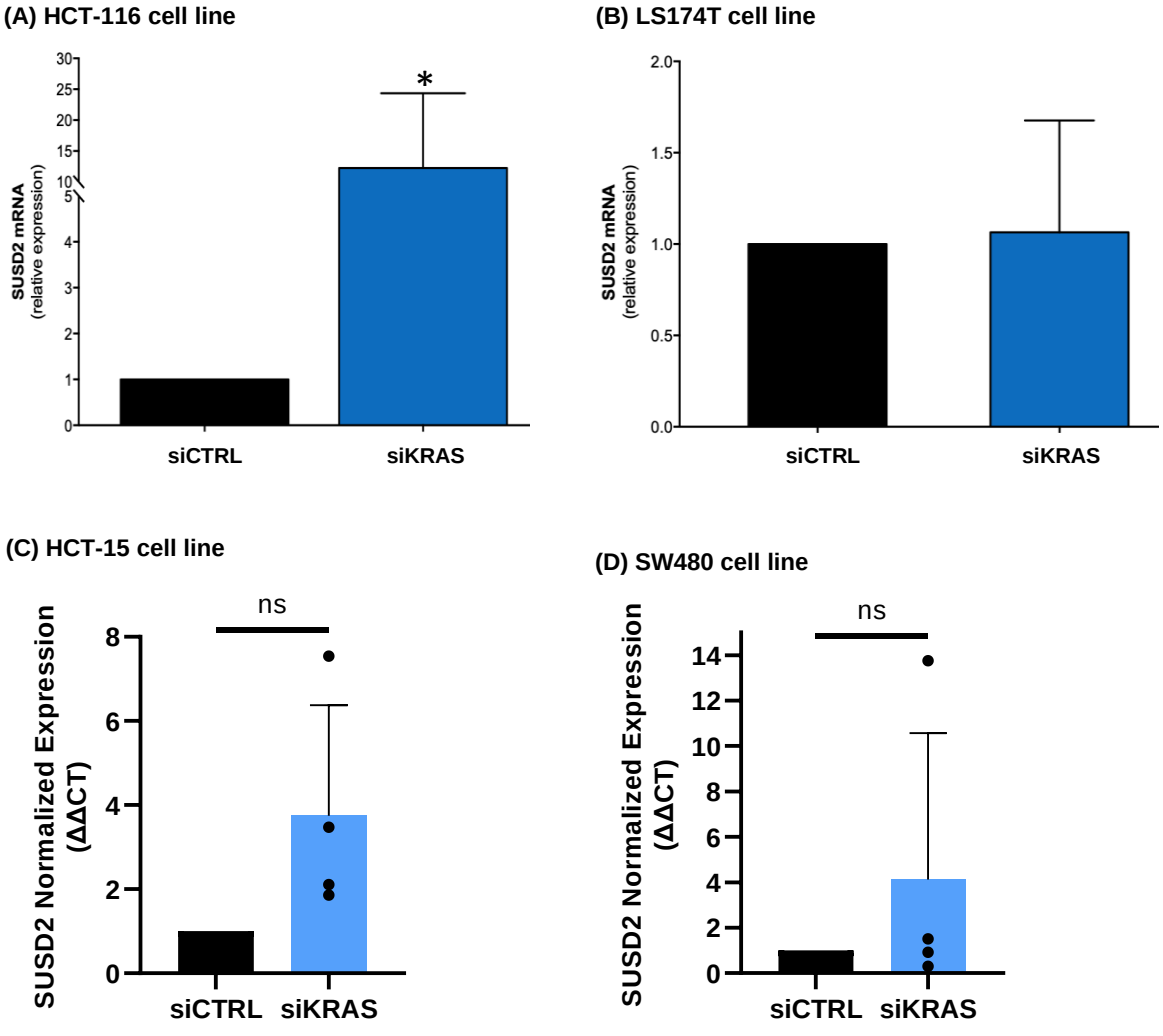
Data analysis was performed with Prism 9.0 (GraphPad Software, USA). All the data are presented as the mean ± standard deviation (SEM), and statistical significance was determined as $P < 0.05$ by Student's t-test, Mann-Whitney test, and One-way ANOVA test. All means were calculated from data of all the independent experiments.

IV. RESULTS

1. Assessment of mRNA expression of SUSD2 upon KRAS silencing in CRC cell lines

Firstly, we intended to determine whether the negative regulation exerted by KRAS on SUSD2 protein expression was also observed at the mRNA level, and whether this effect was in transversal to other cell lines. To do so, we selected a panel of KRAS mutant colorectal cancer cell lines in which the expression of KRAS had been silenced through siRNA. Differences in SUSD2 expression at mRNA level were analyzed between the siCTRL (control) and *siKRAS* groups. *KRAS* silencing was confirmed through Western Blot.

Regarding HCT-116 cell line, the results show a significant difference in the relative expression of SUSD2 between the conditions under study. *KRAS* silencing promoted an increased SUSD2 expression, as shown in **Figure 11**. In the LS174T cell line, no differences in SUSD2 expression were observed. Furthermore, in HCT-15, SW480, and SW620 cell lines an increasing trend in SUSD2 expression upon *KRAS* silencing is also noted. However, SUSD2 expression in these cell lines presented a high variability between biologic replicates, and therefore statistical significance was not achieved.



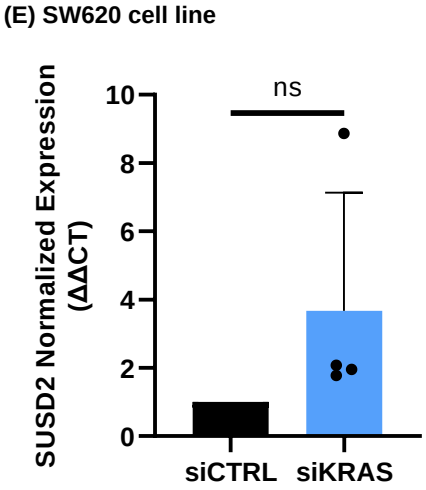


Figure 11. SUSD2 mRNA expression upon KRAS silencing in CRC cell lines. SUSD2 mRNA expression levels were assessed by RT-qPCR in HCT116 (n=5), LS-174T (n=5), HCT-15 (n= 4), SW480 (n= 4), SW620 (n= 4) cell lines; **(A)** HCT-116 cell line; **(B)** LS174T cell line ; **(C)** HCT-15 cell line; **(D)** SW480 cell line; **(E)** SW620 cell line; Mann-Whitney test was performed; * indicates a significantly different result P<0:05; ****Previous result by Patrícia Carvalho in our group**.

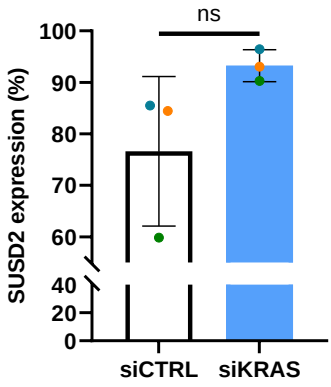
2. Assessment of protein expression of SUSD2 upon KRAS silencing in CRC cell lines

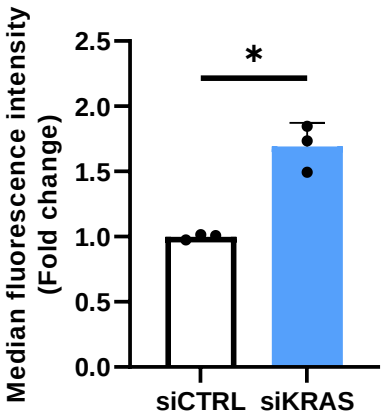
To address whether the effects of KRAS on SUSD2 expression observed at mRNA level extends to protein level, and to evaluate if that regulation occurs intracytoplasmic (IC) or at cell surface (CS), flow cytometry was performed.

Regarding HCT-116 cell line, the results of the CS staining show statistically significant alterations in the number of SUSD2 positive cells upon *KRAS* silencing. Contrarily, in the IC, despite the results showing an increase in the number of SUSD2 positive cells, no statistical significance was reached. Nevertheless, the results show a statistically significant increase in median fluorescence intensity (MFI) of the IC staining **(Figure 12)**.

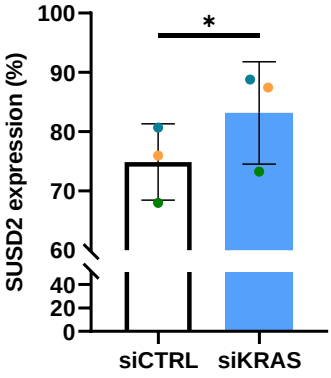
In SW480, SW620, and LS-174T cell lines, no differences were observed in the number of SUSD2 positive cells upon *KRAS* silencing, in both CS and IC stainings. Regarding MFI, the same was also observed. Interestingly, in the CS of SW620 cell line, the results show a statistically significant decrease in MFI in the *siKRAS* condition.

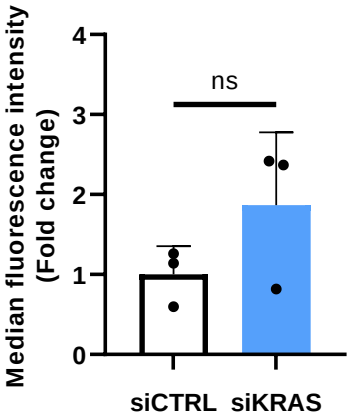
(A) HCT-116 cell line - IC



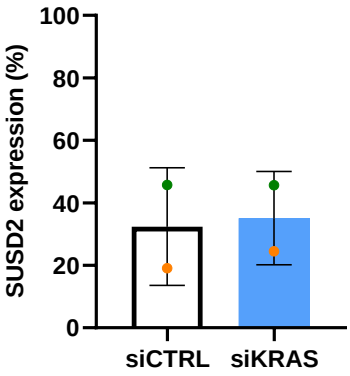


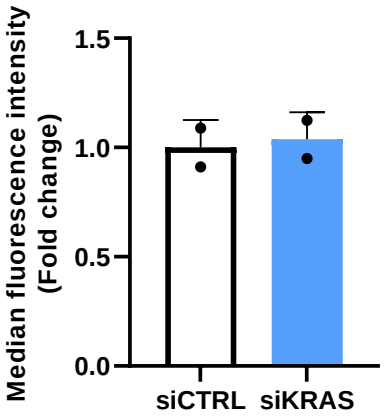
(B) HCT-116 cell line – CS



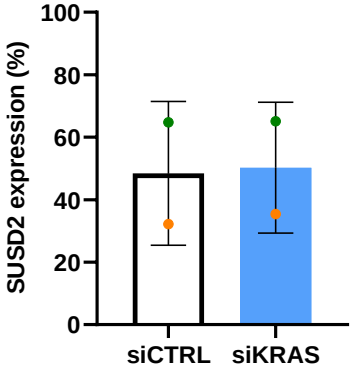


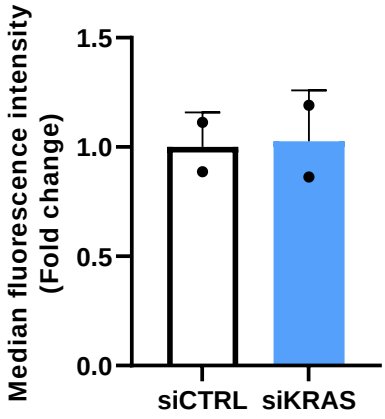
(C) SW480 cell line - IC





(D) SW480 cell line - CS





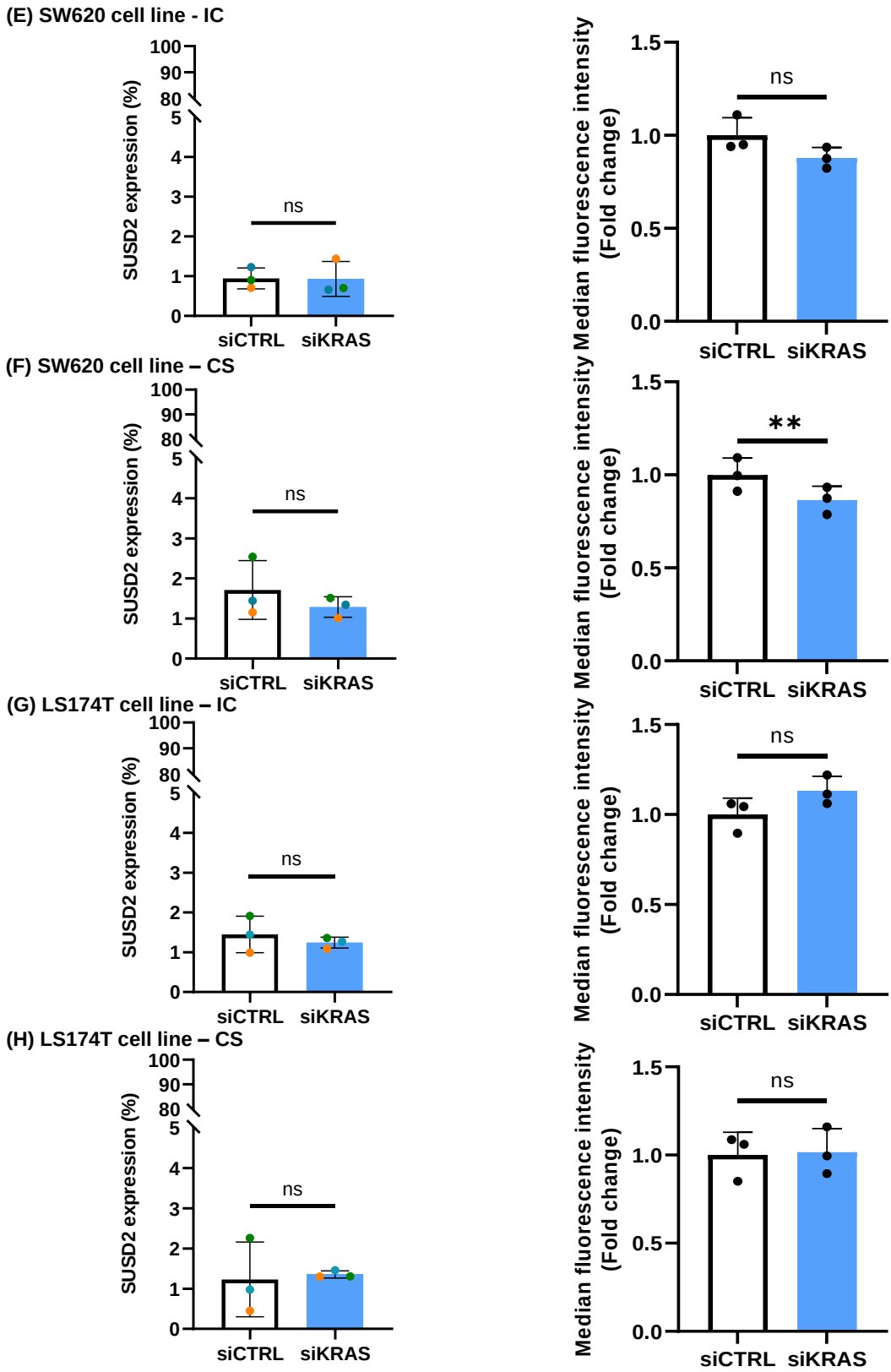


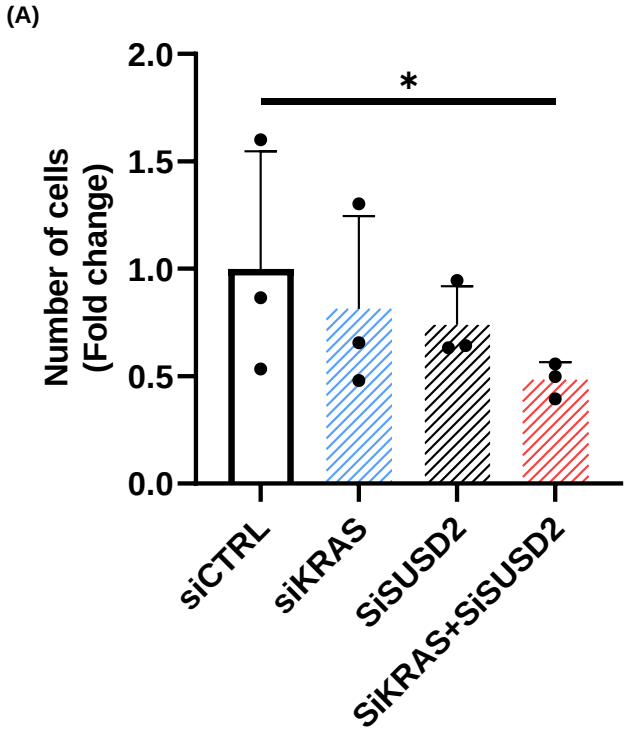
Figure 12. SUSD2 protein expression upon KRAS silencing in CRC cell lines. SUSD2 protein expression levels were assessed by flow cytometry in HCT116 (n=3), SW480 (n=2), SW620 (n= 3), LS174T (n=3) cell lines; **(A)** HCT-116 cell line - IC; **(B)** HCT-116 cell – CS; **(C)** SW480 cell line - IC; **(D)** SW480 cell line - CS; **(E)** SW620 cell line - IC; **(F)** SW620 cell line – CS; **(G)** LS174T cell line – IC; **(H)** LS174T cell line – CS; IC – Intracytoplasmatic; CS – Cell surface; Student's t-test was performed; * indicates a significantly different result P<0:05; ** P<0:01

3. Assessment of SUSD2 functional activity as a downstream target of KRAS

In an attempt to understand how SUSD2 upregulation upon KRAS inhibition in the HCT-116 cell line are translated into a biologic effect, we silenced the expression of *KRAS*, *SUSD2* or both genes using siRNA and evaluated cell growth and apoptosis as well as the expression levels of markers of cell differentiation, immune and cancer stemness. To do so, four different experimental groups were established: (1) the siCTRL group, was created as a negative control; (2) & (3) the *siKRAS* and *siSUSD2* groups, respectively, were created for the purpose of demonstrating how these genes regulate the evaluated markers; (4) lastly, the *siKRAS/siSUSD2* group was established to evaluate the involvement of SUSD2 upregulation in mediating the tumor suppressive effects of *KRAS* silencing. In every experiment, *KRAS* silencing was confirmed through Western Blot, and *SUSD2* silencing through flow cytometry **(Figure S1 and S3)**.

3.1. Assessment of cell growth and apoptosis upon KRAS, SUSD2 and both silenced genes in HCT-116 cell line

The results show a decreasing trend upon *KRAS* and *SUSD2* silencing in the number of cells **(Figure 13.A)**. Furthermore, in the *siKRAS*/*siSUSD2* group, a significant decline in the number of cells was observed.



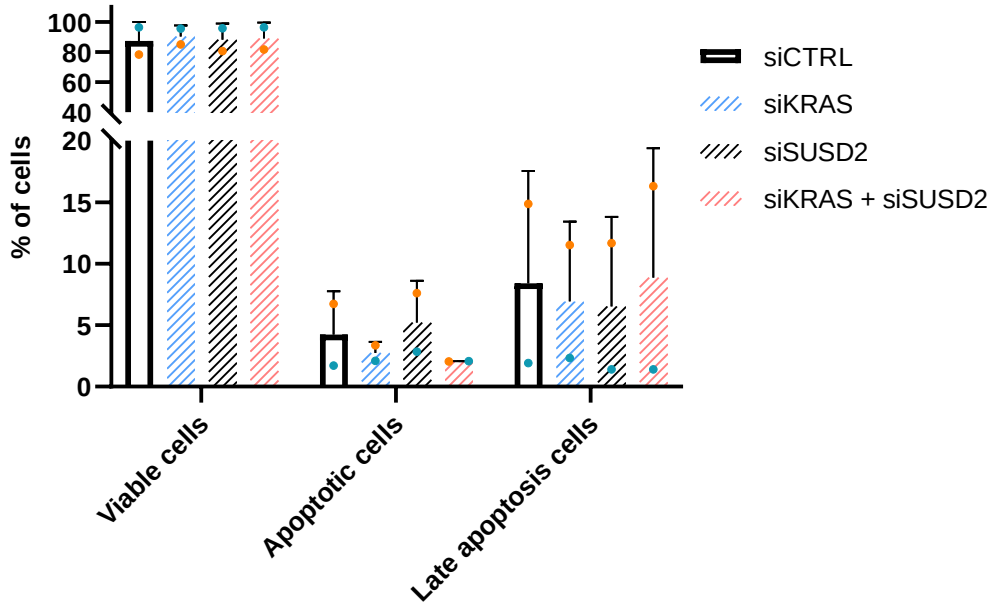


Figure 13. SUSD2 role in proliferation and apoptosis. (A) Number of cells, results are presented as fold change relative to the siCTRL **(B)** Percentage of viable, apoptotic and late apoptosis cells in siCTRL, siKRAS, siSUSD2 and siKRAS/siSUSD2 HCT-116 cells (n=2); One-way ANOVA test was performed; * indicates a significantly different result P<0:05

In order to evaluate a possible regulation of SUSD2 in CRC apoptosis, Annexin V/Propidium Iodide staining was performed in four different groups (siCTRL, *siKRAS*, *siSUSD2*, and *siKRAS*/*siSUSD2*). Data were acquired by flow cytometry. Importantly, given the lower number of replicates, no statistical analysis was performed. The results show no alterations in the viable cells between the groups. Moreover, a viable cells percentage of about 89% was observed in all groups. Apoptotic and late apoptosis cells follow the same tendency **(Figure 13.B)**.

3.2. Assessment of mRNA expression of different epithelial and mesenchymal markers upon KRAS, SUSD2 and both silenced genes in HCT-116 cell line

RT-qPCR was performed to assess the mRNA levels of different epithelial and mesenchymal markers related to CRC progression. The expression levels of *CDH1*, *LGR5*, *NANOG*, *TWIST*, *Vimentin*, and *ZEB1* were evaluated in the four experimental groups. Importantly, given the lower number of replicates, we did not achieve statistical significance for any marker. *KRAS* silencing was confirmed through Western Blot, and *SUSD2* silencing through flow cytometry **(Figure S1 and S3)**.

3.2.1. *CDH1*

The mRNA expression levels of the epithelial marker *CDH1* gene (encoding for E-cadherin protein) were evaluated by RT-qPCR **(Figure 14)**. The results demonstrate that in the *siKRAS* group no changes in *CDH1* expression were observed. Contrarily, in the *siSUSD2* group, the results suggest a decrease in CDH1 expression levels. Lastly, in the *siKRAS*/*siSUSD2* group, *CDH1* expression levels show to be between the expression levels exhibited in the *siKRAS* and *siSUSD2* groups

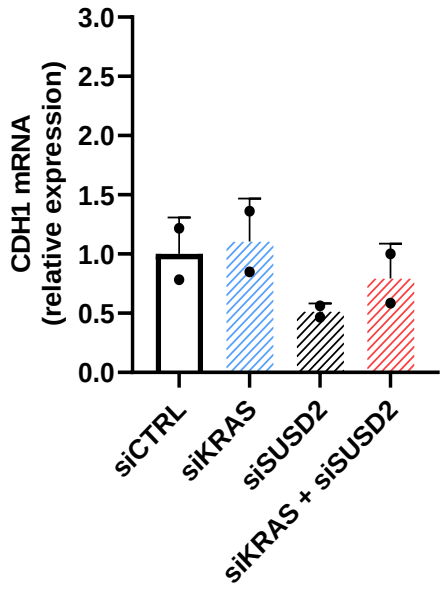


Figure 14. CDH1 mRNA expression in different groups in HCT-116 cell line. CDH1 mRNA expression levels were assessed by qRT-PCR in HCT116 cell line (n=2).

3.2.2. *LGR5* and *NANOG*

The expression levels of the intestinal stem cell markers *LGR5* and *NANOG* genes was also accessed by RT-qPCR **(Figure 15)**. The results suggest an upregulation of *LGR5* mRNA in the *siKRAS* group. In contrast, in *siSUSD2* and *siKRAS*/*siSUSD2* groups, the results tend to a decreased expression of *LGR5*.

The results regarding *NANOG* mRNA suggest a substantial decrease expression of *NANOG* in the *siKRAS* group, whereas no differences were observed in the expression of this marker in the *siSUSD2* group. In contrast, in the *siKRAS*/*siSUSD2* group, *NANOG* expression levels tend to be between the expression levels exhibited in the *siKRAS* and *siSUSD2* groups **(Figure 15.B)**.

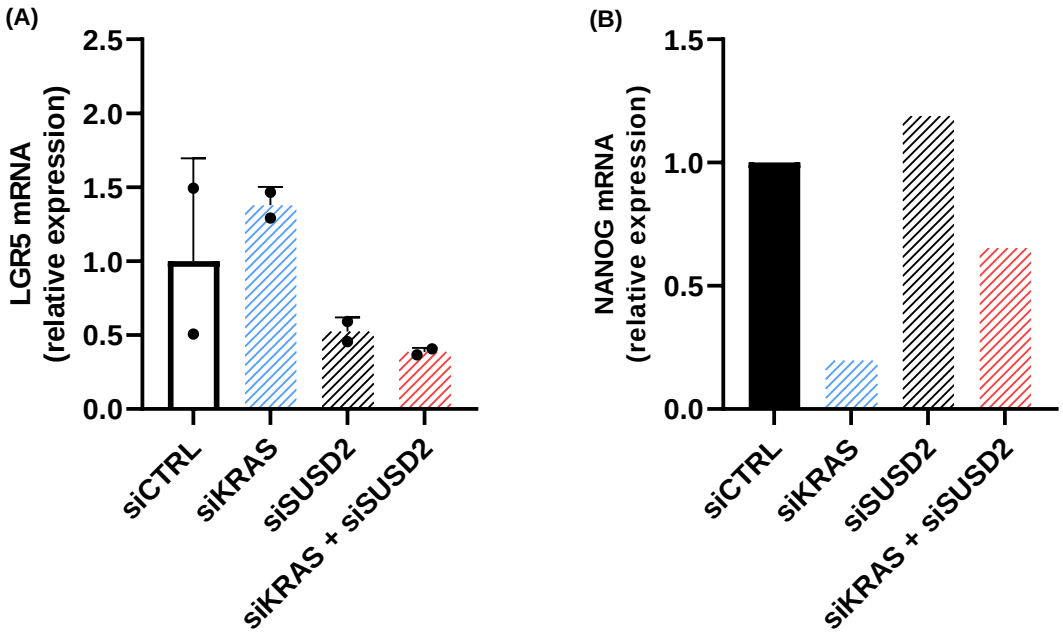


Figure 15. LGR5 and NANOG mRNA expression in different groups in HCT-116 cell line. (A) LGR5 mRNA expression levels were assessed by qRT-PCR in HCT116 cell line (n=2); **(B)** NANOG mRNA expression levels were assessed by qRT-PCR in HCT116 cell line (n=1).

3.2.3. *TWIST1*, *ZEB1* and *Vimentin*

Regarding the expression levels of the epithelial-to-mesenchymal transition markers *TWIST1*, *ZEB1* and *Vimentin* **(Figure 16)**, we observed that in all groups (*siKRAS*, *siSUSD2*, and *siKRAS*/*siSUSD2*), the expression of *TWIST1* decreases. In the *siKRAS* and *siKRAS*/*siSUSD2* groups, *Vimentin* expression tends to decrease. In contrast, in the *siSUSD2* group, no differences were reported. *ZEB1* downregulation was observed in the *siKRAS* group. In contrast, in the *siSUSD2* group, no differences are observed, while in the *siKRAS*/*siSUSD2* group, *ZEB1* expression levels show to be between the expression levels presented in the *siKRAS* and *siSUSD2* groups **(Figure 16.C)**.

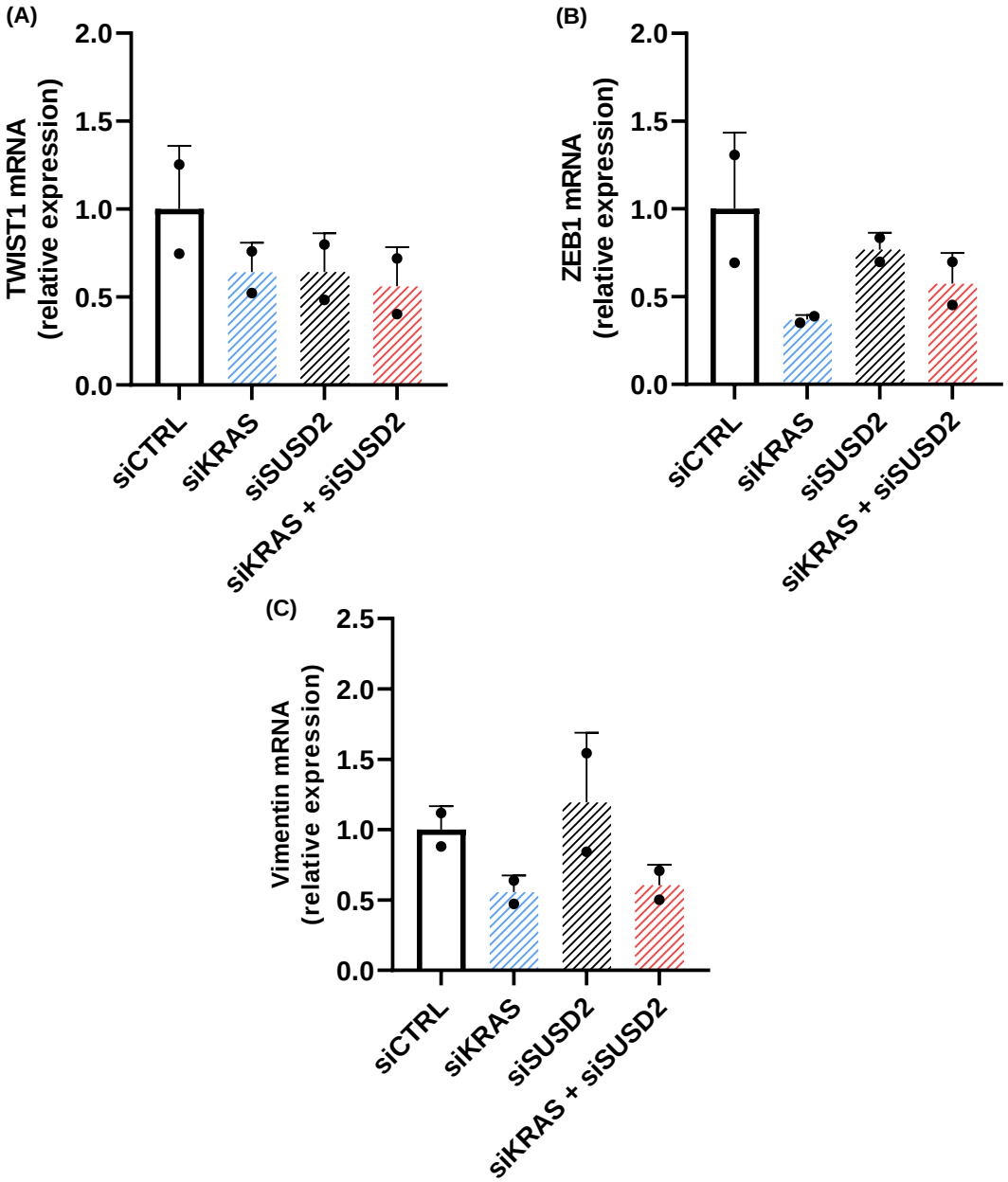


Figure 16. TWIST1, ZEB1 and Vimentin mRNA expression in different groups in HCT-116 cell line. **(A)** TWIST1 mRNA expression levels were assessed by qRT-PCR in HCT116 cell line (n=2). **(B)** ZEB1 mRNA expression levels were assessed by qRT-PCR in HCT116 cell line (n=2); **(C)** Vimentin mRNA expression levels were assessed by qRT-PCR in HCT116 cell line (n=2).

3.3. Assessment of protein expression levels of different molecules related to CRC stemness and immune regulation upon KRAS, SUSD2, and both silenced genes in HCT-116 cell line

In an effort to understand if SUSD2 expression differences in the HCT-116 cell line are translated into a biologic effect, protein expression of different molecules related to distinct oncogenic traits that support CRC progression were accessed by flow cytometry. The expression levels of the CRC stem cell markers CD24, CD44, CD44v6,

CD47, CD133, CD166, and immune modulators HLA-ABC and PD-L1 were evaluated in the four experimental groups (siCTRL, *siKRAS*, *siSUSD2*, and *siKRAS/siSUSD2*). Importantly, given the lower number of replicates, we did not achieve statistical significance for any marker.

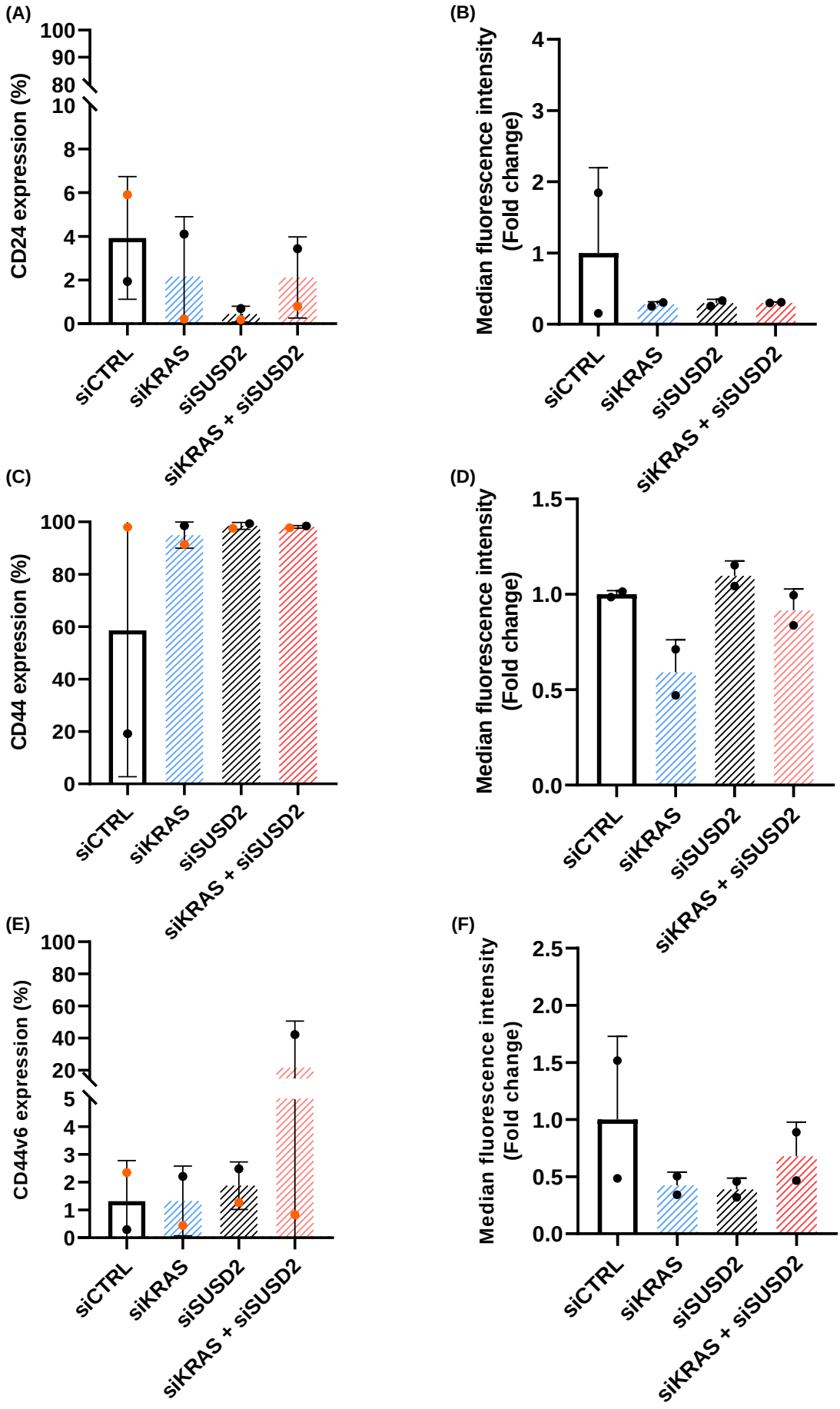
3.3.1. Assessment of SUSD2 impact on CRC stem cell markers

The obtained results show no alterations in the number of CD24 positive cells between the groups. Moreover, the percentage of positive cells is close to 0% in all groups. Nevertheless, the results show a decreasing tendency of MFI in all groups.

Regarding CD44, the results suggest an upregulation of this protein in the *siKRAS*, *siSUSD2*, and *siKRAS*/*siSUSD2* groups. However, this tendency should be due to a defect in the staining in one sample of the siCTRL group. Nevertheless, MFI tends to decrease in the *siKRAS* group. Contrarily, in the *siSUSD2* groups no differences were observed, while, in the *siKRAS*/*siSUSD2* group, CD44 expression levels tend to be between the expression levels exhibited in the *siKRAS* and *siSUSD2* groups **(Figure 17.D)**. The results show no alterations in the number of CD44v6 (a CD44 splicing isoform) positive cells in the *siKRAS* and *siSUSD2* groups compared to the siCTRL group. Moreover, in these three groups, the percentage of positive cells is close to 0%. However, in the *siKRAS*/*siSUSD2* group, the results show an increasing tendency in the number of CD44v6 positive cells. Regarding the MFI, in the *siKRAS* and *siSUSD2* groups, a decreasing tendency was reported, while in the *siKRAS*/*siSUSD2* group no alterations were observed.

The results suggest a CD133 downregulation upon *KRAS* silencing. Otherwise, in the *siSUSD2* group no differences in the number of CD133 positive cells were obtained, while, in the *siKRAS*/*siSUSD2* group, CD133 expression levels tend to be between the expression levels displayed in the *siKRAS* and *siSUSD2* groups **(Figure 17.G)**. Regarding the MFI levels, no differences were observed between the groups.

In all groups, the percentage of CD166 positive cells is close to 100%, and no alterations were found, neither in the number of CD166 positive cells nor in MFI values between the groups.


(A)
CD24 expression (%)
100
90
80
10
8
6
4
2
0
siCTRL
siKRAS
siSUSD2
siKRAS + siSUSD2
(B)
Median fluorescence intensity (Fold change)
4
3
2
1
0
siCTRL
siKRAS
siSUSD2
siKRAS + siSUSD2
(C)
CD44 expression (%)
100
80
60
40
20
0
siCTRL
siKRAS
siSUSD2
siKRAS + siSUSD2
(D)
Median fluorescence intensity (Fold change)
1.5
1.0
0.5
0.0
siCTRL
siKRAS
siSUSD2
siKRAS + siSUSD2
(E)
CD44v6 expression (%)
100
80
60
40
20
5
4
3
2
1
0
siCTRL
siKRAS
siSUSD2
siKRAS + siSUSD2
(F)
Median fluorescence intensity (Fold change)
2.5
2.0
1.5
1.0
0.5
0.0
siCTRL
siKRAS
siSUSD2
siKRAS + siSUSD2

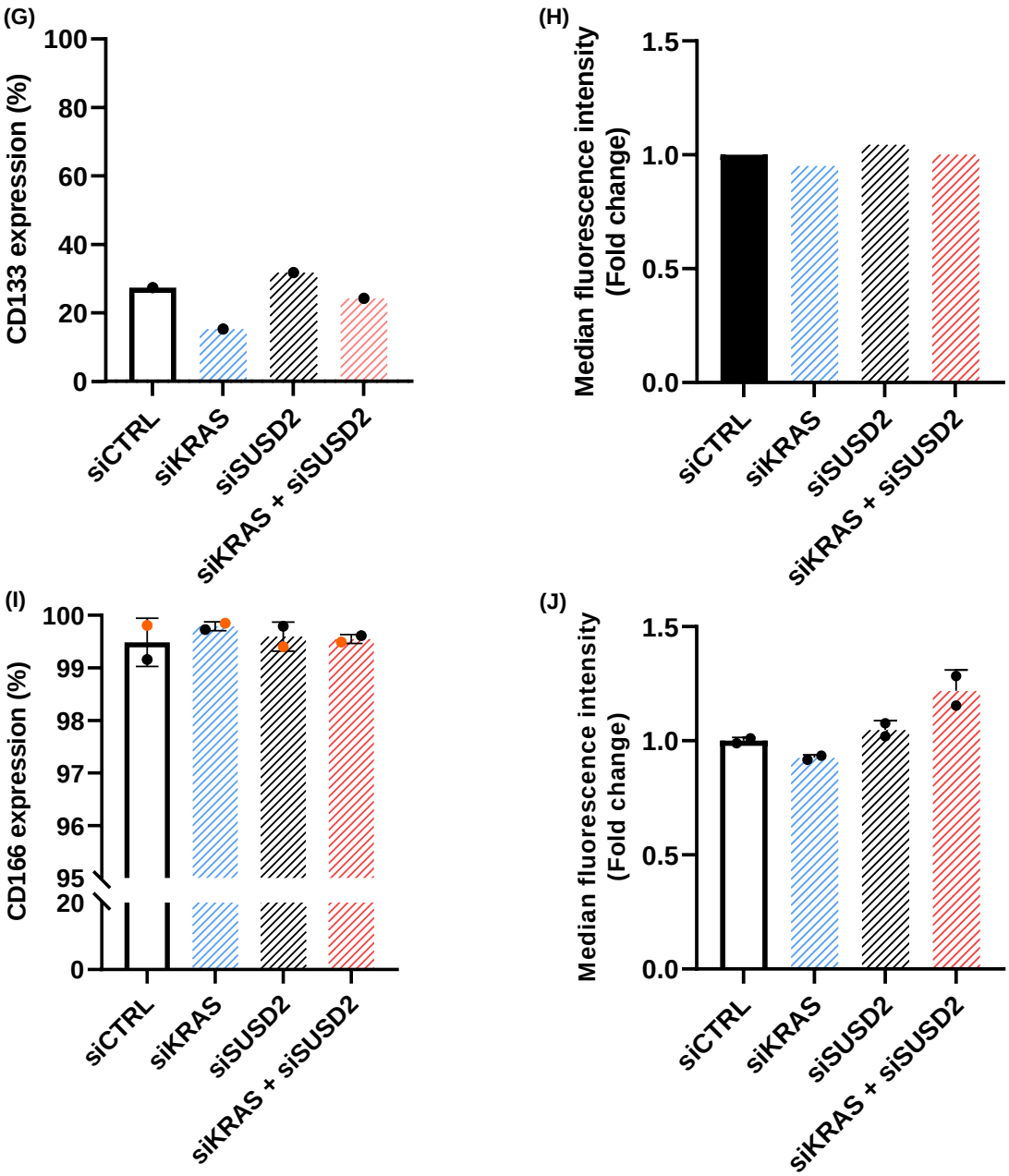
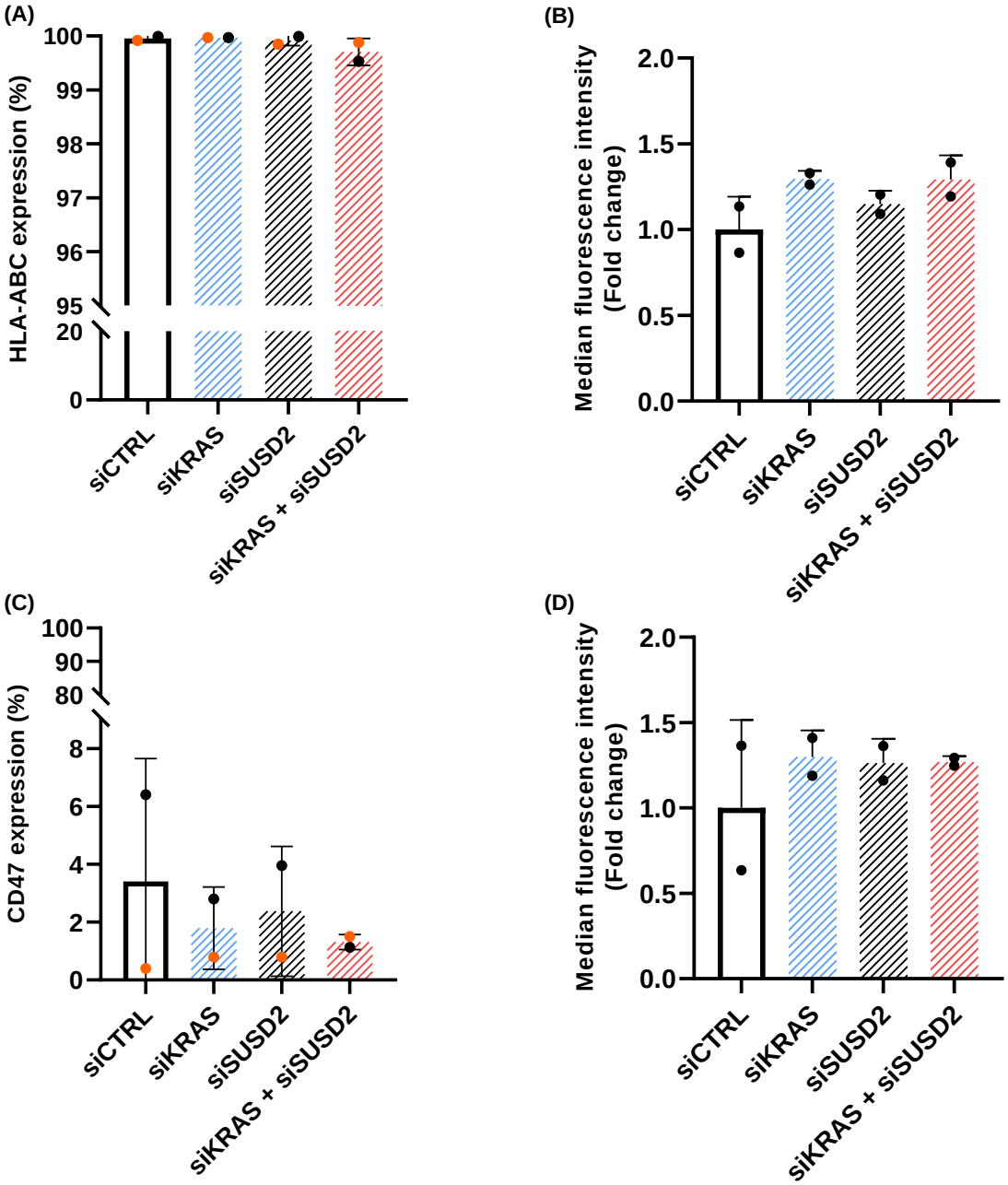


Figure 17. CRC stem cell markers in different groups in HCT-116 cell line. (A) Percentage of CD24-PE positive cells in siCTRL, siKRAS, siSUSD2 and siKRAS/siSUSD2 HCT-116 cells (n=2); **(B)** MFI of the CD24-PE positive cells, results are presented as fold change relative to the siCTRL MFI levels; **(C)** Percentage of CD44-APC positive cells in siCTRL, siKRAS, siSUSD2 and siKRAS/siSUSD2 HCT-116 cells (n=2); **(D)** MFI of the CD44-APC positive cells, results are presented as fold change relative to the siCTRL MFI levels; **(E)** Percentage of CD44v6-APC positive cells in siCTRL, siKRAS, siSUSD2 and siKRAS/siSUSD2 HCT-116 cells (n=2); **(F)** MFI of the CD44v6-APC positive cells, results are presented as fold change relative to the siCTRL MFI levels; **(G)** Percentage of CD133-APC positive cells in siCTRL, siKRAS, siSUSD2 and siKRAS/siSUSD2 HCT-116 cells (n=1); **(H)** MFI of the CD133-APC positive cells, results are presented as fold change relative to the siCTRL MFI levels; **(I)** Percentage of CD166-APC positive cells in siCTRL, siKRAS, siSUSD2 and siKRAS/siSUSD2 HCT-116 cells (n=2); **(J)** MFI of the CD166-APC positive cells, results are presented as fold change relative to the siCTRL MFI levels.

3.3.2. Assessment of SUSD2 impact on the cell surface expression of immune modulators

The results obtained show no differences in the number of HLA-ABC positive cells between the groups. Moreover, the percentage of positive cells is close to 100% in all groups. However, in all groups, there is a tendency to an increased tendency in MFI levels **(Figure 18.B)**.



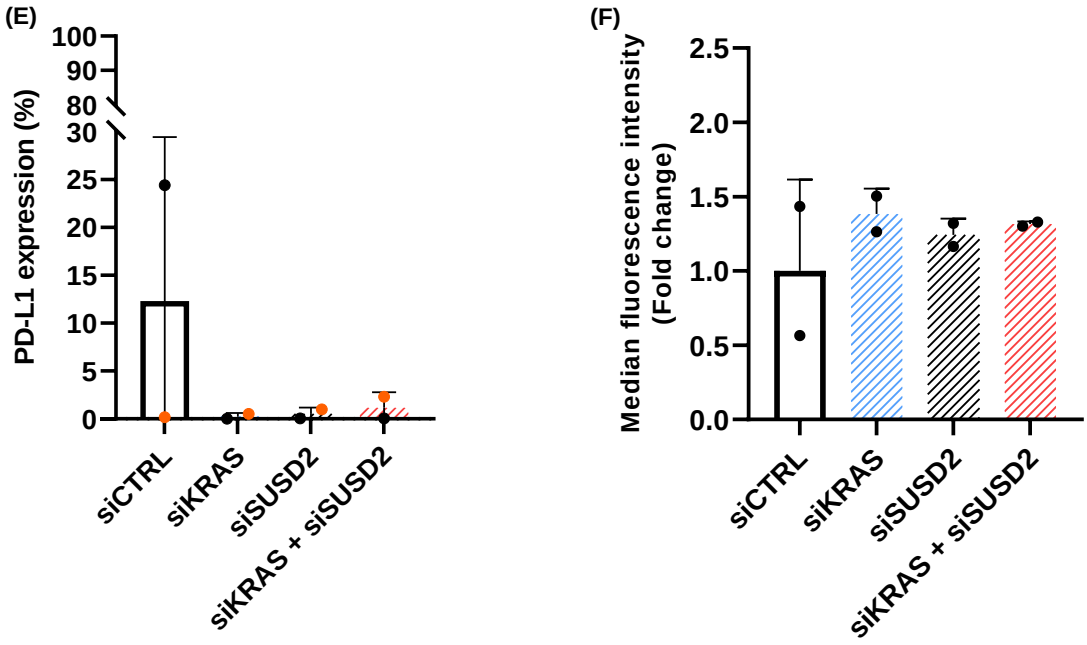


Figure 18. Immune modulators expression in different groups in HCT-116 cell line. (A) Percentage of HLA-ABC-PE positive cells in siCTRL, siKRAS, siSUSD2 and siKRAS/siSUSD2 HCT-116 cells (n=2); **(B)** MFI of the HLA-ABC-PE positive cells, results are presented as fold change relative to the siCTRL MFI levels; **(C)** Percentage of CD47-FITC positive cells in siCTRL, siKRAS, siSUSD2 and siKRAS/siSUSD2 HCT-116 cells (n=2); **(D)** MFI of the CD47-FITC positive cells, results are presented as fold change relative to the siCTRL MFI levels; **(E)** Percentage of PD-L1-FITC positive cells in siCTRL, siKRAS, siSUSD2 and siKRAS/siSUSD2 HCT-116 cells (n=2); **(F)** MFI of the PD-L1-FITC positive cells, results are presented as fold change relative to the siCTRL MFI levels.

The number of CD47 positive cells did not differ between the groups. Furthermore, the percentage of positive cells is close to 0% in all groups. MFI levels follow the same tendency, with no differences between the groups. Furthermore, no alterations in the number of PD-L1 positive cells were observed in the results. Despite the siCTRL group showing an outlier, all groups express residual levels of PD-L1. The same was also observed in the MFI values **(Figure 18)**.

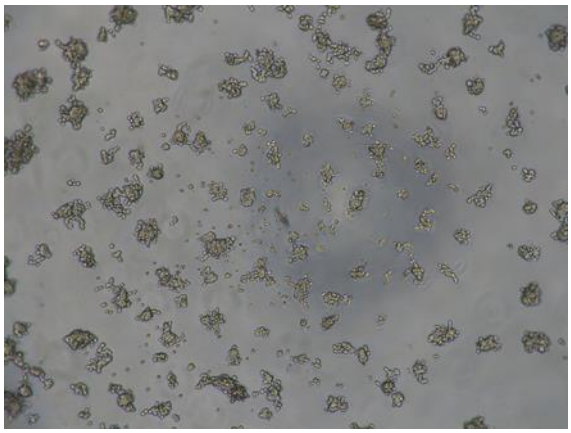
4. SUSD2 silencing promotes HCT-116 cells aggregation

SUSD2 extracellular domains are also found in molecules frequently associated with cancer cell-cell and cell-matrix adhesion. Thus, SUSD2 role in oncogenic events related to these traits was evaluated by aggregation assays. Images after 24 and 48 hours of incubation were acquired. The results show small and losse tumor cell aggregates in all groups (siCTRL, *siKRAS*, *siSUSD2*, and *siKRAS*/*siSUSD2*) in the first 24 hours of incubation. Nevertheless, at the 48-hour timepoint, all groups formed bigger and more compact cell aggregates. Concerning the *siKRAS* group, cell aggregates show to be larger than the siCTRL group **(Figure 19.B)**. A bigger difference was even noticed in the *siSUSD2* group in which tumor cells aggregates are much larger compared to the other groups.

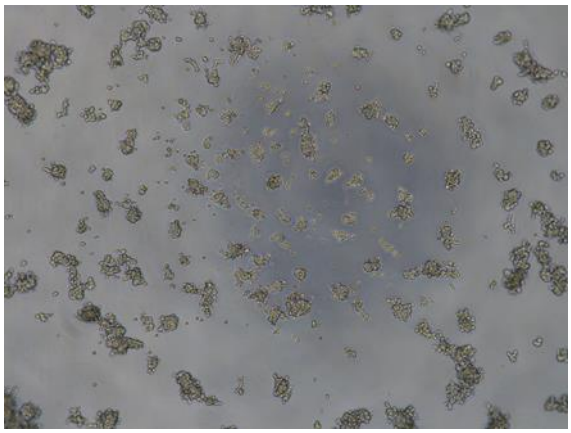
siKRAS/*siSUSD2* double inhibition seems to revert the *siSUSD2* effect, with the formation of smaller aggregates, very similar to the ones found in the *siKRAS* group.

(A) TimePoint: 24 hours

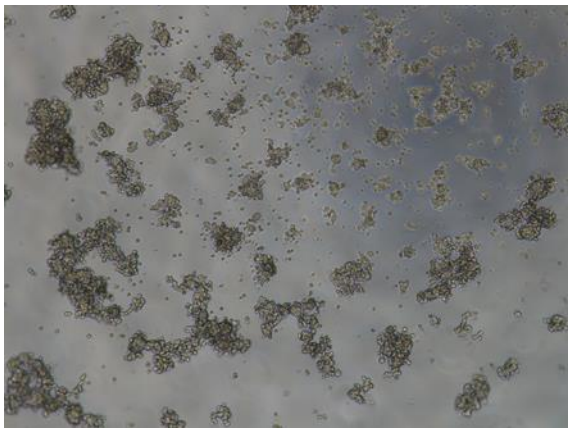
1. siCTRL



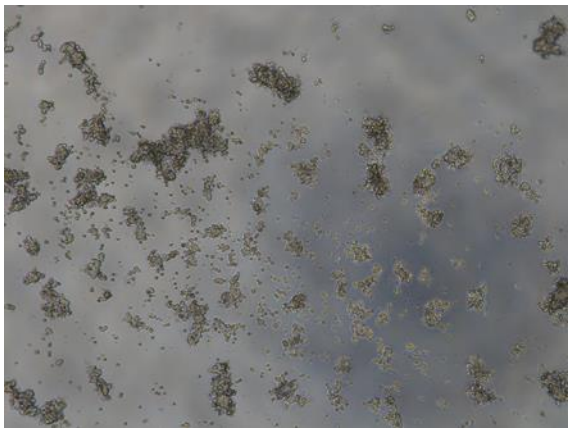
2. siKRAS



3. siSUSD2

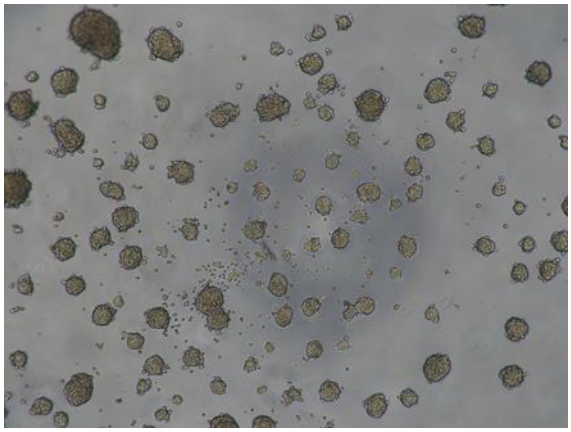


4. siKRAS/siSUSD2

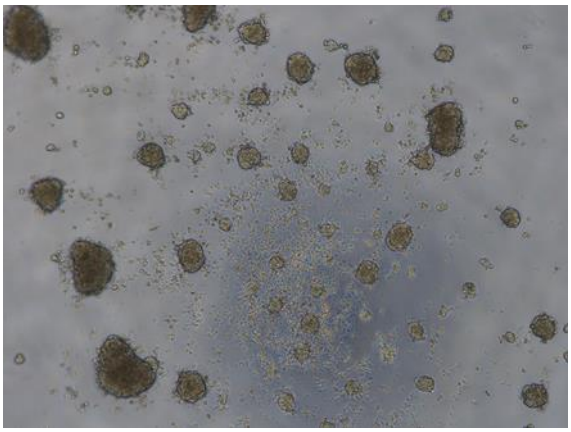


(B) TimePoint: 48 hours

2. siCTRL



1. siKRAS



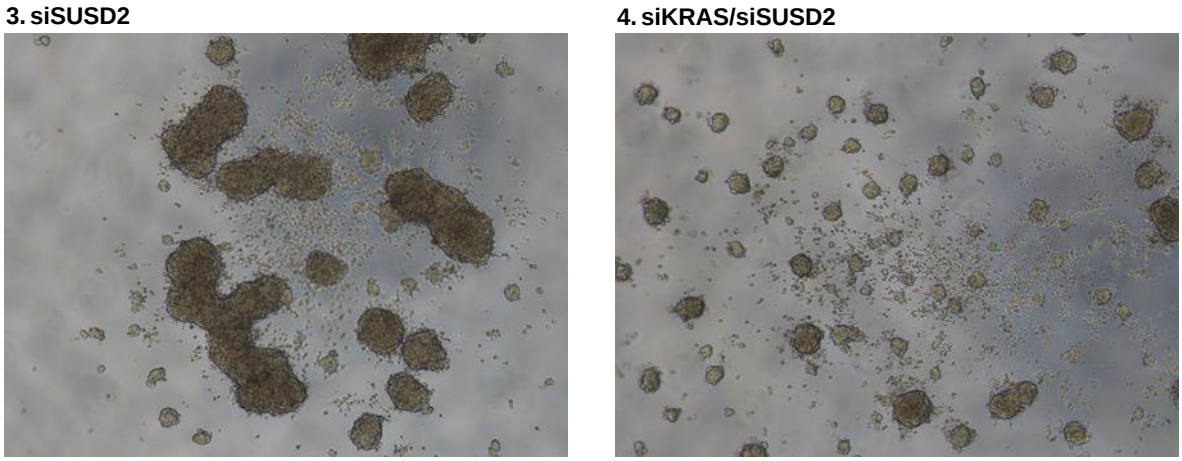


Figure 19. Tumor cell aggregates upon KRAS, SUSD2, and KRAS/SUSD2 silencing in HCT-116 cell line. Representative images of **(A)** Tumor cell aggregates after 24 hours of incubation (n=3); **(B)** Tumor cell aggregates after 48 hours of incubation (n=3).

5. *In silico* analysis of *SUSD2* mRNA expression in colorectal tumor samples

Using the available TCGA data, we performed an *in silico* analysis of the association between *SUSD2* mRNA expression levels and colon and rectum tumor samples **(Figure 20)**. The results show a significant downregulation of *SUSD2* in colon and rectum tumor samples compared to normal samples. The same was found upon separation into colon and rectum tumor samples.

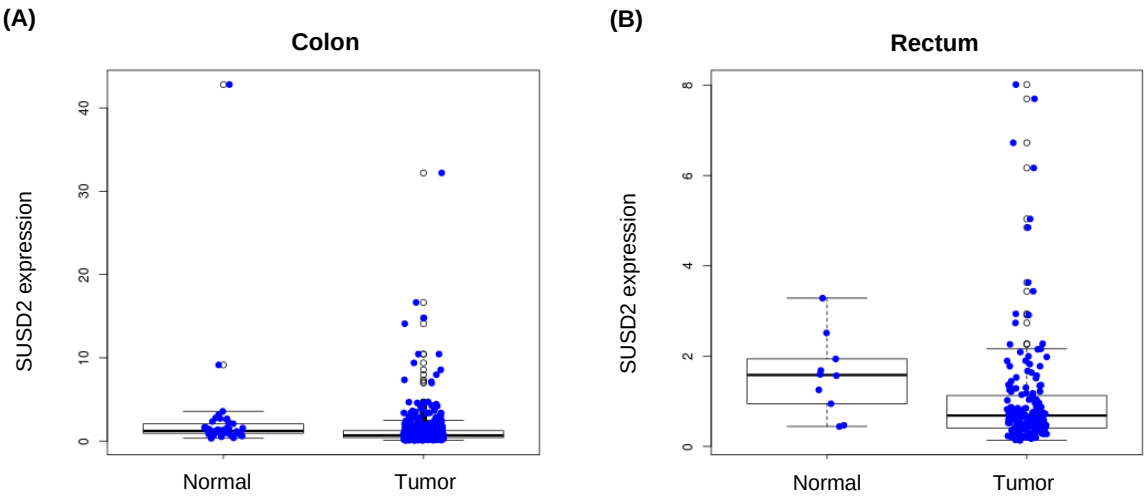


Figure 20. SUSD2 mRNA expression in colon and rectum tumor samples. (A) SUSD2 is downregulated in colon tumor samples (p-value = 2.788e-06); **(B)** SUSD2 is downregulated in rectum tumor samples (p-value = 0.01134). Mann-Whitney test was performed.

Moreover, we assessed *SUSD2* mRNA expression levels in wildtype KRAS (WT KRAS) and mutated KRAS (Mut. KRAS) CRC in order to determine if there was as association between SUSD2 expression and with the presence of KRAS mutations **(Figure 21)**. The results show a significant SUSD2 downregulation in mutated KRAS colorectal tumor

samples. Moreover, SUSD2 downregulation in the mutated KRAS CRC samples. Moreover, SUSD2 downregulation in the mutated KRAS group was still observed when colon or rectum tumor samples were analyzed separately.

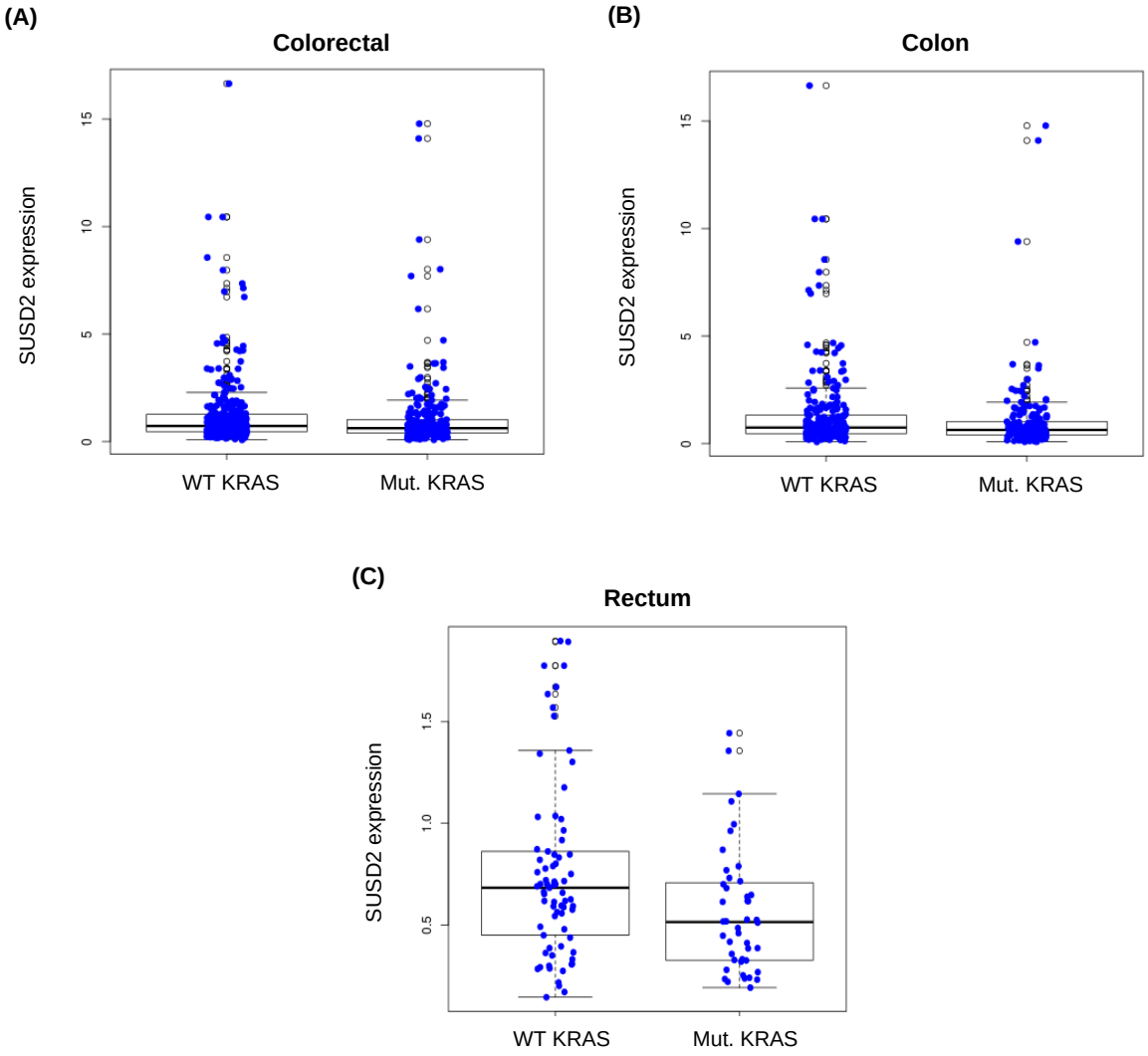


Figure 21. SUSD2 mRNA expression in wildtype KRAS vs mutated KRAS groups in CRC, in general, and in colon and rectum samples. (A) SUSD2 is downregulated in the mutated KRAS group in colorectal tumor samples (n=528; p-value = 0.0190); **(B)** SUSD2 is downregulated in the mutated KRAS group in colon tumor samples (n=396; p-value = 0.0365); **(C)** SUSD2 is downregulated in the mutated KRAS group in rectum tumor samples (n=132; p-value = 0.01143); Mann-Whitney test was performed.

Further stratification for the microsatellite status and disease stage revealed a correlation between SUSD2 downregulation and MSS CRC in mutated KRAS samples. Besides that, regarding the CRC stage, an association between SUSD2 downregulation and KRAS mutation was only found in CRC stage I patients.

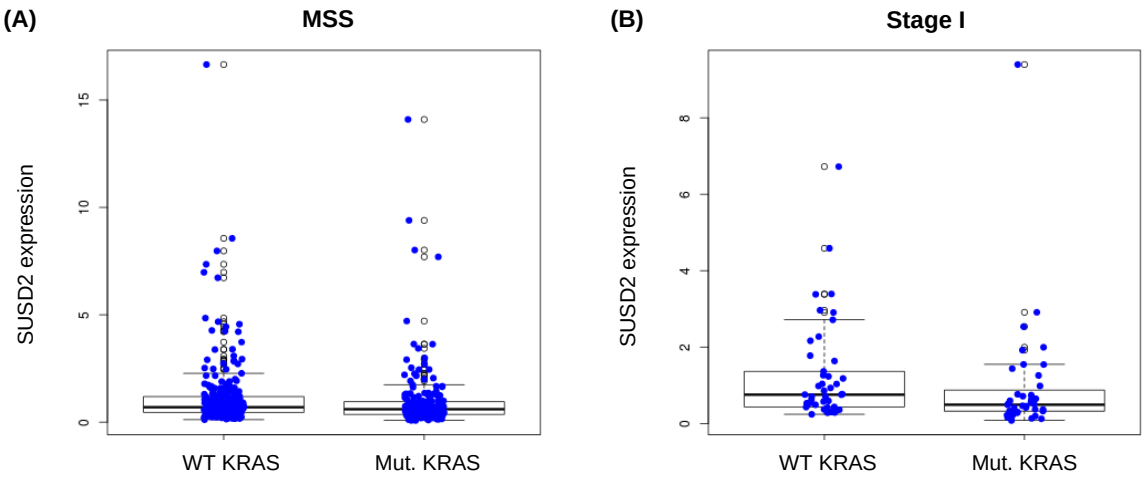


Figure 22. SUSD2 mRNA expression considering microsatellite instability status and staging of colorectal tumor samples. (A) SUSD2 downregulation in the mutated KRAS group is correlated with MSS CRC (n=435; p-value = 0.0062); **(B)** SUSD2 downregulation in the mutated KRAS group is related with CRC stage I patients (n=84; p-value = 0.0222). Mann-Whitney test was performed.

V. DISCUSSION

The growing trend in CRC incidence and mortality makes the study of the underlying molecular mechanisms by which CRC progresses of utmost importance. Unfortunately, due to the lack of symptomatology presented during its evolution, CRC is often diagnosed in advanced stages, in a metastatic stage, where therapeutic options are more restricted. Furthermore, metastatic CRC patients harboring KRAS mutations do not benefit from anti-EGFR therapy, unlike those with wild-type KRAS, where improvements have been reported (58). The importance of KRAS activating mutations in CRC initiation and progression is well-documented. As described throughout this dissertation, KRAS regulates several oncogenic traits, such as proliferation, cell growth, survival, metastasis, and the TME. Thus, KRAS is the ideal target for a therapeutic approach in CRC. However, despite several efforts, this oncogene remains mostly undruggable. Therefore, finding new targets that counteract KRAS oncogenic effects is of absolute importance.

Recently, in our group, a proteomic analysis suggested KRAS mutant regulation on SUSD2 expression in CRC. Supported on it, we attempt to validate this result and evaluate the role of SUSD2 in CRC. We started by validating SUSD2 upregulation at the mRNA level and protein level. For this purpose, a panel of KRAS mutant CRC cell lines was created, and SUSD2 expression levels upon *KRAS* silencing were evaluated. Transcript levels of SUSD2 were significantly higher after *KRAS* silencing in the HCT-116 cell line. In HCT-15, SW480, SW620 cell lines, despite not having achieved a significant upregulation, a trend for higher mRNA expression levels of SUSD2 expression upon *KRAS* silencing was observed. Lastly, in the LS-174T cell line, a non-dependent mutant KRAS signaling cell line, no alterations in SUSD2 expression were observed, suggesting that KRAS regulation on SUSD2 mRNA expression is only observed in mutant KRAS signaling-dependent cell lines. Based on these findings, it was evaluated whether KRAS regulation on SUSD2 expression observed at the mRNA level would also translate into differences in protein expression of SUSD2. The HCT-116 cell line was the only used cell line that demonstrated a significant increase in both the percentage of SUSD2 positive cells and amount of SUSD2 molecules expressed per positive cell (MFI values). In SW480 and SW620 cell lines, where mRNA levels indicated a KRAS regulation on SUSD2, the same trend was not observed at the protein level, with no alterations in the number of SUSD2 positive cells and MFI. Lastly, in the LS-174T cell line, the protein expression levels of SUSD2 agreed with those observed at the mRNA level, with no differences either in the number of positive cells or in the MFI. Therefore, in the first part of this dissertation, mutant KRAS regulation of SUSD2 expression, at both the mRNA and protein levels, was only observed in the HCT-116 cell line. These finds raise the question of whether KRAS regulation of SUSD2 transcription and translation may be dependent on the type of activating mutation. The HCT-116 cell line

harbors the G13D mutation, while the other cell lines used harbor other activating KRAS mutations, as shown in **Table I**, suggesting a KRAS dependency on the KRAS G13D mutation to regulate SUSD2 expression, needing further studies to evaluate that. Unfortunately, we did not analyze SUSD2 protein expression in HCT15 (also harboring a G13D mutation) due to time constraints. This analysis is essential to support our hypothesis.

In the second part of this dissertation, we assessed the role of SUSD2 in CRC. Considering the obtained results, this analysis was conducted only in HCT-116 cell line. In CRC, SUSD2 was described as a tumor suppressor, controlling cell proliferation by interacting with its ligand C10orf99 (127). However, in other types of cancer, SUSD2 has the ability to regulate other oncogenic events such as angiogenesis, cell migration and invasion, metastasis, and the TME. Furthermore, SUSD2 exhibits a curious dual role in cancer. Thus, we aim to decipher the functional effect of SUSD2 upregulation upon *KRAS* mutant silencing in CRC. Our results showed that *SUSD2* silencing decreases HCT-116 cells growth potential. In the *siSUSD2* group, the number of cells was much lower than the siCTRL group. Furthermore, *KRAS* and *SUSD2* silencing promoted a synergistic effect on cell growth inhibition, as in this group a significant higher decline in the number of cells was observed. To elucidate if this decrease was due to SUSD2 role in proliferation or in apoptosis, an Annexin V/Propidium Iodide staining was performed. Apoptosis resistance by KRAS activating mutation is one of the well-described oncogenic events promoted by this GTPase. In contrast, the SUSD2 role in apoptosis in CRC is not fully understood. The results showed no differences in the number of viable, apoptotic, or late apoptosis cells. These results are in concordance with those observed by Pan et al., who reported that SUSD2/C10orf99 interaction did not regulate apoptosis (127). Thus, the decreased number of cells caused by *SUSD2* silencing is likely to be related with an impaired proliferative capacity. This result suggests that SUSD2 upregulation upon *KRAS* silencing could be a mechanism to sustain the proliferative activity of KRAS inhibited cells.

In order to further dissect the impact of SUSD2 upregulation on the malignant traits of KRAS-inhibited HCT-116 cells, several cell differentiation markers were analyzed. *CDH1*, the gene encoding for E-cadherin, is an important epithelial marker, relevant for cell adhesion and invasion. In CRC, *CDH1* loss is associated with cell migration and invasion, promoting metastasis (153). Yet, its expression in CRC stem cells was described to be fundamental to promote higher tumor growth in association with pluripotency factor *NANOG* (154). Nevertheless, a recent report demonstrated the ability of mutant KRAS to suppress *CDH1* expression levels (155). Our results show a residual increase in *CDH1* expression upon *KRAS* silencing, thus corroborating the involvement of mutant KRAS in controlling *CDH1* expression. However, in our work more replicates are needed to assess the role of

mutant KRAS role in *CDH1* regulation. In contrast, *SUSD2* silencing decreased *CDH1* expression levels. Furthermore, simultaneous *KRAS* and *SUSD2* silencing is associated with *CDH1* expression values between those obtained in the *siKRAS* and *siSUSD2* groups, suggesting a possible role of SUSD2 downregulation induced by mutant KRAS on the regulation *CDH1* expression. Vimentin, TWIST and ZEB1 were used as a mesenchymal markers to evaluate a possible role for SUSD2 in EMT. Increased expression of mesenchymal markers such as Vimentin is commonly associated with tumor progression, enabling tumor cells to migrate and invade (156). The results suggest that *KRAS* silencing decreases *Vimentin* mRNA levels, following those reported in the literature (157). Furthermore, a decreasing trend in *Vimentin* expression levels is observed in the *siKRAS*/*siSUSD2* group, whereas *SUSD2* silencing does not affect Vimentin expression levels. Thus, these results suggest an independent ability of KRAS to increase Vimentin levels. Besides, the expression values of other well-known EMT markers such as *TWIST1* and *ZEB1* were assessed. *TWIST1* mRNA levels display a decreasing trend in all analyzed groups, suggesting that the possible regulation of KRAS and SUSD2 on TWIST1 expression levels is independent of each other. At the same time, the results showed a decreased *ZEB1* expression levels upon mutant *KRAS* silencing, while in the *siSUSD2* and *siKRAS*/*siSUSD2* group, levels tend to be restored, yet not to the siCTRL levels, highlighting a possible role of SUSD2 upregulation on *ZEB1* downregulation observed upon *KRAS* silencing **(Figure 23)**. These findings propose an opposite role of SUSD2 in regulating ZEB1 levels to that reported by Umeda et al., who in gastric cancer demonstrated that SUSD2 overexpression promotes ZEB1 upregulation (132).

LGR5 is a well-described cancer stem cell marker and a target of Wnt/β-catenin signaling. Besides, LGR5 regulates β-catenin cytoplasmic concentration, promoting it signaling pathway activation, supporting cell proliferation (158). In this dissertation, the expression values of LGR5 were assessed to evaluate the possible role of SUSD2 in tumor stemness. In CRC, LGR5 expression is not linked to KRAS mutation (159). However, upon *KRAS* silencing, the results suggest increased *LGR5* mRNA levels. In contrast, in the *siSUSD2* and *siKRAS*/*siSUSD2* group, *LGR5* levels tend to decrease, suggesting that SUSD2 induces LGR5 expression. Although its role as a stem cell marker, LGR5 has also been demonstrated to function as an independent prognostic marker for better clinical outcomes in CRC patients (159). Therefore, the biological significance of LGR5 upregulation upon *KRAS* silencing remains to be determined. Yet, as *siKRAS* cells also express more *CDH1*, we postulate that the combination CDH1 and LGR5 upregulation may associate with a better outcome **(Figure 23)**. Next, we analyzed the expression of the embryonic pluripotency gene *NANOG*. In CRC, NANOG upregulation is associated with

greater tumor cells aggressiveness, promoting metastasis (160, 161). At the mRNA level, *KRAS* silencing promoted a decrease in *NANOG* expression, while in the *siSUSD2* group, data suggest that its expression is restored. Furthermore, simultaneous *KRAS* and *SUSD2* silencing is associated with *NANOG* expression values between those obtained in the *siKRAS* and *siSUSD2* groups. This finding suggests a possible role played by SUSD2 upregulation in NANOG downregulation in the *siKRAS* group.

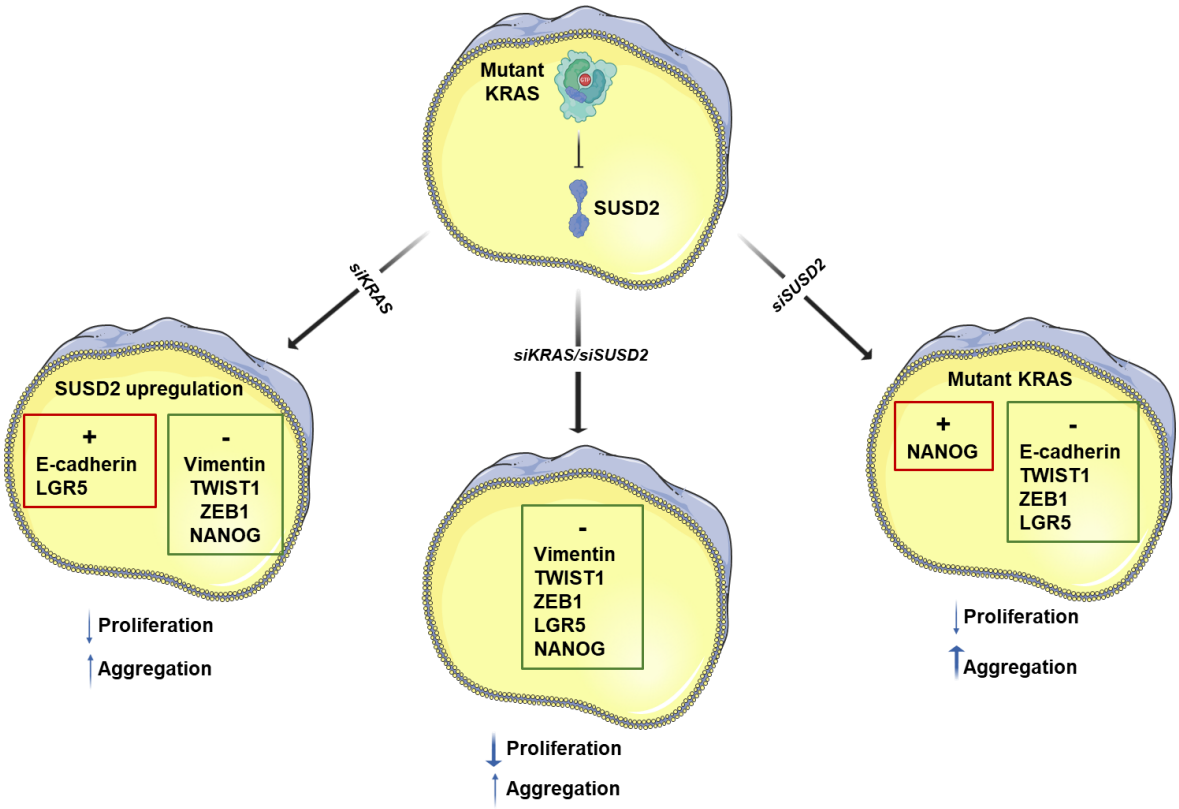


Figure 23. The influence of KRAS and SUSD2 on different epithelial, mesenchymal, EMT, and cancer stem markers. Created with BioRender.com.

Since KRAS has been shown to promote CRC stem cell features, we investigated whether this effect could be mediated by SUSD2. To do so, we analyzed the expression of several CRC stem cell markers. CD24 is a highly expressed protein in several cancers that promotes tumor progression by regulating migration, cell invasion, and metastasis. Furthermore, CD24 has been reported a pro-tolerogenic molecule. CD24 interaction with Siglec-10 has been shown to promote an immunosuppressive phenotype in immune cells (162). Besides that, in CRC, CD24 is described as a CSC marker and regulator of metastasis and angiogenesis (163, 164). We demonstrate that CD24 is barely expressed in the HCT116 cell line, as described in the literature (165). Besides that, no alterations in the percentage of CD24 positive cells were observed in all groups. Nevertheless, MFI values suggest that all groups exhibit a decreased number of CD24 molecules on the cell surface.

CD44 is a well-recognized CSC marker in CRC (166). Furthermore, CD44 regulates cell proliferation and survival (167). We demonstrate that CD44 is highly expressed in the HCT-116 cell line, as described in the literature (166). Although no changes in the number of CD44 positive cells were observed, the MFI values suggest that *KRAS* silencing decreases the number of CD44 receptors on the cell membrane. This finding is in line with Jeong et al. report, which describes a mutant KRAS ability to induce CD44 expression in CRC CSC (107). Furthermore, *SUSD2* silencing reversed the effect observed by *KRAS* silencing, suggesting that the upregulation of KRAS on CD44 may be due to the SUSD2 downregulation caused by this GTPase. CD44v6 expression, a splicing isoform of CD44 gene, was also evaluated. CD44v6 has been reported as a regulator of proliferation, migration, EMT, and metastasis in CRC (168, 169). The percentage of CD44v6 positive cells showed no alterations after *KRAS* and *SUSD2* silencing. However, in the *siKRAS*/*siSUSD2* group, one of our replicates showed an increase in the number of CD44v6 positive cells. Regarding MFI values, *KRAS* or *SUSD2* silencing decrease the number of proteins expressed in the membrane.

CD133 is a pro-tumoral protein and a marker of CSC that supports a pro-angiogenic environment and promotes cell growth and survival (170, 171). *KRAS* silencing tends to decrease the number of CD133 positive cells. This finding follows the reported literature since KRAS mutations are associated with this CSC marker expression (107). Furthermore, in the *siKRAS*/*siSUSD2* group (in which SUSD2 expression is higher compared to *siSUSD2* probably due to *KRAS* silencing that contra balances *siSUSD2*), the number of CD133 positive cells was restored, highlighting a possible role of SUSD2 upregulation in the regulation exhibited by the mutant KRAS on CD133 expression. Moreover, CD166 expression levels, another CRC CSC marker, were evaluated. Interestingly, CD166 downregulation enables CRC cells to metastasize (172). Jeong et al. demonstrated that mutant KRAS is important for CD166 expression in CSC (107). Thus, CD166 downregulation by *KRAS* silencing was an expected outcome. However, we didn't observe alterations in CD166 expression levels, showing the presence of alternative mechanisms adopted by CSC for CD166 expression. Furthermore, no differences in the percentage of CD166 positive cells and MFI values were observed after *SUSD2* silencing.

Throughout this dissertation, the role of KRAS and SUSD2 as two regulators of TME has been highlighted. However, SUSD2 effects on the CRC TME have never been investigated. Thus, we assessed the protein expression levels of immune modulator molecules that display an important role in the TME.

HLA-ABC downregulation in cancer is a well-known mechanism of tumor progression. Previously described, mutated KRAS has been shown to inhibit surface expression of

MHCI, allowing tumor cells to evade the cytotoxic attack triggered by $CD8^+$ T cells (89). Although *KRAS* silencing did not change the percentage of HLA-ABC positive cells, MFI values tended to increase, suggesting that *KRAS* silencing promotes an increase in the number of HLA-ABC expressed molecules. The *siKRAS*/*siSUSD2* group follows the same trend, while silencing of *SUSD2* has been shown to alter neither the number of HLA-ABC positive cells nor the MFI values, demonstrating that SUSD2 does not regulate HLA-ABC expression. CD47, also known as the “don’t eat me signal” is expressed on the cell surface and inhibits macrophage phagocytosis. Its upregulation is common in many types of cancer. In CRC, CD47 promotes cell proliferation and metastasis (173). We show that *KRAS* and *SUSD2* silencing does not alter the number of HCT-116 CD47 positive cells. Moreover, the MFI values follow the same tendency.

PD-L1 upregulation is a well-described mechanism acquired by tumor cells to evade immune surveillance (102). As mentioned before, mutant KRAS regulates PD-L1 expression in lung and pancreatic cancer (103). However, in CRC, mutant KRAS ability to regulate PD-L1 is not observed (102). In line with the literature, we show that basal PD-L1 expression in HCT-116 cell line is very low. Moreover, we did not observe alterations were on PD-L1 positive cells, nor the MFI values in the analyzed groups.

To assess a SUSD2 role in the HCT-116 cells aggressiveness, aggregation assays were performed. Tumor cell aggregates are usually related to less tumor aggressiveness, while the opposite is associated with greater tumor aggressiveness. The ability of tumor cells to aggregate is associated with an epithelial phenotype, while the inability associated with a mesenchymal phenotype. The acquisition of mesenchymal features and epithelial loss is a major mechanism involved in EMT and tumor to metastization. It is known that SUSD2 extracellular subdomains are also found in molecules frequently associated with cancer cell-cell and cell-matrix adhesion, migration, homing (123, 124, 174). As our results showed that SUSD2 upregulation in the context of *KRAS* inhibition is likely to induce a more epithelial phenotype, we sought to determine whether it would promote cell-cell aggregation. All the conditions tested promoted aggregation, yet at different levels. We found that silencing *KRAS* induces more and bigger aggregates when compared to siCTRL. To determine whether this could be mediated by the consequent upregulation of *SUSD2* that occurs upon *KRAS* silencing, we silenced both oncogenes. Yet the aggregation results showed that the aggregates formed with simultaneous inhibition were comparable to the one formed in the *siKRAS* condition, suggesting that SUSD2 upregulation does not mediate aggregation of *KRAS* silenced cells. Additionally, and contrarily to our expectations, the biggest aggregates were found in *siSUSD2* condition.

Finally, we wanted to assess whether SUSD2 downregulation is also observed in human colorectal cancer samples and whether it associates with mutant KRAS. Colon and rectum tumor samples show significant downregulation of SUSD2 levels compared to normal mucosa tissues. Furthermore, we were able to observe a significant decrease in SUSD2 expression in colorectal tumor samples harboring KRAS mutations. Finally, to evaluate a correlation between SUSD2 downregulation in CRC harboring KRAS mutations and some pathological features, the MSI status, and patients' stage were observed. The results show a correlation between the downregulation of SUSD2 in colorectal tumor samples with KRAS mutations and MSS. However, the MSS CRC sample size was much larger than MSI CRC, which was expected since KRAS mutations are frequently observed in MSS CRC, which may explain these differences (45). Moreover, the finding that mutant KRAS and SUSD2 downregulation are mostly associated with stage I indicates that the interplay between both genes may be more relevant for malignant transformation than for tumor progression.

VI. CONCLUSION AND FUTURE PRESPECTIVES

Throughout this study, we demonstrate that SUSD2 is a downstream target of KRAS, and the mutant KRAS possesses the ability to reduce its expression levels in CRC. By validating SUSD2 upregulation upon mutant *KRAS* silencing, a possible association between the type o *KRAS* mutation and the ability of this oncogene to regulate SUSD2 expression was hypothesized. HCT-116 cell line was the only cells harboring G13D mutations where SUSD2 protein levels were assessed. The remaining cell lines had different KRAS-activating mutations. Thus, in the future, we intend to understand if mutant KRAS ability to downregulate SUSD2 is dependent on the type of KRAS mutation. Moreover, it would also be interesting to characterize the functional effects of SUSD2 using cell lines in which SUSD2 protein expression is not controlled by mutant KRAS

Furthermore, the results obtained suggest an unexpected dual role played by SUSD2 in CRC. We started this dissertation with the aim of evaluating the involvement of SUSD2 upregulation in mediating the tumor suppressive effects of *KRAS* silencing. The results demonstrated that SUSD2 upregulation might be a pathway by which *KRAS* silencing induces increased levels of *CDH1* and decreases of *ZEB1*, CD44, and CD133, highlighting a tumor suppressor role in CRC, as described in the literature. However, the results also suggest that SUSD2 upregulation increases *LGR5*, *TWIST1*, CD24, and CD44v6 levels, reporting an oncogenic role of *SUSD2*. Besides, we found that SUSD2 expression decreases the ability of tumor cells to aggregate. This finding may also support an oncogenic role of *SUSD2*. Further studies are needed to elucidate the SUSD2 role in CRC aggressiveness (175). In the future, we plan to perform cell invasion and migration assays to further detail the role of SUSD2 as downstream target of mutant KRAS.

VII. BIBLIOGRAPHY

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VIII. SUPPLEMENTARY MATERIAL

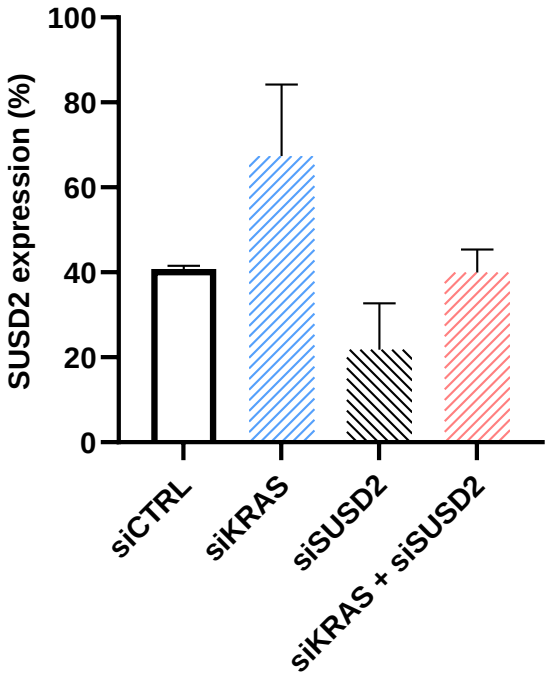

Figure S1. Confirmation of SUSD2 silencing. Flow cytometry analysis show a decreased expression of SUSD2 in the siSUSD2 group.

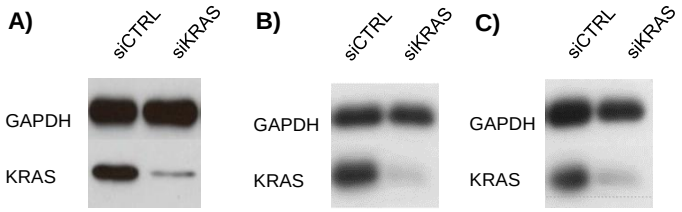

Figure S2. Confirmation of KRAS silencing. (A) Western blot analysis revealed a significant decrease of KRAS protein levels after silencing (siKRAS) when compared with the negative control (siCTRL).

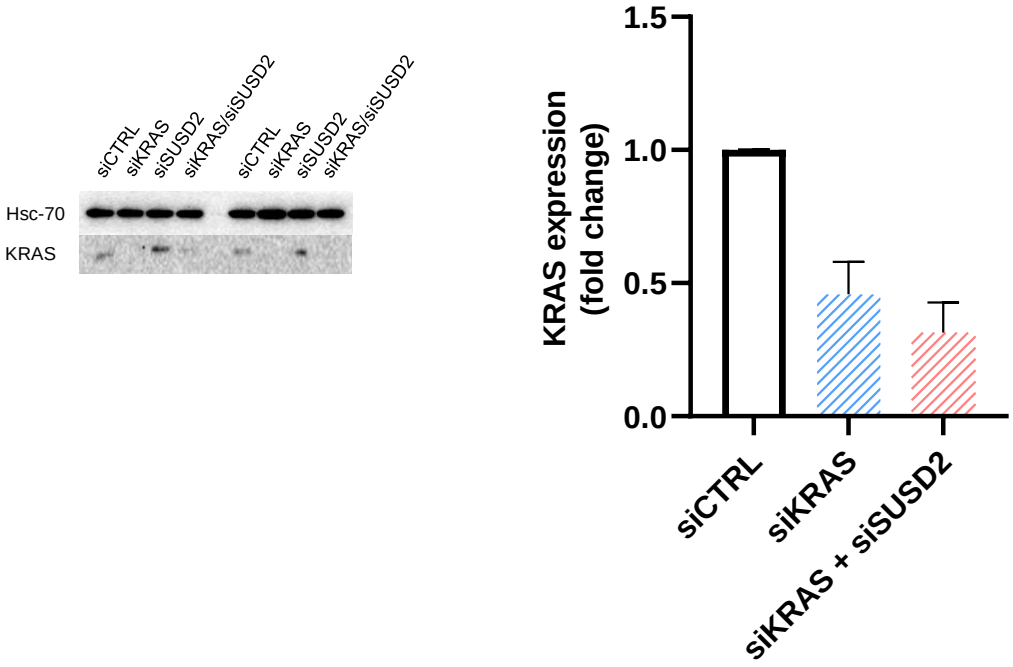

Figure S3. Confirmation of KRAS silencing. (A) Western blot analysis revealed a significant decrease of KRAS protein levels after silencing (siKRAS and siKRAS/siSUSD2) when compared with the negative control (siCTRL). **(B)** Histogram represents the silencing confirmation revealed by the decrease of KRAS protein on the used samples.

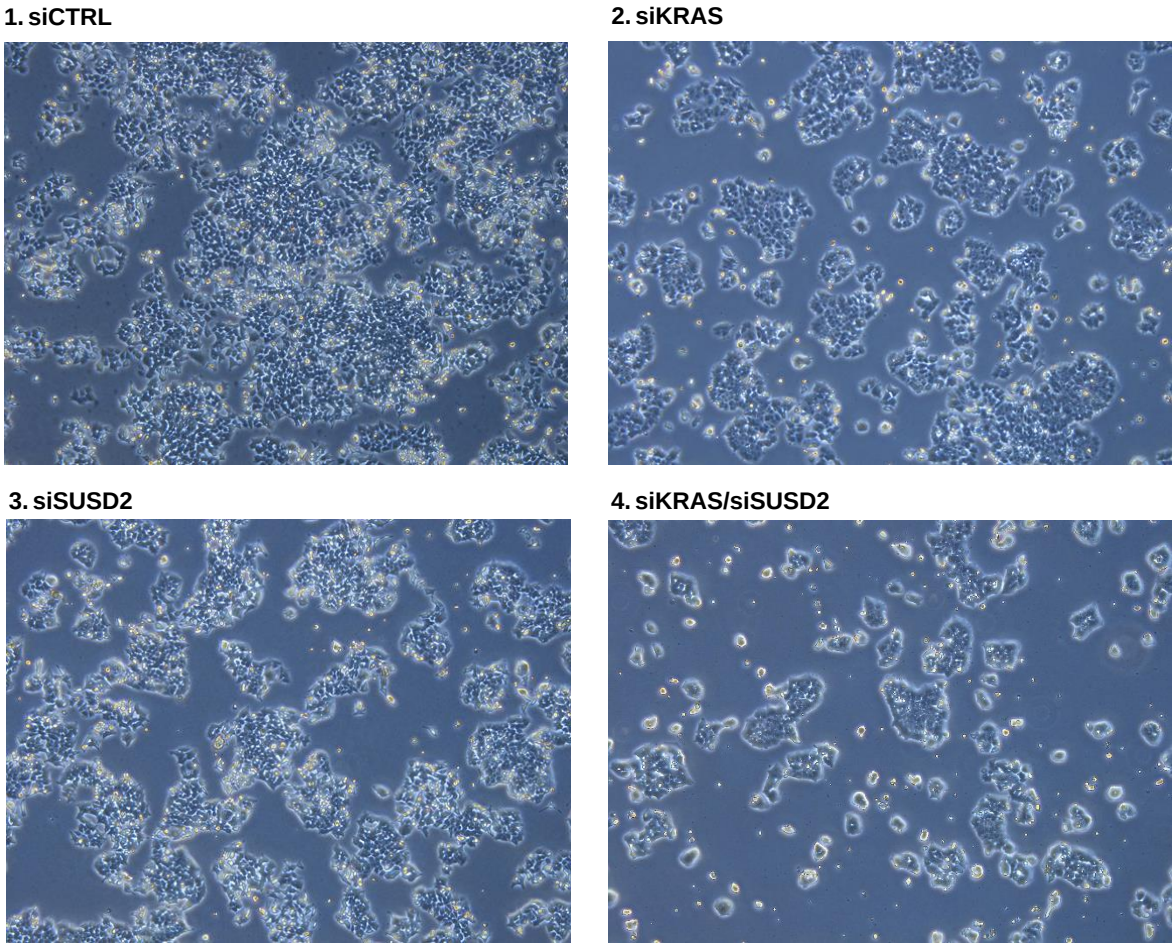

Figure S4. Cell aspect after KRAS, SUSD2 or KRAS/SUSD2 silencing. HCT-116 control and upon KRAS, SUSD2 and KRAS/SUSD2 silencing.

Table SI. Resources

PRODUCT	MANUFACTURER	CATALOG N°
RPMI MEDIUM	Life Technologies	72400-021
DMEM MEDIUM	Life Technologies	41965-039
FETAL BOVINE SERUM	GE Healthcare	SV30160.03
PENICILLIN/STREPTOMYCIN	Life Technologies	15140-122
TRYPSIN	Invitrogen	25300-062
LIPOFECTAMINE RNAIMAX	Invitrogen	13778-150
OPTI-MEM	Life Technologies	11058-021
PROTEASES INHIBITORS COCKTAIL	Roche	11836170001
PHOSPHATASES INHIBITORS COCKTAIL	Sigma-Aldrich	P0044-1ML
DC PROTEIN ASSAY KIT	BioRad	#500-0114
NITROCELLULOSE BLOTTING MEMBRANES	GE Healthcare	106000002
PONCEAU S SOLUTION	Sigma-Aldrich	P7170-1L
CLARITY WESTERN ECL KIT	BioRad	102030695
TRIPLEXTRACTOR	GRiSP Research Solutions	GB23
CHLOROFORM	Merck Millipore	1024311000
ISOPROPANOL	Fisher Scientific	P/7555/17
RNASE-FREE WATER	Invitrogen	10977-035
QSCRIPT™ CDNA SUPERMIX	Quanta Bioscience	95161-100
TAQMAN UNIVERSAL PCR MASTER MIX	Applied Biosystems	4324018
FORMALDEHYDE SOLUTION 37%	Merck Millipore	10400131000
ANNEXIN V/PROPIDIUM IODIDE APOPTOSIS ASSAY KIT	BD Biosciences	556547