UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXIII CICLO

DEL DOTTORATO DI RICERCA IN

BIOMEDICINA MOLECOLARE

AP3M2, NEW MOLECULAR TARGET IN COLORECTAL CANCER THERAPY

Settore scientifico-disciplinare: BIO/11 - BIOLOGIA MOLECOLARE

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ABSTRACT

Annually, around 1 million cases of CRC are diagnosed, with 50% death rate, representing 8% of cancer-related deaths worldwide. The implementation of early diagnosis screening programs contributed to the reduction of the incidence and the mortality rates. The combination of chemotherapeutic regimens (5-FU, oxaliplatin and irinotecan) and targeted drugs (EGFR and VEGF inhibitors) have improved patients' prognosis. However, the combination of some types of chemical and biological therapies failed in the fourth line treatment and resistance to targeted therapies is the major limit in CRC.

Under these circumstances, we have conducted an RNAi screening aiming to identify new targetable oncogenes. Starting from the studies conducted by the Cancer Genome Atlas that identified a list of amplified genes in CRC, we analyzed putative genes that their Knockdown could negatively influence cell viability in different CRC cell lines, indicating the possible involvement in CRC. After a careful bioinformatic analysis I found that one gene called AP3M2 could be a successful candidate. AP3M2 (adaptor related protein complex 3 subunit mu 2) encodes for the neuronal Mu subunit of the heterotetrameric adaptor-related protein complex 3 (AP-3), which recognizes tyrosine-based sorting signals within the cytoplasmic domains of transmembrane cargo proteins and is involved in the biogenesis of lysosome-related organelles.

Deletion of this gene in mice was associated with susceptibility to drug-induced seizures and fewer synaptic vesicles. In the other hand, AP3M2 amplicons were observed in the primary tumor and maintained in at least two passages of breast cancer xenograft. AP3M2 is also in the list of genes in recurrent amplicons associated with reduced survival in breast cancer.

The new "druggable" gene was found to be altered in 6% of CRC patients and was associated with reduced survival rate according to the TGCA data. Our experiments confirmed it to be overexpressed in colon cancer patient tissues and cancer cell lines and proved to be a potent oncogene by promoting the colorectal cancer cell lines viability, adhesion and colony formation. Interestingly, AP3M2 levels affects the expression of other CRC associated protein such as P62, ATG7and ARF6 involved in autophagy and influenced ROS levels in colorectal cancer cell lines.

1. INTRODUCTION

1.1 Colorectal cancer

1.1.1 Epidemiology

Colorectal cancer (CRC) represents one of the leading causes of cancer worldwide, with a prevalence of more than a million cases every year. Despite the decreasing incidence in the last few years, thanks to the implementation of early diagnosis screening programs, the mortality rate is still about of 50%. New therapeutic opportunities to treat CRC patients are requested especially in the case of metastatic tumors (mCRC) [1].

In 2019, more than one and a half million new cancer cases were estimated and 600 thousand cancer deaths in the United States alone, being the third cause of death related to cancer.

CRC incidence in many western countries have been stable or increased slightly. However, CRC incidence increased rapidly in several areas at low risk, including Spain, and a number of countries in Eastern Asia and Eastern Europe [2].

CRC risk was associated to several unhealthy behaviors related to physical inactivity, diet, smoking, and obesity. Men and African American are at higher risk than women and Whites. Age is a major risk factor for sporadic CRC. In addition, heredity accounts for a high percentage of CRC syndromes.

However, socioeconomic inequalities are accounting for notable gaps and barriers in the most preventable cancers. Low socioeconomic condition is also associated with a high risk for the development of CRC. Mortality rates in poor countries account up to the double of those in richer countries for different types of cancer [3].

In 2018, around 243000 mortalities were related to colorectal cancer just in Europe [4].

However, mortality rates could be reduced if the disease is diagnosed at early stages; advanced stages are associated with metastatic involvement of lymph nodes or other organs.

1.1.2 Screening programs and prevention

1.1.2.1 Screening

In ***Table 1*** is shown the updated table of CRC screening [5]. In ***Table 2*** is shown the updated CRC screening in 2019, and recommendations for in average-risk individuals between of 50 and 75 years [6].

Average-risk individuals are recommended to start regular CRC screening at age 45 for earlier CRC screening. Recommended tests for CRC screening can be classified as visual/structural tests and stool-based tests [7].

- Visual/structural tests consist of colonoscopy every 10 years, and every 5 years a CT (computed tomography) colonography and flexible sigmoidoscopy.
- Stool-based tests consist of yearly fecal immunochemical test or immunochemical-based fecal occult blood test (FIT or iFOBT) and high-sensitivity guaiac-based fecal occult blood test (gFOBT) and every 3 years a multitarget stool DNA (MT-sDNA or FIT-DNA).

For the Food and Drug Administration (FDA), only approved stool DNA test is Cologuard, it incorporates KRAS and β-actin mutants, BMP3 methylation and promoter regions of NDRG4 gene and fecal immunochemical test for human hemoglobin.

After any positive results by flexible sigmoidoscopy or CT colonography or any of the stool-based tests, colonoscopy is performed [8].

In 2014, capsule colonoscopy, a procedure that uses a pill-size wireless camera to take pictures, was approved by the FDA to detect colon polyp only for patients with incomplete colonoscopy [9].

Methylated SEPT9 assay, the first blood test approved by the FDA for CRC screening, based on PCR (polymerase chain reaction), and is up to 92% accurate [10].

Table 1. CRC Screening Recommendations by MSTF in 2017

Average-risk individuals	**Recommended tests**	**Family history of CRC**
First grade tests	• Colonoscopy each 10 years • Annual FIT	• People with one first-degree relative of CRC or recorded advanced adenoma • diagnosed < 60 years • People with two first-degree relatives with advanced adenoma
Second grade tests	• CT colonography each 5 years • FIT-fecal DNA test each 3 years • Flexible sigmoidoscopy each 5- 10 years.	• People with a single first-degree relative diagnosed at ≥ 60 years with CRC or an advanced adenoma
Third grade test	• Capsule colonoscopy each 5 years.	

CRC: colorectal cancer; MSTF: Multi-Society Task Force; FIT: fecal immunochemical test; CT: computed tomography.

Screening by: MSTF represents American College of Gastroenterology (ACG), American Gastroenterology Association (AGA) and American Society of Gastrointestinal Endoscopy.

Table 2. CRC Screening Recommendations update by American College Physicians (ACP) in 2019

Test recommended for average-risk individuals
FIT or gFOBT every 2 years.
Colonoscopy every 10 years.
Flexible sigmoidoscopy every 10 years plus FIT every 2 years.

CRC: colorectal cancer; ACP: FIT: fecal immunochemical test; gFOBT: guaiac-based fecal occult blood test

1.1.2.2 Prevention

1.1.2.2.2 Primary prevention

Knowing more about risk and prevention have potential capabilities to reduce CRC risk factors. Measures to adapt lifestyles are potential for primary prevention. Risk factors, including, alcohol consumption, smoking, obesity, and other chronic diseases, should be included in primary prevention strategies.

Evidences demonstrated that exercising could enhance health-related quality of life in survivors of colorectal cancer. In addition, smoking showed adverse effect on disease-specific and reduced overall survival. Therefore, it is suggested to support smoking cessation [11].

Other studies based on clinical trials, evidenced effective chemoprevention of CRC using specific drugs, such as aspirin or hormone therapy. However, adverse effects of these drugs on other health should be taken into consideration and might be restrictive in primary prevention outside specific risk groups. Other studies showed that vitamin D has a potentially promising effect for chemoprevention could be preventive CRC [12].

1.1.2.2.2 Secondary prevention: Screening Colonoscopy

CRC screening, availability of better treatment and removal of adenomatous colon polyps, decreased incidence and mortality. Colonoscopy has shown to reduce up to 75% the risk of death of CRC [13].

The disparity in treatment in right and left parts of colon and rectum is multifactorial. Absence of polyp or lesion in the right colon is an important factor. Some cases, has shown that incidence of right-sided CRC is higher in females and in older subjects, and can be explained by biologic factor including difficulty in visualizing flat serrated adenoma in the right colon which contains BRAF V600E that can lead to microsatellite instability (MSI) [14] [15]. Therefore, measures should be taken to enhance the quality of colonoscopy screening of right-sided colon polyps. The adenoma detection rate (ADR) is the proportion of individuals undergoing a screening colonoscopy, to find one or more adenomas, or polyps, detected. The highest the ADR percentages, the lowest the detected colon cancer cases, its recommended percentage average is 25% in total (20% or more in females, 30% or more in males) [16].

1.1.2.3 Penetrability of Colonoscopy in General Population

Colonoscopy is an effective screening tool of detecting and limiting pre-cancerous lesions development into colon cancer. However, not all people at risk accept to undergo this test, they consider it as a heavy test probably because of colon cleansing, pain and complications and probably the high cost. Thirty percent of target patients aged 50 to 75 who need screening colonoscopy are not performing the procedure done. Nonetheless, in the US, CRC screening in the high-risk population increased from 58% in 2013 to 63% in 2015, [17] thanks to different medical society guidelines, national media coverage, instructions by the physicians, digital marketing and social media. Colon cleansing regimens effectiveness should proceed with tolerance and superior compliance [18]. Patients should be aware of colonoscopy prep instructions to improve compliance. Cleansing of colon mucosa should proceed adequately for higher detection of colon polyps.

1.1.3 CRC, Risk Factors and Development

1.1.3.1 Risk Factors for the CRC

Several environmental and genetic factors could increase the risk of CRC. These factors could be related to age, socioeconomic class, obesity, lifestyle, gut microbiota, smoking, alcohol intake, diet and red meat consumption, diabetes mellitus, long-term immunosuppression, androgen deprivation therapy, family history of CRC or colorectal adenoma, and other history related diseases.

One non- family related factor is the gut microbiota, a key component of the human body that is mutually beneficent to host epithelium and bacteria. Gut microbes contain over 1000 different species and possess a large diversity of enzymes and diverse metabolic functions that produce potential products to supplement host metabolism, regulate host signaling pathways or induce immune responses [19].

Therefore, any change in microbial composition may disrupt intestinal homeostasis and colonic inflammation. The latter is implicated in inflammatory bowel disease (IBD), sporadic colorectal cancer and colitis-associated colon cancer [20].

Diet is an important contributor to human health and metabolism, it can change the composition and function of the gut microbiota [21].

Diets rich in fruits, vegetables, whole grains, and legumes reduced colon cancer risk, being rich in fibers and antioxidants, they improve the beneficial gut microbiota that produce healthy metabolites such as short-chain fatty acids and phenolics [22].

Thus, altered microbial composition through diet may be a potential therapeutic method for colon cancer.

In addition, some dietary elements such as red and processed meat, contain hetero-cyclic amines, hemes and N-nitroso compounds that can be carcinogenic, they can damage intestinal mucosa and induce CRC [23]. Smoking and alcohol consumption lead to modifications in the stomach microbiota and affect Helicobacter pylori-related disorders such as the risk of gastric cancer. In the colon alcohol induces depletion of beneficial bacteria with anti-inflammatory activity, resulting in intestinal damage. Smoking also causes changes in the intestinal microbiome with a protective role toward ulcerative colitis and deleterious for Crohn's disease [24].

The history related diseases include:

- Familial adenomatous polyposis (FAP),
- Long-time inflammatory bowel disease (IBD)
- Lynch syndrome and Muir-Torre syndrome or mutated MMR gene syndromes like hereditary nonpolyposis colorectal cancer (HNPCC)
- Hamartomatous polyposis syndromes like Peutz-Jeghers syndrome
- Cowden syndrome and Juvenile polyposis syndrome
- Non-inherited polyposis syndromes like serrated polyposis syndrome (SPS) and Cronkhite-Canada syndrome.

Though, in the past 25 years, CRC incidence has been increasing in young adults aged between 30 and 40 years in rich countries like the USA, Australia and some European countries. However, the highest incidence of CRC is among adults aged above 50 [25]. Generally, young people that develop late stage CRC is because of ignoring their symptoms. Main factors inducing CRC in this category comprise smoking, alcohol consumption, obesity, male sex and family history of CRC. In 35% of CRC young cases are linked to genetic mutations, whereas in total CRC cases, 3-5% have genetic mutation. Thus genetic studies should be taken into consideration in young subjects with CRC even without any family history of colon cancer [26].

1.1.3.2 High-Risk Individuals for CRC

People having average-risk individuals of developing CRC do not have any risk factor such as polyposis syndrome, or familial history of colorectal adenoma or CRC, or hereditary colorectal cancer syndrome, or Bowel disease. These individuals can develop CRC after the age of 50 years.

Other Individuals have high risk of developing CRC at an earlier age and are advised to follow a specific screening and surveillance program. These people include:

- Family history of CRC:
  - One first-degree relative with CRC or advanced adenoma, diagnosed before 60 years age.
  - Two first-degree relatives with CRC or advanced adenoma at any age [5].

- Patients with classical germline mutation of Adenomatous polyposis coli (APC) gene should be screened for CRC annually by colonoscopy or flexible sigmoidoscopy until colectomy. The onset age of polyposis is around 16 years and the age of development of CRC is around 25 years later.

The time of colectomy is based on the degree of developing symptoms including, size and number of adenomas, presence of cancer or high-grade dysplasia. In early twenties, colectomy is optional if the number is less than 10 of adenoma or small-sized < 5 mm adenomas. It should be performed earlier if the patient is symptomatic with gastrointestinal bleeding, if there are many 6 -10 mm polyps that cannot be cleared by polypectomy or if there is an increasing number of colon polyps in consecutive endoscopies [27].

The need of colectomy is more urgent if there is adenoma with high-grade dysplasia or suspected or documented CRC.

After surgery, surveillance should be continued as there is a risk of developing adenoma or adenocarcinoma in the rectal cuff or in the ileostomy site [28]

Patients with attenuated FAP, bearing a germline mutation of APC gene near its 3' end or 5' end, develop 100 or less, predominantly right-sided, adenomatous colon polyps at 20 to 25 years old with a tendency to reach the rectum and develop CRC at 55 years. Screening should start at age 20 to 25 [29].

- HNPCC: Patients according to the Amsterdam or Bethesda criteria, should have their CRC tested for MSI, looking in the tumor cells for variability in number of nucleotides in DNA, and MMR proteins by immunohistochemical staining. If the tests become positive, patients should get the genetic testing for HNPCC. All the family members positive for this genetic testing should proceed screening colonoscopy at first, every 2 years starting age 20 to 25 until age 40, then annually [30].
- SPS: represents the most common polyposis aberrance with a prevalence of 1 in 3,000. According to WHO criteria for SPS include:
    - At least 5 serrated polyps adjacent to the sigmoid colon, with 2 or more of those being ≥ 1 cm
    - Any number of serrated polyp adjacent to the sigmoid colon in a person who has a first-degree relative with SPS
    - More than 20 serrated polyps of any size distributed throughout the colon [31].

In SPS, the progressive incidence of CRC is 7% in 5 years with endoscopic surveillance [32]. Colonoscopy surveillance is recommended annually in all patients with SPS.

- Long-standing IBD: In IBD patients, the incidence of CRC is 6 times more than that in general population, leading to death in 10-15% of IBD patients [33]. The risk factors for developing CRC in IBD include disease duration, extent and severity of colitis, presence of primary sclerosing colitis (PSC) and family history of IBD [34]. Ulcerative colitis (UC) and Crohn's disease (CD) have an equivalent risk of developing CRC [35].

In case of UC, colonoscopy for screening is recommended 10 years after the diagnosis of colitis. Surveillance colonoscopy should be performed every 1 to 2 years depending on the presence or absence of dysplasia [36].

The high-risk individuals for CRC with current recommendations for screening and surveillance are summarized in **Table 3.**

Table 3. Screening for CRC in High-Risk Individuals

High-risk individuals for CRC	**Recommendations**
1. Family history	Screening colonoscopy every 5 years starting from 40 years old or 10 years earlier than the youngest patient in the family.
2. Classical FAP	Colonoscopy each year or flexible sigmoidoscopy beginning at age 12 to 14 years until performing colectomy.
3. Attenuated FAP	Screening should start at age 20 to 25 without any limit to stop the surveillance.
4. HNPCC	All positive members for the genetic testing should start at 20-25 years old to get screening colonoscopy every 2 years, until age 40, then annually.
5. SPS	Surveillance colonoscopy each year.
6. IBD	Screening colonoscopy is recommended 10 years after the diagnosis of colitis as well as Crohn's colitis involving at least one third of the colon.

CRC: colorectal cancer; FAP: familial adenomatous polyposis; HNPCC: hereditary non-polyposis colorectal cancer; SPS: serrated polyposis syndrome; IBD: inflammatory bowel disease.

1.1.3.3 Molecular classification and development of CRC

Sequential multistep mutational processes (schematized in **Figure 1**), play crucial roles in the development of CRC:

- 70% of CRC arise from adenocarcinoma sequence seen in sporadic adenoma and FAP [37].
- The remaining 30% arises from other pathways including MMR gene defect detected in Lynch syndrome, BRAF mutation detected in sessile serrated polyps and base-excision repair (BER) gene impairment seen in MYH-associated polyposis syndrome [38].

In adenocarcinoma sequence, loss of function of tumor suppressor genes: adenomatous polyposis coli (APC) gene, p53 gene and deleted in colon cancer (DCC) gene on chromosome 18q and in the other side, activation of oncogene KRAS lead to the formation of CRC. Loss of function of APC gene is crucial in the first step in the adenocarcinoma sequence. Later, loss of function of DCC gene induces late stage of adenoma progression, while the loss of p53 gene is at the last stage of adenocarcinoma sequence. In the other hands, KRAS oncogene activation occurs in up to 45% of CRC, and is associated with increased aggressiveness of CRC, reduced responsiveness to selected chemotherapeutic agents in metastatic colon cancer, and poor survival [39].

Microsatellite instability (MSI) and chromosomal instability (CIN) are predominant tumorigenic pathways in CRC. CIN leads to high mutation rates in genes strictly linked to the development of CRC, and WNT pathway such as APC, KRAS, β-Catenin, AXIN, SMAD4, PI3KCA, SOX9, ARID1A, FAM123B and TP53, which lead to the development of CIN tumors [40].

MMR genes are involved in repairing the mistakes made during DNA replication. MMR deficiency leads to accumulation of many DNA mutations and may lead to MSI and cancer. This deficiency occurs mostly in Caucasians, middle-aged to older population with no family history of CRC, mostly in the right colon and is associated with relatively good prognosis.

Aberrant DNA methylation of CpG islands, is widely classified in CRC. CpG island methylator (CIMP)-associated methylation of MLH1 induce sporadic cases of MMR deficiency. Germline mutations in the MMR genes such as hMSH2, hMLH1, hPMS1 and hPMS2, occur in HNPCC and account for 3-6% of total CRC [41].

In the other hands, a study suggested that the simultaneous methylation of P16 and MGMT genes is correlated with better prognosis of disease and lower aggressiveness and longer overall survival in patients with CRC [42].

BRAF is a proto-oncogene, functions as serine/threonine kinase, is associated with a gene mutation of valine-to-glutamate change at the residue 600 - V600E is found in 10% of CRC. This mutation causes sessile serrated adenoma (SSA) and classical serrated adenoma (TSA). CRC caused by BRAF mutation are usually right-sided, occurring in older ages, with higher recurrence in females and associated with MSI. In addition, CRC caused by BRAF mutation with MSI is generally associated with better prognosis but right-sided CRC carries a bad prognosis [43]. Generally, all cases of BRAF-mutated CRC are CIMP-positive [44].

So CIMP-positive tumors are tumors arising from serrated adenoma. Approximately, 50% of CIMP-positive tumors have microsatellite instabillity. 20 to 30% of all CRC are CIMP-positive and 10-12% of all CRC are CIMP-positive and microsatellite unstable.

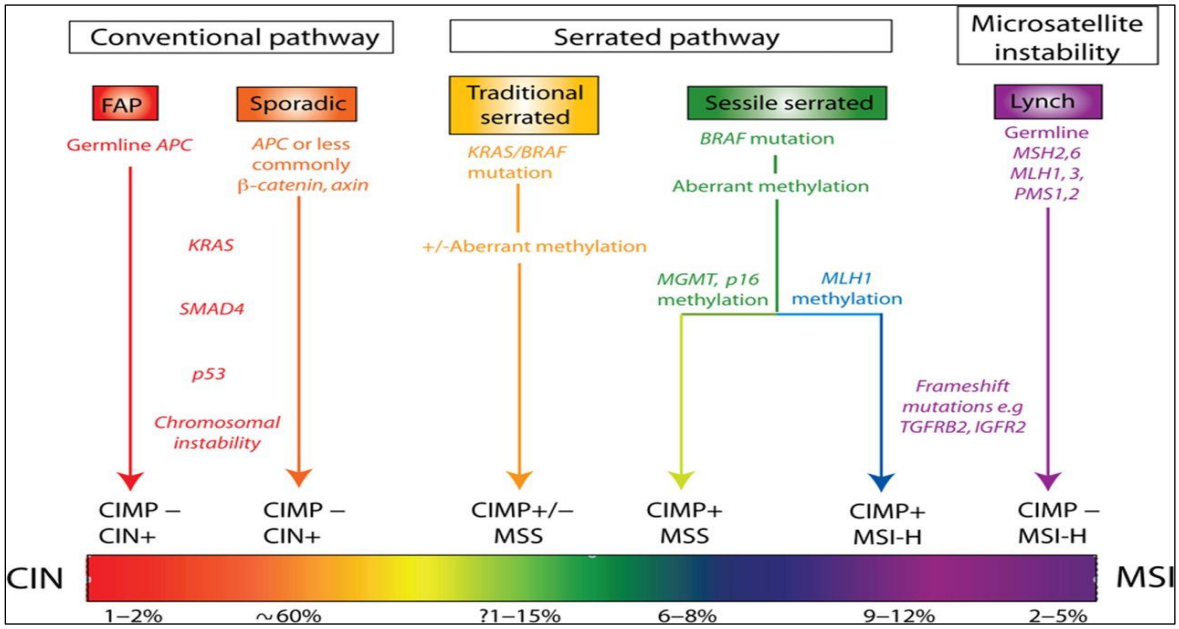


Figure 1: Molecular classification of colorectal cancer [45]

Considering the spectrum of colorectal cancer (CRC)—conventional adenomas progress by the sequential accumulation of genetic mutations and chromosomal instability causing microsatellite stable (MSS) tumors. Microsatellite instability (MSI) is the result of defective DNA repair through inactivation of mismatch repair genes and is epitomised by the germline mutation of

Mis-Match Repair (MMR) genes seen in Lynch syndrome (hereditary non-polyposis coli). The sessile serrated neoplasia pathway is often initiated by genetic mutation of BRAF or KRAS genes but then progresses by methylation of tumour suppressing genes (CpG island methylator phenotype (CIMP)). Both MSS and unstable tumors can result depending on the genes epigenetically silenced as the lesions progress. Comparatively, little is known about the traditional serrated pathway, but evidence is accumulating that this is a distinct molecular subtype. FAP, familial adenomatous polyposis.

1.1.4 Staging and therapy

Different staging criteria have been used to assess the degree of cancer penetration in the colon and extracolonic spread (**Figure 2**).

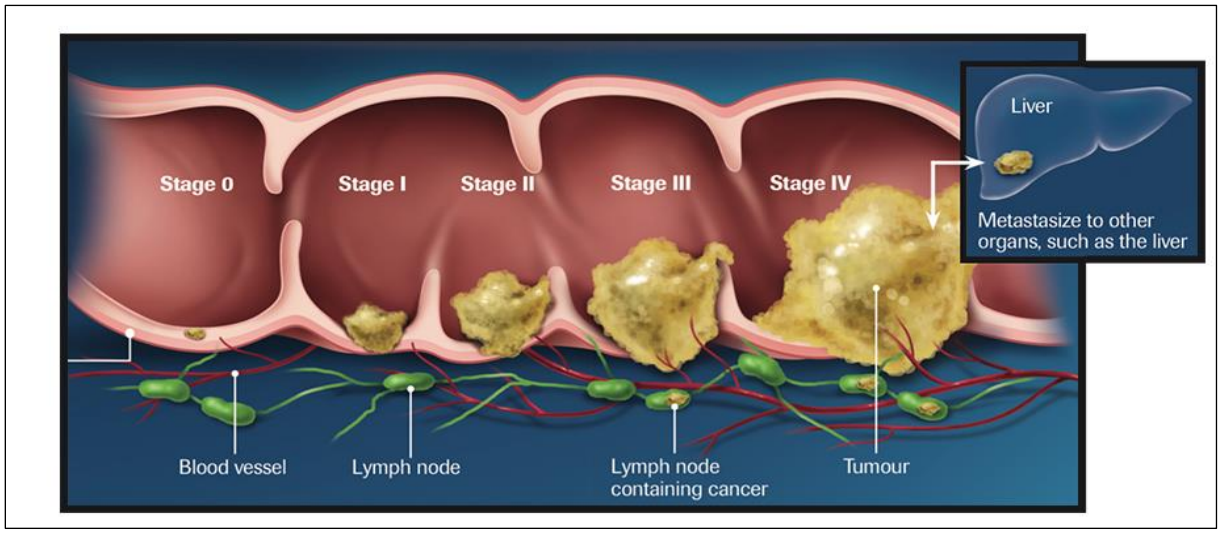


Figure 2: Stages of CRC

Stage 0: Tumor is located in the inner layer of the colon or rectum. Stage I: Tumor has grown into the second layer of the colon or rectum or its muscle layer. Stage II: Tumor has spread completely through the colon or rectal wall but has not invaded nearby lymph nodes. Stage III: Tumor has reached nearby lymph nodes. Stage IV: Tumor has spread to one or more distant structure(s), include the liver, lungs, or reproductive organs. from: https://www.guidetocrc.ca/subpages/stages-of-colorectal-cancer.html

A commonly used method for colon cancer staging (summarized in **table 4**) is based on the TNM (tumor/node/metastases) system, approved by the American Joint Committee on Cancer (AJCC) [46].

Other classification methods exist such as Dukes System: A, tumor confines to intestinal wall; B, tumor invades through the intestinal wall; C, tumor involves lymph node; D, tumor metastases to distant sites. Astler-Coller System describes: A, Tumor restricted to mucosa; B1, tumor beyond muscularis mucosa but not muscularis propria; B2, tumor within muscularis propria; C1, B1, lymph node metastases; C2, B2, lymph node metastases; D, Distant metastases [46].

Table 4: Cancer classification of CRC stages according to American Joint Committee

AJCC stage	**TNM stage** tumor/nodes/ metastases	**Tumor Node Metastases**
Stage 0	Tis N0 M0 carcinoma in situ	Tumor confined to mucosa
Stage I	T1 N0 M0	Tumor invades submucosa
Stage I	T2 N0 M0	Tumor invades muscularis propria
Stage IIA	T3 N0 M0	Tumor invades subserosa
Stage IIB	T4 N0 M0	Tumor invades adjacent organs
Stage IIIA	T1-2 N1 M0	Tumor metastases to 1-3 nodes
Stage IIIB	T3-4 N1 M0	Tumor metastases to 1-3 node
Stage IIIC	Any T, N2, M0	Tumor metastases to 4 or more nodes
Stage IV	Any T or N, M1	Tumor metastases to distant sites

In the first stage of CRC, the disease appears to be completely resected without any subsequent spread to the adjacent organs, lymph nodes or distant sites and they are often characterized as "curative". Later, the pathology could develop, and malignant cells might evade into the peritoneum or other nearby organs, lymph nodes, or distant sites, including the liver. However, these cells with disease localized in the colon reflect a high risk for subsequent development of colon polyps that could lead to more aggressive stages of CRC. Therefore, "early stage" disease requires more colonoscopic surveillance to control the disease progression.

In addition, precise staging, is critical, not only in evaluating the requirement for adjuvant therapy, but also for the need for continued surveillance [47].

In the following stage I, surgical resection of the tumor and the local lymph nodes are necessary to treat CRC. Checking the adequate number of lymph nodes during surgery is essential for correct staging of CRC [48].

At this stage, malignant polyps may appear benign looking endoscopically, account for 12% of polyps in polypectomy series, they are T1 lesions and cancer cells have invaded the muscularis mucosae into the submucosa [49].

In stage II CRC, the treatment consists of surgery alone and without using common adjuvant chemotherapy, only in critical cases such as poorly differentiated cancer, lympho-vascular invasion, perineural invasion, lymph nodes number, bowel obstruction, localized perforation and positive margins, The European Society of Medical Oncology (ESMO) recommends adjuvant chemotherapy [50]. The adjuvant chemotherapy includes 6 months course of one of the conventional chemotherapy regimens: 5-flurouracil with leucovorin, capecitabine, or combination of 5-flurouracil with leucovorin and oxaliplatin (FOLFOX) or capecitabine and oxaliplatin (Capeox).

In stage III, CRC treatment consists of surgical resection of the tumor succeeded by adjuvant chemotherapy consisting of six cycles of FOLFOX or Capex [51].

In USA, second and third stages of CRC are treated by neoadjuvant chemoradiation; infusion of 5-flurouracil with leucovorin accompanied with radiation therapy for more than 5 weeks followed by surgery (low anterior resection or abdominal perineal resection) [52].

In Stage IV CRC can be managed according to the location of primary CRC and classification of the disease; a specific or a combination of chemotherapy, immunotherapy/inhibitor therapy, biologic targeted therapy, palliative surgery, radiotherapy and radiofrequency ablation could be used. The classification is based on tumor MSI, KRAS/NRAS and BRAF mutation status. Patient's general health profile and history, goals of treatment and patient's preferences are also considered [53]. In addition, patients' situation should be taken into consideration to relief pain and maintain quality of life and increase overall survival. Commonly used chemotherapeutic agents in stage IV CRC are FOLFOX (5-flurouracil, leucovorin and oxaliplatin), FOLFIRI (5-flurouracil, leucovorin and irinotecan), CAPIRI (capecitabine and irinotecan), CAPOX (capecitabine and oxaliplatin), 5-flurouracil with leucovorin, irinotecan, capecitabine and trifluridine plus tipiracil (Lonsurf). In **Table 5** are summarized the different treatment regimen according to each stage of CRC.

In combination with chemotherapy is also given the targeted therapy. Targeted therapy is a kind of personalized treatment, it depends the CRC patient's profile. For example, KRAS could be either mutated or wild type. The targeted therapy in this case, includes some angiogenesis inhibitors to target tumor angiogenesis and tumor blood vessels formation, it consists of:

- Bevacizumab (monoclonal antibodies against vascular endothelial growth factor (VEGF))
- Ramucirumab (monoclonal antibody against vascular endothelial growth factor receptor (VEGFR))
- Cetuximab and panitumumab (monoclonal antibody against epidermal growth factor receptor (EGFR)
- Regorafenib (angiogenic, stromal and oncogenic kinase inhibitor)
- Aflibercept (VEGF A inhibitor and placental growth factor inhibitor) [54] [55].

Targeted therapy increases the efficacy of chemotherapy agents by ensuring and targeting their delivery to the CRC.

Several studies showed that this combination of chemotherapy and chemotherapy had a beneficial impact in CRC treatment. For instance, bevacizumab plus FOLFIRI given to patients with metastatic CRC, increased the overall survival (20.3 vs. 15.6 months) [56].

The anti-EGFR agents showed improved survival only in KRAS wild type CRC, in stage IV CRC as well [57].

In addition, up to 20% of stage II and stage III CRC have MMR-deficiency (dMMR) or microsatellite instability-high (MSI-H) and they show a better prognosis compared to proficient mismatch repair (pMMR) or microsatellite stable (MSS) CRC, while 3.5% of stage IV CRC are dMMR or MSI-H and are associated with poor prognosis [58] [59].

In dMMR or MSI-H cases of CRC, there is an upregulation of checkpoint inhibitory proteins:

- PD1, (PDL1programmed cell death protein 1) and its ligand
- CTLA-4 (cytotoxic T lymphocyte-associated antigen 4)
- LAG3 (lymphocyte activation gene-3 protein)
- IDO (indoleamine 2,3-dioxygenase)[60].

These inhibitors have been found to be helpful in these patients; cases with metastatic tumors were treated with nivolumab plus ipilimumab.

PD1 inhibitors pembrolizumab and nivolumab have been approved by the FDA for the treatment of dMMR or MSI-H metastatic CRC.

Colorectal obstruction and bleeding might be treated by palliative surgery, colonic stent is also placed to avoid major surgery and shorten hospital stay [61 62].

In some patients with CRC with liver metastasis, that could be detected using different imaging techniques such as positron emission tomography (PET)/CT imaging surgical, resection is helpful before or after chemotherapy, to prolog patient's survival.

Other methods of treatment of liver metastasis include radiofrequency ablation, hepatic intra-arterial chemotherapy infusion, trans-arterial chemoembolization (TACE), radioembolization, external beam radiation and stereotactic radiation; nonetheless, surgery exhibit the best treatment without relapse [63].

Table 6. Treatment of Different Stages of CRC

Stages of CRC Treatment modalities	**Treatment modalities**
Stage 1	Resection of malignant polyp by endoscopy or resection of tumor and local lymph nodes by surgery.
Stage 2	Surgery alone. Adjuvant chemotherapy may be needed in presence of high-risk characteristics only.
Stage 3	Surgery supplemented with adjuvant chemotherapy.
Stage 4	Chemotherapy, biologic targeted therapy, immunotherapy, palliative surgery, radiotherapy, radiofrequency ablation and coloning stenting.

1.1.4.1 Outcome of staging

Evidence have shown that earlier cancer stage is correlated with a better clinical outcome and vice versa.

Patients having localized disease limited to the submucosa or muscularis propria, have up to 70% overall 5-year survival. More the disease develops and spreads through the subserosa into

adjacent or distant structures, peritoneum, lymph nodes, the estimated 5-year survival, decreases to 30%. In addition, 5-year survival might decrease in some early stage colorectal cancer, bowel perforation from the tumor itself or anastomotic leakage following surgery [64].

Early detection of CRC is important for patients at increased risk.

Colonoscopic surveillance represents a prominent proof that morbidity and mortality can be improved, being an important tool for surveillance for high risk groups to detect colon cancer that include patients with bowel disease or familial history of colon cancer or colon adenoma [65].

Additionally, CRC mortality is consequently related to polypectomy, malignant colorectal polyps with localized submucosal invasion and advanced adenomas therefore, checkup is useful to keep the progress of CRC under control [66].

1.2 The TCGA project and RNAi

1.2.1 TCGA

TCGA, The Cancer Genome Atlas, is a reference for cancer genomics, characterizing at the molecular level more than 20,000 samples from primary cancer and matched normal parts in 33 cancer types. This project between the National Cancer Institute and the National Human Genome Research Institute began in 2006. Over the next dozen years, TCGA generated over 2.5 petabytes of genomic, epigenomic, transcriptomic, and proteomic data. The data, which has already led to improvements and ability to diagnose, treat, and prevent cancer, is publicly available for anyone in the research community to use (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga).

1.2.2 TCGA genomic characterization of colorectal cancer

In this project, colorectal tumor samples were collected with the corresponding germline DNA samples from 276 patients for sequencing of 224 cancers compared to the corresponding normal samples. Information about DNA SCNA (somatic copy number alteration) analysis, promoter methylation, messenger RNA (mRNA) and micro RNA (miRNA) resulted from these studies. The value of this study in both clinical and pathological characteristics, could be helpful to bypass the anatomical limits by giving further insights into the molecular pathology of CRC [40].

The resulting classification systems allow better prognostication and help to direct post-surgical adjuvant treatment, including immune checkpoint blockade.

Biologically CRC is categorized into microsatellite instable (MSI) or microsatellite stable (MSS) but chromosomally unstable (CIN).

After this analysis, CRC could be divided into two major groups according to mutation rate; non hypermutated and hypermutated cancers which match with the CIN and MSI pathways.

The hypermutated category was subdivided in two subgroups, the majority of tumors in this group (~17 %) were hypermutated due to defective mismatch repair (dMMR). The other group of nonhypermutated cancers mostly demonstrated a high SCNA frequency (~84 %) of colorectal adenocarcinomas that were MSS due to an intact MMR pathway.

Colon and rectal cancers combined, showed no distinguishable molecular differences by analyzing the non-hypermutated MSS group.

Hypermutated MSI cancers had less SCNAs, but a similar pattern of chromosomal arm gains and losses.

The most frequently altered pathways were classified according to gene mutations, deletions, amplifications and translocations, they included:

- The activation of the WNT, PI3K and MAPK signalling pathways.
- The deactivation of the TGF-β and P53 inhibitory pathways [67].

1.2.3 Colorectal cancer gene expression profiling (CMS Classification)

An international expert consortium[68] described the four consensus molecular subtypes (CMS) after analyzing 18 different CRC gene expression datasets, including data from TCGA:

- CMS1 (MSI-immune, 14 %) represents hypermutated CRC due to defective DNA mismatch repair, while having a low number of SCNAs. Affected genes induce strong immune activation. Patients with the CMS1 had a very poor survival rate after relapse.
- CMS2 (canonical, 37 %) represents CRC with predominantly with epithelial signatures, prominent WNT and MYC signalling activation, loss of tumor suppressor genes and copy number gains of oncogenes. Patients had a better survival rate after relapse.
- CMS3 (metabolic, 13 %) represents CRC with fewer SCNAs and more hypermutated/MSI samples than CMS2 and CMS4, with frequent KRAS mutations.

Affected genes in CMS3 have an epithelial signatures and metabolic dysregulation in a variety of pathways.

- CMS4 subtype (mesenchymal, 23 %) represents CRC with high expression of EMT genes and genes implicated in inflammation, matrix remodeling, stromal invasion and angiogenesis. Patients with the CMS4 subtype had the worst survival and the worst relapse-free survival.

Other samples have mixed features (13 %) possibly due to transition phenotype or intra-tumoral heterogeneity [67].

1.2.4 RNAi screening

RNA interference (RNAi) has been widely used for gene silencing allowing genome-wide functional screenings both *in vitro* and *in vivo*. The information deriving from a number of genes' screening led to the identification of new cellular pathways and potential drug targets [69].
RNAi is a physiological pathway that reduces, without totally eliminating mRNA target expression by double strand RNA (dsRNA), that could be found in the cells, in a sequence-specific manner [70]. The application of this process as a molecular biology tool, have led to impressive new research in several fields. In fact, RNAi has been used for downregulation of mRNA levels, contributing to large-scale studies in functional genomics applied in a wide variety of cells and organisms, such as mammalian cells, fruit fly and mouse [71 72].

1.2.5 Applications

RNAi technologies are applied in genomic sequence databases; genome-scale libraries of RNAi reagents have been designed and have been applied in high-throughput screenings (HTS) in multiple types of cells [73].
This tool allowed to bypass technical limitation and proved to be powerful by allowing systematic functional analyses in several cells or animal models. One of the applications of RNAi include genome-scale cell-based RNAi screening in mammalian cells [74]. In fact, several biomedical discoveries became possible using RNAi, including the identification of novel oncogenes and potential targets to design new therapeutics [75].

1.2.6 Methodology

The application of RNAi made possible the loss-of-function genetic screening. Thanks to RNAi, a gene can be knock down to analyze its importance in a specific function. The gene function would be affected by the absence of the gene, therefore reflecting the normal phenotype, that could be assessed by a conventional test for the specific function [76]. This unique mechanism of action improved the analysis of both small-scale studies and HTSs. For example, HTSs permitted the simultaneous analysis of a wide number of gene functions, allowing the identification of various members of functional pathways or revealing the involvement of new genes in a known function or process [77].

These types of RNAi screenings are usually subdivided in two categories:

- The pool format, where the gene silencing occurs randomly in the cells
- The arrayed format, where each gene is targeted for silencing by the appropriate molecule in a multi-well plate.

The importance of pool format is allowing the screening of a library of RNAi reagents, usually against the whole genome by introducing randomly only one gene in one cell. The readout of these screens is based on selecting a phenotype displayed by the cells such as, the survival or the expression of a particular protein [78].

In arrayed formats the set of genes to analyze is more adaptable, it could be from few hundreds to the entire genome. The RNAi reagents are delivered each well of the multiwell plate, facilitating both the manipulation and the readouts. The phenotype is usually detected by assessing cell viability, the response to a drug or the localization of a protein, by fluorescence or luminescence readouts [73] [79]. Additionally, the advantage of arrayed formats is the ability to assess multiple phenotypes in each gene or well in a single screen, in order to improve the specificity of the system.

1.2.7 The role of bioinformatics

The emerging bioinformatics scanning approaches allow researchers to simultaneously investigate the changes in genome-wide genes in certain biological conditions. These high-throughput technologies generate large gene lists of biological research interest as final data. Bioinformatics favors complete analysis of biological experiments in a shorter time.

These tools made all the data available online and very efficiently able to reach, modify and replace the complete data in a very shorter period. In molecular biology experiments require lots of analysis and generate large outputs thus bioinformatics was first applied to molecular biology integrated by almost all fields of biological research [80].

The crucial information obtained from RNAi screenings could be integrated with bioinformatic approaches. The performed bioinformatic analyzes on the entire genome resulted in identifying a subset of candidate genes, which can furtherly be tested with the experimental setup of RNAi reagents. For instance, a subset of candidate genes sharing a biochemical function such as kinases have been analyzed, revealing the role of JNK, as transcription factor, that could be regulated by several upstream and unrelated kinases [81].

This approach can be used indirectly as well, to find more information about a specific group of genes from the data available in the literature. These *in silico*-based analyses are followed by the screening of the obtained gene subset. For example, the identification new regulatory mechanisms of hematopoietic stem cell activity by RNAi screening, by evaluating a set of genes involved in stem cell homeostasis [82].

Additionally, the further complementation of RNAi screenings with bioinformatic platforms and the information obtained from gene expression studies or protein-protein interaction experiments can highlight the complexity of gene networks [83].

1.2.8 cBioPortal

The cBioPortal for Cancer Genomics (http://cbioportal.org) is a Web resource for exploring, visualizing, and analyzing multidimensional cancer genomics data. The portal reduces molecular profiling data from cancer tissues and cell lines into readily understandable genetic, epigenetic, gene expression, and proteomic events. The query interface combined with customized data storage enables researchers to interactively explore genetic alterations across samples, genes, and pathways and, when available in the underlying data, to link these to clinical outcomes. The portal provides graphical summaries of gene-level data from multiple platforms, network visualization and analysis, survival analysis, patient-centric queries, and software programmatic access. The intuitive Web interface of the portal makes complex cancer genomics profiles

accessible to researchers and clinicians without requiring bioinformatics expertise, thus facilitating biological discoveries.

Large-scale cancer genomics projects, such as The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC), are generating an overwhelming amount of cancer genomics data from multiple different technical platforms, making it increasingly challenging to perform data integration, exploration, and analytics, especially for scientists without a computational background. The cBioPortal for Cancer Genomics was specifically designed to lower the barriers of access to the complex data sets and thereby accelerate the translation of genomic data into new biological insights, therapies, and clinical trials. The portal facilitates the exploration of multidimensional cancer genomics data by allowing visualization and analysis across genes, samples, and data types. Users can visualize patterns of gene alterations across samples in a cancer study, compare gene alteration frequencies across multiple cancer studies, or summarize all relevant genomic alterations in an individual tumor sample. The portal also supports biological pathway exploration, survival analysis, analysis of mutual exclusivity between genomic alterations, selective data download, programmatic access, and publication-quality summary visualization.

Genomic data types integrated by cBioPortal include somatic mutations, DNA copy-number alterations (CNAs), mRNA and microRNA (miRNA) expression, DNA methylation, protein abundance, and phosphoprotein abundance. Currently, the portal contains data sets from 10 published cancer studies, including the Cancer Cell Line Encyclopedia (CCLE), and more than 20 studies that are currently in the TCGA pipeline. For each tumor sample, data may be available from multiple genomic analysis platforms. The portal's simplifying concept is to integrate multiple data types at the gene level and then query for the presence of specific biological events in each sample (for example, genetic mutation, gene homozygous deletion, gene amplification, increased or decreased mRNA or miRNA expression, and increased or decreased protein abundance). This allows users to query genetic alterations per gene and sample and test hypotheses regarding recurrence and genomic context of gene alteration events in specific cancers [84].

1.3 The adaptor protein complexes

1.3.1 Adaptor proteins and vesicular trafficking

Cargo proteins designed for transport to different locations are assembled into vesicles to be delivered to their target sites by vesicular trafficking by the endocytic and secretory pathways. The adaptor protein (AP) complexes have a key role in this process. They recognize and bind to sorting signals located in the cytoplasmic tails of the cargo proteins, consequently they will recruit clathrin or other accessory proteins, and finally they pack the cargo proteins in vesicular carriers to be transported from the donor membrane to the destinated organelle membrane (**Figure 3**).

To date, five AP complexes have been characterized, they include: AP-1, AP-2, AP-3, AP-4 and AP-5. Each complex is composed by:

- two large subunits: one each of γ /α/δ/ε/ζ and β1, β 2, β 3, β4 and β 5 respectively
- one medium-sized subunit: μ1, μ 2, *μ3, μ4* and μ 5
- one small-sized subunit: σ1, σ 2, σ 3, σ 4 and σ 5.

Some subunits have multiple isoforms encoded by different genes:

- AP-1: has two γ (γ1 and γ 2), two μ (μ1A and μ1B) and three σ *is*forms (σ1A, σ1B and σ1C)
- AP-2: has two α isoforms (αA and αC)
- AP-3: has two β (β3A and β3B), two μ (μ3A and μ3B) and two σ isoforms (σ3A and σ3B).

The different assembly between different subunit isoforms generates diverse AP heterotetramers, that may exhibit tissue-specific expression and function [85]. For example, AP-1A containing μ1A is ubiquitously expressed, whereas AP-1B containing μ1B is epithelial-specific. AP-3 also exists as either ubiquitous or tissue-specific isoforms: AP-3A having, μ3A is ubiquitous, whereas AP-3B formed with μ3B is neuron-specific (**Table 7**).

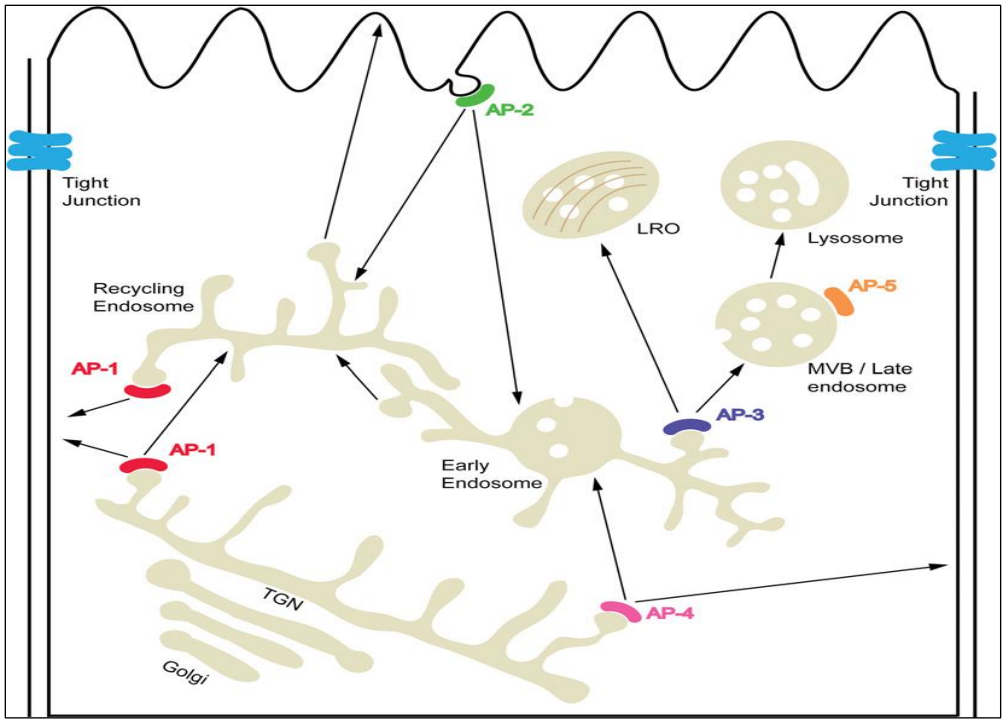


Figure 3: Localization and trafficking of AP complexes [86]

AP-1 is localized to TGN and REs, mediates bidirectional transport between them. AP-1 also mediates basolateral sorting in epithelial cells. AP-2 plays a role in clathrin-dependent endocytosis from the plasma membrane. AP-3 is localized to endosomes, and responsible for the LRO biogenesis. AP-4 is localized to TGN and mediates vesicle trafficking from TGN to endosomes or basolateral plasma membrane. AP-5 is localized to late endosomes, and its function is largely unknown.

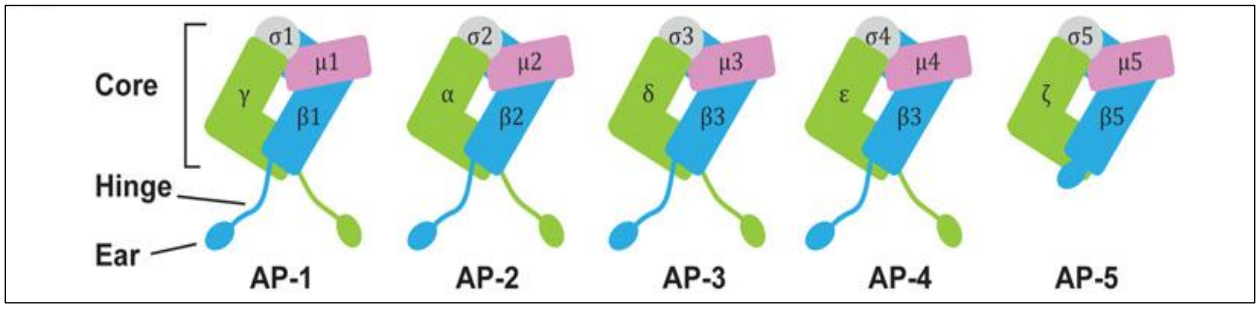


Figure 4: Structures of AP complexes [86]

Diagrams of heterotetrameric AP complexes. All AP complexes consist of core, hinge, and ear domains, except AP5 that lacks hinge domain. Core domains are responsible for the cargo protein binding and membrane localization. Hinge and ear domains are important to the interaction with coat proteins and regulatory/accessory proteins.

1.3.2 Localization, function and regulation of the AP complexes

Protein trafficking across membranes is a highly dynamic and efficient process orchestrated by a number of connected proteins. AP complexes play a crucial role in this process. Indeed, each AP complex performs the specific sorting function, specific to intracellular organelles (**Figure 3**). AP-1 located in TGN (*trans*-Golgi network) and endosomes is associated with clathrin-coated vesicles [87]. AP-1 operates in the biogenesis of secretory granule and in the bidirectional transport between TGN and endosomes.

AP-2 is the most studied family member; it localizes to plasma membrane, facilitating clathrin-mediated endocytosis of a large number of proteins such as, receptors, adhesion molecules and viral proteins [88].

AP-3 is mainly found on a tubular endosomal compartment where it mediates cargo transport from tubular endosomes to late endosomes and participates in the biogenesis of lysosome-related organelles (LROs). The neuron-specific AP-3B plays an important for the formation and release of exocytic organelles such as large dense core vesicles, synaptic vesicles, etc. [89].

AP-4 preferentially localized to the TGN, regulates the cargo transport from the TGN to endosomes in a clathrin-independent manner [90]. Besides its role in TGN sorting, AP-4 is involved in polarized sorting in epithelial cells and neurons as well [91] (**Table 7**).

Both AP-3 and AP-4, found in TGN and/or endosomal membranes are recruited to the membrane by Arf1 [92 93].

The most recently identified AP-5 complex, localized to late endosomes, functions in endosomal sorting [94]. AP-5 is also clathrin-independent [95], implicated in a sorting step out of late endosomes to Golgi [96].

Table 7: AP complexes

Adaptors	Subunits	Localization	Functions
AP-1	γ 1/2 β1 μ1A/B σ1A/B/C	TGN/endosomes	TGN↔endosomes Polarized sorting
AP-2	αA/C β2 μ2 σ2	Plasma membrane	Clathrin-dependent endocytosis
AP-3	δ β3A/B μ3A/B σ3A/B	Endosomes	LRO biogenesis
AP-4	ε β4 μ4 σ4	TGN	TGN↔endosomes Polarized sorting
AP-5	ζ β5 μ5 σ5	Late endosomes	Late endosome-to-Golgi Retrieval

1.3.3 The AP3 complex

1.3.3.1 The genetics of the AP-3 complex: Neuronal functions versus non-neuronal AP-3 complexes

The AP-3 complex is formed by four subunits δ, β3, μ3 and σ3. β3, μ3 and σ3 each exist as two isoforms A and B (**Figure 5**). The called B isoforms of both β3 subunit and μ3 subunit [97 98], are largely restricted to neuronal and neuroendocrine tissues, whilst both σ3A and σ3B are ubiquitously expressed. Therefore, a neuronal AP3 isoform assembles δ with either σ3A or σ 3B subunits to β3B and μ3B. In the other hands, β3A and μ 3A assemble with δ, and σ 3A or σ 3B subunits to form the ubiquitous AP-3 isoform expressed in all cells, including neurons [99].

The AP-3 isoforms have different sorting functions:

The ubiquitous AP-3 regulates the biogenesis of neuronal lysosomes whereas neuronal AP-3 plays an exclusive role in synaptic vesicle proteins trafficking. Indeed, mice selectively lacking neuronal AP-3 (*Ap3b2–/–* or *Ap3m2–/–*) [100 101] exhibit an epileptic phenotype but mice lacking the ubiquitous AP-3 isoform do not (*Ap3b1–/–*) [102]. In contrast, a closer inspection of the Ap3b1–/– (gene encoding for β3A) mouse brains reveals that to a certain extent, AP-3 complexes assembled with β3B and may recognize similar cargoes, suggesting that subtle neurological phenotypes may occur in case of β3A deficiency.

The reason after β 3A or β 3B containing complexes could generate different phenotypes even if both can recognize an overlapping synaptic vesicle proteins, is explained by differences in the range of synaptic vesicles recognized by AP3A or AP3B, the subcellular localization of AP3A and AP3B complexes, and/or the destination of the vesicles generated by these adaptor complexes. For example, vesicles generated by AP3A-containing complexes could target synaptic vesicle to degradative compartments [101].

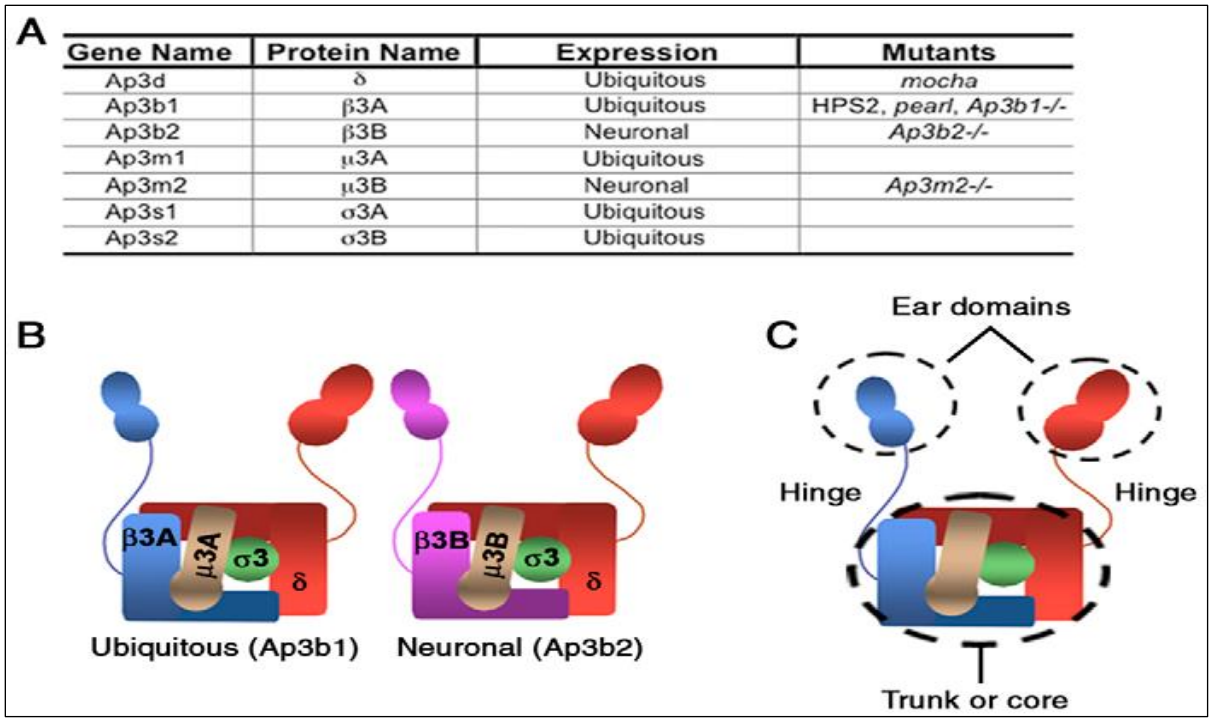


Figure 5: Nomenclature and structure of AP-3 subunit isoforms [99]**.**

(A) AP-3 subunit genes and their corresponding gene products. The pattern of expression of each subunit is described as neuronal, corresponding to neurons and neuro-endocrine tissue, or ubiquitous, for subunits expressed in all cells including neuronal tissues. Mice carrying natural or engineered AP-3 subunit deficiencies are listed. (B) Proposed subunit composition of the neuronal and ubiquitous AP-3 isoforms. Both AP-3 complexes can carry either a σ3A or a σ3B subunit. (C) Adaptor complexes possess three defined domains. The ears correspond to the C-terminal domains of δ and β3. The trunk or core is composed of a protease-resistant core formed by fragments of β3 and δ as well as full-length μ3 and σ3 subunits. The ears and trunk are connected by hinges.

1.3.3.2 Regulation of AP-3 function

Diverse types of proteins are involved in the recruitment of AP-3 to membranes and in the AP-3-dependent membrane protein sorting, they include clathrin [103], kinases [104], GTPases [105], intermediate filament proteins [106] and accessory proteins [107]. The most studied category is the Arf GTPases, they exist as GTP- and GDP-bound forms, commanding the recruitment and release of coat proteins from membranes. Brefeldin A is a negative regulator of this cycle leading to the

accumulation of the GDP form, preventing the binding of coats such as AP-3 to membranes [108]. Arf1 mutants locked in their GDP form inhibits the binding of AP-3 to organelles [109]. GTP-bound Arf1 directly interacts with the AP-3 complex through the σ3 subunit or the δ-σ3 AP-3 subcomplex [110] and consequently σ3 becomes exposed partially to the cytosolic environment. AP- 3 is capable to discriminate between different Arf GTPases.

The intrinsic GTPase activity of Arf1 occurs only in the presence of members of a diverse family of GTPase activating proteins (ArfGAPs) [111] auch as AGAP1 which selectively affects Arf-dependent recruitment of AP-3 to membranes by binding to inositol phospholipids [112]. The AGAP1 functionally links inositol phospholipids with AP-3 δ-σ3 subcomplex. AGAP1 regulates the levels of membrane-bound AP-3 by sensing membrane concentrations of PtdIns(4,5)*P*2 and/or PtdIns(3,4,5)*P*3. The phosphatidylinositol 4-kinase type II alpha (PI4KIIa), phosphorylates phosphatidylinositol (PtdIns) generating phosphatidylinositol (4)-phosphate, a precursor of both PtdIns(4,5)*P*2 and PtdIns(3,4,5)*P*3 [113]. PI4KIIa, present in the endosomes, is a major component of AP-3-derived vesicles [114] and is targeted to vesicles by an AP-3-dependent mechanism, thus behaving as an AP-3 cargo [115]. Beside acting as a passive cargo, the kinase expression affects the subcellular localization of AP-3 to endosomes hence, AP-3-dependent vesicle generation [115].

1.3.3.3 The neuronal form of adaptor protein-3 is required for synaptic vesicle formation from endosomes

Neuronal AP-3 is specifically involved in vesicle formation from endosomes, possibly for recovering such vesicles that have escaped the conventional recycling path. The main pathway of synaptic vesicles formation directly from the plasma membrane is AP-2 and clathrin whereas AP-3 plays an minor role in this pathway [116].

In addition, one possible function of AP-3 is the retrieval of synaptic-like microvesicles (SLMVs) that escape the normal, non-endosomal route to be recaptured and internalized into axonal endosomes (Figure 4 summarizes the roles of AP-3 in neurons).

The AP-3-mediated retrieval in neurons would be into specialized endosomes in the axons exocytotic sites but not immediately adjacent to them. In case of dysregulation of retrieval

mechanism in absence of neuronal AP-3, the result is deficiencies in synaptic transmission in case of high synaptic demands.

Another important role in endosome-derived synaptic vesicles is recapturing components of membrane from large dense core vesicles (LDCVs) that are neo- exocytosed [117]. A membrane expressing mutant proteins that could not be targeted to the SLMVs are degraded rapidly by lysosomes. The neuronal AP-3 could recapture protein components of LDCV proteins and sequester selected LDCV proteins from a degradative pathway, allowing them to release their contents at the plasma membrane regions away from sites of synaptic vesicle exocytosis. This step could and allow LDCV proteins to be integrated into the standard synaptic vesicle recycling mode (**Figure 6**).

The distribution of neuronal AP-3 in the brain reveals that the retrieval pathway or LDCV membrane recycling are frequently engaged in some neuronal pathways than others [89].

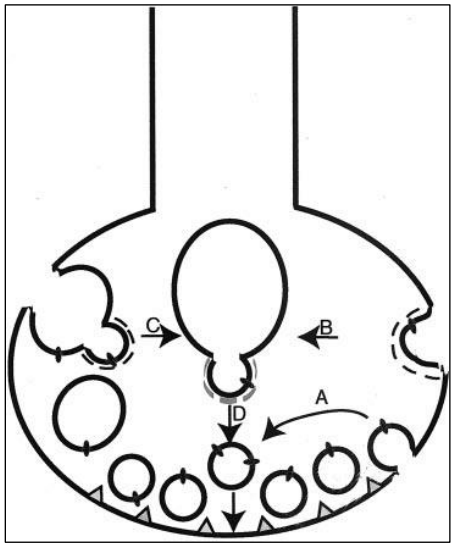


Figure 6: Neuronal AP-3-mediated pathway of synaptic vesicle biogenesis from endosomes [89].
Synaptic vesicles that cluster in the active zone (the triangles at the plasma membrane) undergo a cycle of exocytosis and recycling. Synaptic vesicle proteins normally recycle via the AP-2/clathrin pathway of endocytosis (arrow A) but escape recovery at the plasma membrane and may recycle via the AP-3 pathway. Such synaptic vesicle proteins may be retrieved into specialized axonal endosomes that use neuronal AP-3 to bud synaptic vesicles (arrow B). The endosomal pathway of synaptic vesicle production also may function to recycle components of

large dense core vesicles (LDCVs). LDCV proteins recycle via an endosomal intermediate, and some proteins may get sorted into synaptic vesicles. Neuronal AP-3 could recognize and bud such cargo into SLMVs from this endosomal intermediate (arrow C). Axonal endosomes that contain synaptic vesicle, as well as some LDCV membrane proteins, use neuronal AP-3 to produce synaptic vesicles, which are competent to fuse with the plasma membrane and release their contents (arrow D).

1.3.3.4 Mutations affecting AP-3 subunits and their physiological roles in vertebrates

Deficiencies caused by the ubiquitously expressed AP-3A are detected in pearl mice mutant for β3A, they display abnormal coat and eye color pigmentation and abnormal LROs, but it does not affect the neurological system [102]. In human the molecular alterations of the β3A subunit cause HPS-2 (Hermansky–Pudlak syndrome type 2) [118], a disorder characterized by LRO impairments, such as decreased eye pigmentation causing oculocutaneous albinism, recurrent infections due to defects of innate immunity and platelets abnormality causing bleeding problems. The pleiotropic nature of the phenotypes is due to the diversity of organelles regulate by the vesicles generated by the AP-3 machinery which carry membrane proteins bound to different types of secretory organelles, such as lysosomes and lysosome-related organelles including melanosomes, platelet dense granules, etc. [118].

In contrast, the neuronal form AP3B mutant mice show exclusively neurological defects. For instance, the μ3B-deficient mice experience spontaneous epileptic seizures [100] whereas the β3B knockout mice suffer from complex neurological and behavioral defects [101]. Moreover, a study involving Japanese epilepsy patients, suggested that a number of AP3M2 mutations are possible candidates for several unmapped disorders including epilepsy, febrile seizure, and other neuronal developmental disorders caused by functional abnormalities of GABAergic transmission [119].

In mocha mice, the lack of both AP-3A and AP-3B, due to homozygous null allele of AP-3 δ subunit, leads to the phenotypes obtained in both AP-3A- and AP-3B-deficient mice; lysosomal abnormalities, color defects and neurological defects [120].

1.3.3.5 The Mu subunit encoded by AP3M2 gene

A cDNA clone encoding for an approximately 47 KDa protein was first isolated from a library deriving from the electric lobe of the ray *Discopyge ommata.*

Two rat homologs, p47A also called μ3A (encoded by the ap3m1 gene) and p47B also called μ3B (encoded by the ap3m2 gene), were subsequently isolated, sharing around 80% amino acid identity to each other and around 27-30% amino acid identity to mouse orthologues.

Rat p47A mRNA is expressed in all the tissues such as brain, heart, kidney, liver, lung, muscle and spinal cord whereas Rat p47B mRNA is found only in brain and in the spinal cord [98].

The AP3M2 gene encodes for the neuron-specific adaptor protein complex 3 medium subunit, that is also called clathrin assembly protein complex 3 mu-2 medium chain (https://www.uniprot.org/uniprot/?query=ap3m2).

The best-characterized sorting signals recognized by the μ3 subunits are tyrosine-based (YXXØ), thus the structure of μ3A subunit of AP-3 complex was solved with TGN38 sorting signal peptide bearing this signal [86].

1.3.3.6 AP3M2 and cancer

Several studies showed the involvement of AP3M2 in different types of cancer.

Indeed, Ap3m2 amplicons were observed in the primary tumor and were maintained in at least two passages of human breast carcinoma xenograft deriving from a woman carrying a germline BRCA2 mutation [121]. Another study to assess the accuracy with which breast patients can be stratified by analyzing gene expression and genome copy number and the predictors of reduced survival duration, listed AP3M2 with the genes in recurrent amplicons associated with reduced survival duration in breast cancer. These genes were classified as therapeutic targets for treatment of patients that respond poorly to current aggressive therapies [122].

In addition, a molecular characterization aiming to improve the prediction of treatments, identified a predictive model for the responders to FOLFOX therapy. The research covered 83 colorectal cancer patients of which 42 were responders to FOLFOX therapy and 41 non-responders. AP3M2 gene was upregulated in the non-responder patients resistant to FOLFOX therapy [123].

Lastly, a study investigating the link between the deregulation of autophagy and cancer, identified novel autophagy regulators that could have potential therapeutic intervention. AP3M2 was listed with the genes whose loss was associated with the impairment of p62 elimination having a putative role as autophagy stimulator being a core components of the endocytosis and protein trafficking machinery [124].

1.4 Overview of Autophagy

Macroautophagy also known as autophagy is a physiological process performed by the cell to degrade and eliminate misfolded proteins and damaged organelles, it plays an important role in adaptation to starvation, cell death, development and tumor suppression [125].

Autophagy is involved in important mechanisms including, intracellular degradation pathway through double membrane vesicles, the autophagosomes. They deliver degraded cytoplasmic organelles and components to the lysosome to be recycled under stress. These stress factors may include hypoxia which regulates autophagy, that alleviate the oxidative stress [126].

Autophagy plays a protecting role, they protect cells from damaged proteins, and preserve organelles from toxins, they promote cell survival by maintaining cell metabolism and homeostasis. This evolutionarily conserved intracellular recycling system, is a response to a range of cellular processes, including nutrient deprivation, cytoplasmic organelles damage, and abnormal protein deposition and can be associated to death and survival [127] (**Figure 7**).

Enhanced autophagy during nutrient deprivation, increases cell survival, to maintain the supply of energy such as important proteins and other nutrients [128].

Autophagy can be either selective or non-selective. Non-selective (general) autophagy packages fractions and components of the cytoplasm into autophagosomes to be delivered then to lysosomes for degradation. Whereas, selective autophagy recognizes specific cellular targets, including damaged organelles, protein aggregates, and pathogens. Thus, defects of autophagy are associated with metabolic stress, genomic damage, and tumorigenesis [129].Moreover, autophagy has been linked both to cancer initiation as a regulator of many oncogenes and tumor suppressor genes and inhibition of cancer. Therefore, being an important target of cancer therapy [130].

In addition, autophagy is an important factor in maintaining the unique properties of stem cells, their differentiation and self-renewal capabilities [131].

Autophagy is highly regulated by a set of signaling events. Formation and turnover of the autophagosome involves conserved genes called autophagy related (ATG) genes [132]. and is divided into different stages:

- Initiation
- Nucleation of the autophagosome
- Expansion and elongation of the autophagosome membrane,
- Closure and fusion with the lysosome
- Degradation of intra-vesicular products

Initiation begins with activation of the ULK1 (known as ATG1) complex, which activates PI3K complex consisting of different proteins, which are scaffolded by a putative tumor suppressor Beclin1. The process continues with the ATG5-ATG12 complex conjugation with ATG16 to expand the autophagosome membrane. Members of the LC3 and GABARAP families of proteins are conjugated to the lipid phosphatidylethanolamine (PE) to be recruited to the membrane. ATG4B, coupled with ATG7, conjugates LC3-I and PE to form LC3-II. At last, the autophagosome fuses with the lysosome, the contents are degraded, macromolecular precursors are recycled or used as energy supply in metabolic pathways. The adaptor protein sequestoome1, known as p62 that guides specific substrates to autophagosomes and LC3II are degraded along with other cargo proteins and recycled to be used as a measure of autophagic flux [133] [134].

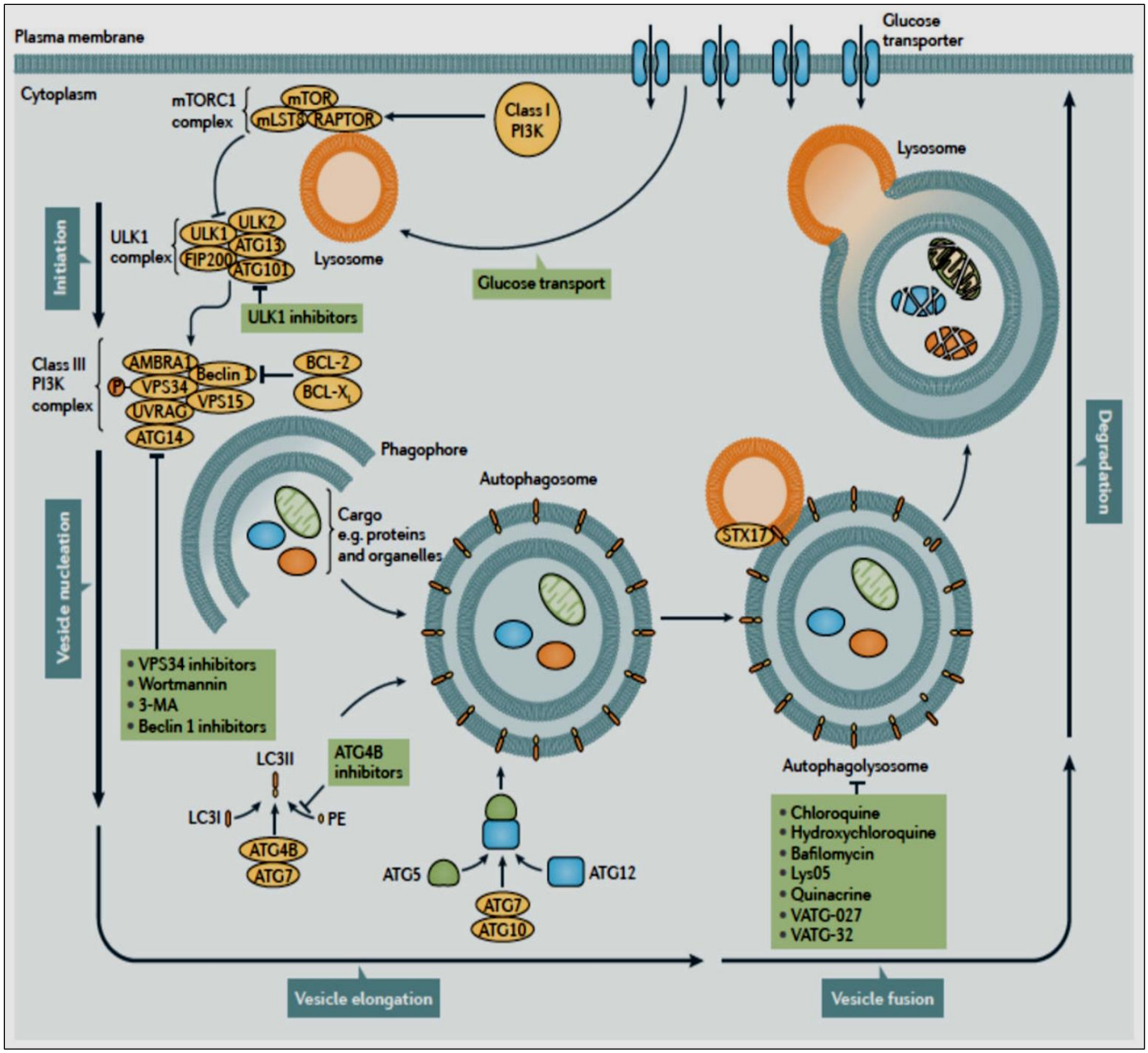


Figure 7. Autophagy can be inhibited at multiple stages [135].

The process of autophagy is divided into five distinct stages: initiation, vesicle nucleation, vesicle elongation, vesicle fusion and cargo degradation. Nonspecific macroautophagy is initiated by upstream activation through either nutrient starvation or growth factors. Under starvation conditions, a drop in glucose transport results in a release of mTOR inhibition of the ULK1 complex, allowing for the progression of autophagy. The ULK1 complex (comprising ULK1, ULK2, FIP200, ATG101 and ATG13) induces vesicle nucleation which is then mediated

by a class III PI3K complex consisting of multiple proteins. Beclin 1, a BCL-2 homology (BH)-3 domain only protein, is phosphorylated by ULK1 and acts as an overall scaffold for the PI3K complex, facilitating localization of autophagic proteins to the phagophore. BCL-2 and BCL-XL interact with Beclin 1 at the BH3 domain to decrease the pro-autophagic activity of Beclin 1 by interrupting the Beclin 1–VPS34 complex formation and decreasing the interaction of Beclin 1 with UVRAG. Additional negative regulation of this process occurs with the phosphorylation of VPS34 (also known as PIK3C3), which decreases its interaction with Beclin 1. In contrast, AMBRA binds Beclin 1 and stabilizes the PI3K complex. ATG14 and UVRAG also bind Beclin 1 to promote interactions between Beclin 1 and VPS34 and phagophore formation. VPS15 is required for optimal VPS34 function by enhancing VPS34 interaction with Beclin 1. The growing double membrane undergoes vesicle elongation to eventually form an autophagasome: a process mediated by two ubiquitin-like conjugation systems. The first system involves the conjugation of phosphatidylethanolamine (PE) to cytoplasmic LC3-I to generate the lipidated form, LC3-II which is facilitated by the protease, ATG4B, and the E1-like enzyme, ATG7, whereby LC3-II is incorporated into the growing membrane. The second conjugation system is mediated again by ATG7 as well as the E2-like enzyme, ATG10, resulting in an ATG5-ATG12 conjugate. Subsequently, the SNARE protein, syntaxin 17 (STX17) facilitates autophagosome fusion with the lysosome, resulting in an autophagolysosome. The low pH of the lysosome results in degradation of the autophagosome contents. This process can be targeted pharmacologically upstream by means of direct ULK1, VPS34, or Beclin 1 inhibition. It can also be targeted by wortmannin and 3-methyladenine (3-MA) which act as PI3K inhibitors. Downstream targets include direct ATG4B inhibitors as well as chloroquine or hydroxychloroquine and bafilomycin, which act to prevent autophagosome fusion with the lysosome. PE, phosphatidylethanolamine.

These steps in the autophagy pathway could be potential druggable targets to regulate autophagy. Chloroquine (CQ) or the related hydroxychloroquine (HCQ), were used to inhibit autophagy based on inhibiting the lysosome. Other inhibitors against different autophagy regulators, can inhibit tumor cell growth or induce tumor cell death in vitro, they include VPS34, ULK1 and ATG4B [135]. SAR405 is a kinase inhibitor of Vps18 and Vps34, induces lysosomal dysfunction, and affects the interaction between the late endosome and lysosome. A combination of SAR405

with everolimus inhibits the cancer proliferation in renal cancer cell lines, indicating that SAR405 has anticancer therapeutic effects [136].

Bisaminoquinoline is a Lys05, a water-soluble analog of HCQ, inhibits autophagy and impairs melanoma and colorectal adenocarcinoma development is in preclinical mouse models. Lys05 has a greater deacidification effect of the lysosome and is more potent than HCQ [137]. Novel potent lysosomal inhibitors such as quinacrine and VATG-027 and VATG-032 are also effective in melanoma cells with BRAF mutation [138].

Other autophagy-related drugs for anticancer therapy include Spautin-1 which inhibits autophagy via the induction of proteasomal removal of PI3K kinase complexes. Spautin-1 plays also a pro-apoptotic effect mediated by GSK3β targeting a crucial downstream effector of PI3K/Akt [139].

Another form of autophagy called microautophagy, can be also selective and non-selective. The non-selective type of microautophagy, is mediated by direct engulfment of cytoplasm and cytoplasmic components by membranous invaginations into lysosomes. Selective microautophagy consists of direct targeting of specific organelles into lysosomes such as peroxisomes, nonessential components of the nucleus and mitochondria (micromitophagy). Microautophagy is implicated with the development of neurodegenerative disorders such as Alzheimer disease as well as lysosomal glycogen storage diseases such as Pompe disease.

Chaperone-mediated autophagy (CMA) is implicated in cancer, it's a type of selective autophagy where cytosolic proteins with motifs related to the pentapeptide KFERQ are recognized by Heat Shock cognate Protein, known as HSPA8 forming a chaperone complex, to translocate into the lysosome via LAMP2A the lysosomal-associated membrane protein 2A. Drugs targeting the lysosome could affect all types of autophagy [140].

1.4.1 The Role of Autophagy in Cancer

The major roles of autophagy is to degrade the damaged organelles and unfunctional old proteins tomaintenance cellular homeostasis [141].

In cancer, autophagy can be both tumor promoter and tumor suppressor by regulating cell proliferation.

Tumor suppression is negatively regulated by mTOR and AMPK, that induce autophagy and inhibit cancer initiation [142]. In the other hands, oncogenesis can be promoted by mTOR, class I PI3 and AKT, they suppress autophagy and promote cancer formation [143].

Basal autophagy is cancer-protective factor, impairments in autophagy lead to the degradation of damaged components by oxidative-stressed mechanism, inducing cancer [144]. Important autophagy proteins, such as BIF-1 proteins that are related to BECN1, are protective against cancer development, when mutated or absent they can be implicated in several cancer types such as colorectal and gastric cancer [145].

Another protein UVRAG related to BECN1, is an autophagy regulator. The mutation of UVRAG reduces autophagy, resulting in increased cancer-cell proliferation in colorectal cancer cells [146].In contrast, autophagy is reduced in several types of RAS-activated cancer as in pancreatic cancers[147] . Deficiency in hepatic autophagy after deletion of ATG5 and ATG7 in mice is toxic, it promotes cell death of hepatocytes and inflammation which are known drivers of liver cancer [148]. Other studies have shown that the deficiency of autophagic regulators, such as ATG3, ATG4, ATG5, ATG9, is associated with oncogenesis [149].

Oxidative stress induced by autophagy deprivation, induces the DNA damage response, and genome instability, promoting cancer initiation. This increase in oxidative stress induces the nuclear factor, erythroid-2–like 2 (NRF2), an antioxidant stress protein [150].

The autophagy substrate p62/SQSTM1 deficiency reduces toxicity and tumorigenesis in Genetically engineered mouse models (GEMMs) for $KRAS^{G12D}$-driven lung cancer by defective autophagy [151]. Therefore, p62 expression promotes oxidative stress and tumor growth and is found to be implicated in clear cell renal cell carcinoma [152].

Cancer cells are more autophagy dependent than normal cells due to the deficient microenvironment and the increased metabolic demands for the increased proliferation [130]. Autophagy is upregulated in RAS-driven cancer cells, promoting their tumorigenesis [153].In addition, stress caused by mitochondrial defects, leads to autophagy deficiency in RAS-related cancers.

A study aimed to determine the causative role of autophagy in lung cancer in an immune-competent setting, they deleted gene *Atg7* in tumor cells, an important autophagy gene, simultaneously with $KRAS^{G12D}$ activation in non–small-cell lung cancer (NSCLC) GEMMs. The

result was accumulation of dysfunctional mitochondria and to premature induction of p53, proliferative arrest [154].

However, deficient autophagy limits tumor growth by other mechanisms independent of p53[155]

Another study on BRAF-driven cancers, *Atg7* was deleted in a mouse model of $BRAF^{V600E}$-induced lung cancer in the presence or absence of *Trp53*. *Atg7* deletion induced oxidative stress and enhanced tumor proliferation. Deletion of both *Atg7* and *Nrf2* had the same effect, suggesting that these two genes promote first stages of tumorigenesis by regulating oxidative stress, p53 activation occurs at later stages [150]. Furthermore, Human melanomas with inducive BRAF mutations, have accelerated basal autophagy proportional to the disease aggressiveness and sensitivity to autophagy inactivation [156].

The dual role of autophagy in cancer is schematized in **Figure 8**.

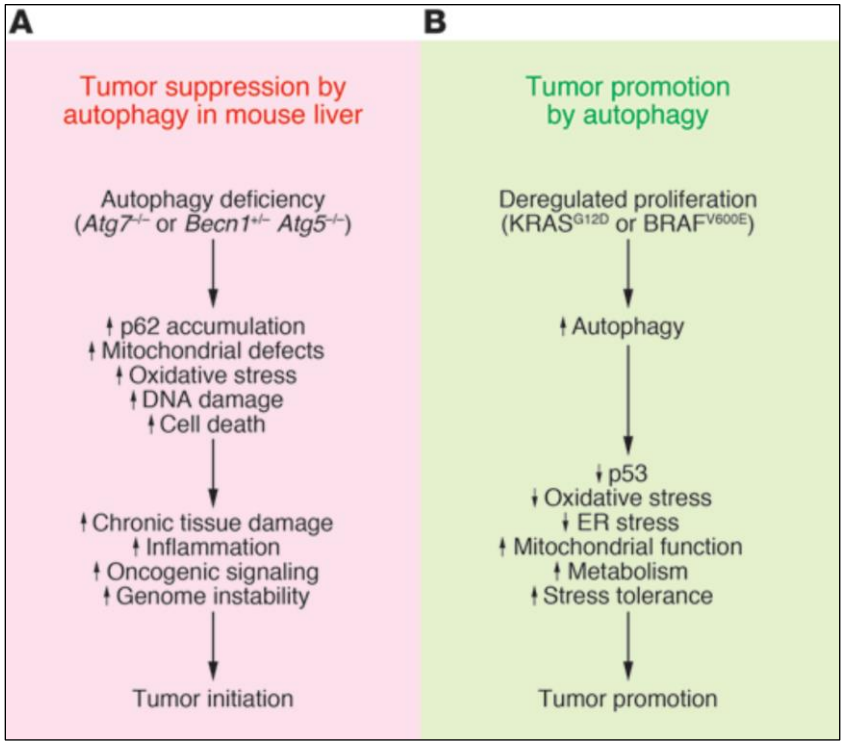


***Figure 8: Autophagy in cancer* [141]**

(A) Proposed mechanisms by which autophagy may suppress tumorigenesis. Autophagy defects impair tissue health, leading to chronic tissue damage and regeneration that may create an environment that promotes cancer. (B) Proposed mechanisms by which autophagy promotes cancer by limiting stress responses and supporting metabolism and survival.

1.4.2 Interaction between Autophagy and Cancer Microenvironment

Autophagy in cancer cells play an important regulator cancer microenvironment such as hypoxia, inflammation, and cytokines [157].Autophagy is a cellular supplier of energy and protector from cytotoxicity under stressful conditions. Previous studies showed that many tumors are exposed to hypoxic conditions [158] that may affect the autophagy pathway to adapt cancer cells to survive in these conditions by activating the stress response pathway, such as hypoxia-inducible factor-1 alpha (HIF-1α) which induces activation of autophagy in cancer by regulating several target genes. Autophagic process processes promote glucose metabolism by HIF-1 α [159]. Autophagy associated with glucose and amino acid deprivation can be independent from HIF-1 α and is activated by the induction of AMPK and inhibition of mTOR [160].

Inflammation is highly activated in the cancer microenvironment by inflammation regulators such as the high levels of ROS (reactive oxygen species) in tumor cells, and immune cells. These cells secrete chemokines and cytokines, including interleukins, tumor necrosis factors, and transforming growth factors into the tumor microenvironment [157]. These cytokines enhance chronic inflammation to protect from cancer, but they can contribute to cancer progression via inflammation as well. Indeed, inflammation is involved in tumorigenesis by reducing autophagy activation. Autophagy suppresses inflammation by blocking NLRP3 formation and autophagy-related protein, and ATG16L [161].

The current knowledge on the generalized cancer chemoprotective mechanisms includes by antioxidant activity:

- Reactive oxygen species (ROS) include free radicals such as hydroxyl radical, peroxy radical, superoxide anion radical and other unavoidably radicals produced in the body as a result of naturally occurring stresses, environmental pollutants, changing atmospheric conditions, and lifestyle stressors. Oxidative stress is created when the balance between ROS levels and antioxidants (or endogenous enzymes) is disrupted, inducing potential cell damage.
- Reactive nitrogen species: (RNS) are formed in cells starting with the synthesis of nitric oxide by nitric oxide synthase. Nitric oxide reacts with superoxide to form peroxynitrite anion a stronger oxidant that reacts with other molecules to generate other RNS [162].

- Reactive carbonyl species (RCS) produced through lipid peroxidation and advanced glycation are generated in physiological systems of high glucose concentration have a strong relationship with ROS.

Multiple clinical studies have shown the correlation between oxidative stress induced by ROS and pathophysiology associated with various diseases including cancer [163]. ROS can effectively induce DNA damage such as base alterations, strand breakage and mutations within normal cells promoting oncogenic transformation [164]. Evidence showed that ROS possess the capacity to activate intracellular signaling cascades and contribute to tumor development and metastasis by regulating cellular proliferation, death and motility. Consequently, human tumor cells have been shown to produce more ROS than non-transformed cell lines *in vitro* [165]. *In vivo*, tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) increased the production of ROS in certain organs. Polyphenols can effectively prevent free radicals-induced DNA damage or carcinogenic agents through radical scavenging, chelating divalent cations, and modulating enzymes related to oxidative stress [166].

1.4.3 Autophagy as a Regulator of Cancer Metastasis

Metastasis is the process that enable cancer cells to invade and colonize new tissues and organs via the circulation systems. During metastasis, cancer cells' motility in primary sites increases to migrate to secondary sites. The dual role of autophagy as pro-metastatic and anti-metastatic make it important regulator of tumorigenesis [167]. Autophagy limits metastasis by limiting cancer necrosis and inflammation responses in early stages of cancer metastasis from primary sites where invasion and migration of cancer cells is also reduced. However, in secondary sites, autophagy acts have a pro-metastatic role by promoting of cancer survival and colonization.

The downregulation of autophagy-related genes, Beclin1 and LC3, inhibited proliferation, migration, and invasion, causing apoptosis in breast Cancer [168]. Knockdown of ATG5, a crucial regulator of autophagy, promotes cancer-cell proliferation in early stage cancer and was found to reduce survival rate in 158 primary melanoma patients [169]. Additionally, the decreased expression of mTOR signaling induces cell-death by autophagy and inhibits metastasis in gastric tumors

Having also a pro-metastatic role, autophagy promotes cancer cells survival and proliferation in the absence of extracellular matrix (ECM) and their circulation to reach secondary sites [170].

Autophagy enables ECM-detached cancer cells to bypass anoikis, an anchorage-dependent programmed cell death, and survive [171]. For instance, the inhibition of autophagy reduced hepatocellular carcinoma (HCC) metastasis in a lung metastasis model [172].

Metastasis of cancer is enhanced by epithelial mesenchymal transition (EMT), a process leading to polarity and cell-adhesion characteristics loss of epithelial cells, and gain of migratory and invasive properties to become multipotent mesenchymal stem cells that can differentiate into a variety of cell types [173]. This transition is relevant in embryonic development and affects wound healing and cancer progression. Cancer cells activating EMT can survive stressful conditions during metastasis due to high level of autophagy. Several studies highlighted the role of autophagy in EMT induced metastasis. For instance, nutrient deprivation and mTOR inhibition [174]. Knockdown of autophagy-related proteins Beclin 1, ATG5, and ATG7
affected migration and invasion of glioblastoma cells [175].

1.4.4 Autophagy as Drug-Resistant Factor of Tumors

Resistance of cancer cells to a variety of anticancer drugs is a common burden that can increase via upregulation of autophagy. Autophagy plays a protective role in cancer cells subjected to anticancer therapy. Cancer chemotherapy and anticancer drugs are common treatment strategies for cancer, they target dividing cells and interrupt cancer-cell division, but the effectiveness of chemotherapy is limited due to chemoresistance. Particularly, protective autophagy is challenging cancer therapy. The 5-Fluorouracil (5FU) is an anticancer drug used in solid tumors, including breast, pancreatic, and colorectal cancer [176]. This compound inhibits thymidylate synthetase enzyme leading to the inhibition of DNA synthesis. However, protective autophagy reduces the efficacy of treatment with 5FU is restricted, resulting in chemoresistance in various cancer cells. Protective autophagy is induced by beclin-1, followed by transformation of LC3I to LC3II. Later, autophagic flux increases after the activation of JNK-mediated protective autophagy and an increase of BCL2, inducing chemoresistance.

Another primary treatment drug for many solid tumors is cisplatin, it acts by the generating DNA damage and mitochondrial apoptosis. However, the success of this treatment is limited due to chemoresistance [177].In ovarian cancer, the modulation of ERK pathway and overexpression of Beclin 1 are the underlying mechanisms leading to cisplatin-mediated resistance [178] A different study in esophageal cancer, demonstrated that protective autophagy in cisplatin treatment is

promoted via the upregulation of Beclin 1, transformation of LC3 proteins and increase of ATG7 expression [179].

The efficacy of chemotherapeutic agents in many cancers is limited by the unexpected protective autophagy, and to overcome the resistance to various chemotherapeutic agents, regulation of autophagy pathway should be understood.

Additionally, autophagy interplays between immunity and anticancer therapy. The tumor releases a new antigen into the surrounding environment, allowing to T cells to recognize it, and therefore cancer cells are killed through T cell-mediated cytotoxicity[180]. Different immune related cells can also be induced to eliminate cancer cells by presenting neoantigen via the generation of interferon- and perforin [181].Autophagy is associated with T-cell survival. However, hypoxia-mediated autophagy leads to resistance of T-cell cytotoxicity via activation of Signal transducer and activator of transcription 3 (STAT3). In effect, the inhibition of autophagy by silencing of Beclin 1 and ATG5 reduced hypoxia-induced activation of STAT3 and re-established cancer cell sensitivity to T-cell cytotoxicity [182].

Alteration of autophagy is a promising strategy to enhance cancer therapy. In fact, drugs design for targeting all steps of the autophagic processes, since the initiation of the autophagosome till the degradation step can be helpful in cancer therapy Different studies showed that autophagy facilitates cancer-cell survival and induces drug resistance to anticancer drugs and maintains stem cell-like properties in hepatocellular carcinoma [183]. and the suppression of autophagy promotes of apoptosis and therapeutic effects of anticancer reagents. In addition, the use of chloroquine, an inhibitor of autophagy, can enhance apoptosis and the therapeutic effects of photososan -II-mediated photodynamic therapy (PS-PDT) in colorectal cancer cells [184].

2. RESULTS

2.1 Scientific Background

Colorectal cancer is one of the global leading causes of cancer-related mortality.

Despite the decreasing incidence in the last few years, thanks to the implementation of early diagnosis screening programs, the mortality rate is still about of 50%. New therapeutic opportunities to treat CRC patients are requested especially in the case of metastatic tumors (mCRC)[4].

A comprehensive molecular characterization including recurrent somatic copy-number amplifications[40] and gene-expression classification[68] could be useful in these tumor subtypes to reveal "druggable" genes and to improve the therapeutic outcome of poor responder CRC patients.

In our lab, we screened the level of expression of the 214 genes found to be altered by the TCGA (The Cancer Genome Atlas) and we found that 116 were amplified (**Figure 9**). STRING tool for retrieval of Interacting Genes/Proteins (STRING) database shows the interactions between the queried genes list. RNAi screening on the amplified potential oncogenes identified several new molecular markers and potential targets in CRC. The TCGA study analysed 257 cancer genomes, of which 97 were analyzed in further detail for somatic copy number variation (SCNV).

The online STRING (http://string-db.org/) database [185] is a biological database and web resource for known and predicted protein-protein interaction (PPIs). This tool allows the visualization of the protein interaction relationship network and the analysis of central proteins, which form the important nodes with many interaction partners.

In STRING, each PPI is annotated with one or more scores that are not indicative about the strength or the specificity of the interaction, they rather indicate the level of confidence showing how likely STRING judges an interaction to be true, given the available evidence.

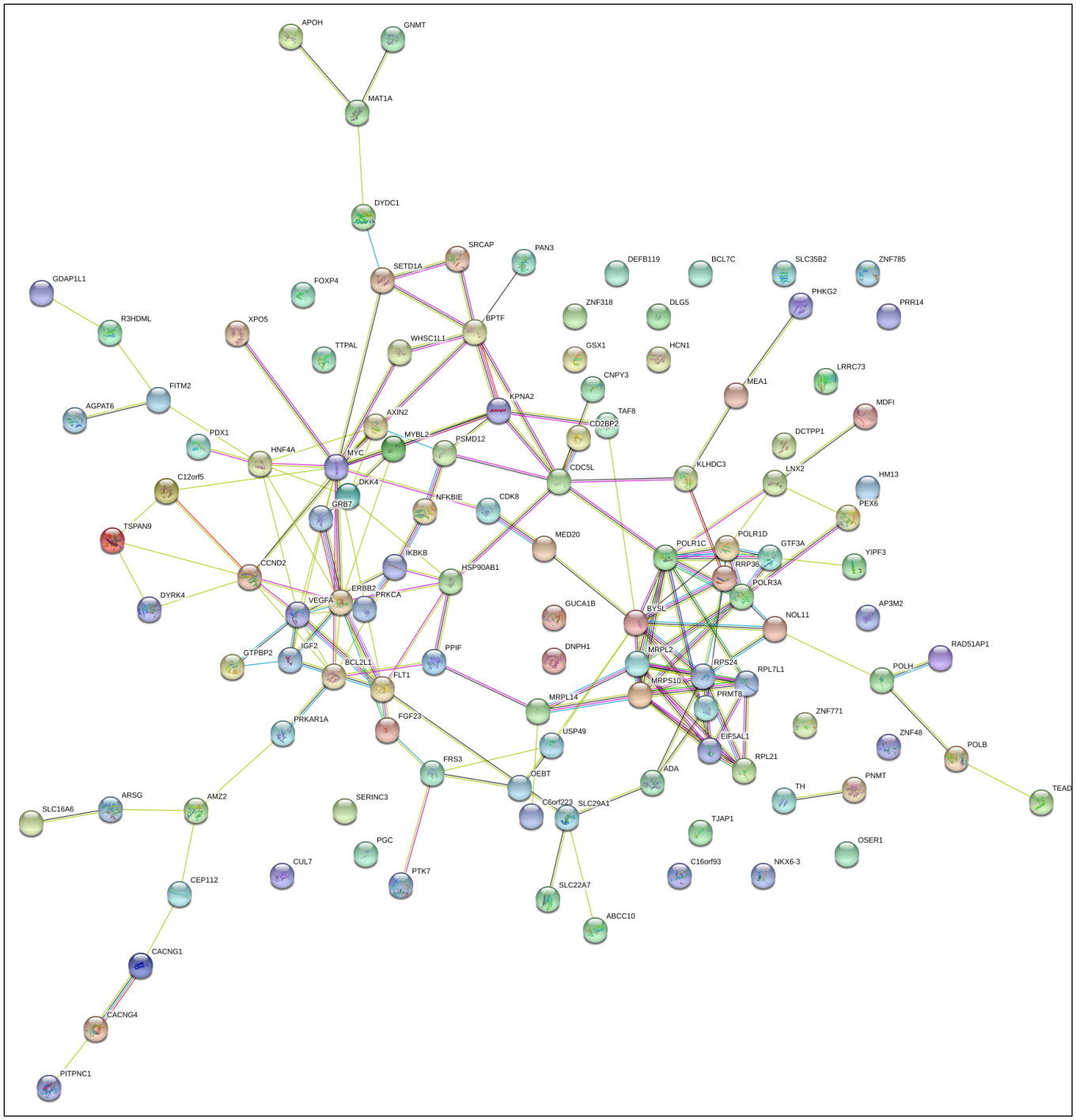


Figure 9: The connections between the genes that are overexpressed in CRC

The network contains 116 nodes and 164 edges; Red line - indicates the presence of fusion evidence, Green line - neighborhood evidence, Blue line - cooccurrence evidence, Purple line -

experimental evidence, Yellow line - textmining evidence, Light blue line - database evidence, Black line - coexpression evidence. (STRING: http://version10.string-db.org)

2.2 Screening of specificity and activity of shRNA library: preliminary data present in the laboratory

To interrogate the gene set, in order to identify potential oncogenes, we have chosen to proceed by carrying out a RNAi screening in an arrayed format, since it has already been proven a useful tool to identify genes involved in oncogenesis. The RNAi reagents chosen were short hairpin RNA (shRNA), by which a more stable and reproducible gene silencing could be obtained [83]. RNAi has an intrinsic issue with specificity, also known as off-target effect (OTE), which can be successfully reduced when RNAi is integrated with gene expression data [186]. Based on this, it was analysed the expression of the gene set by qPCR in parallel with specificity of the silencing. By the evaluation of mRNA levels (**Figure 10**), it was identified that in HCT116 cell line about 70% of the genes were indeed expressed and specifically silenced by the action on shRNA compared to the control (cell transduced with PLKO empty vector), considering an arbitrary cut-off of 50% silencing.

Considering the levels of reduction of cell viability, the druggability of the genes (i.e. the presence of enzymatic active sites) and the relative involvement in cancer (i.e. number of publications retrieved), it was identified a set of genes, which, after further validation, I choose AP3M2. AP3M2 (adaptor related protein complex 3 subunit mu 2) encodes for the neuronal Mu subunit of the heterotetrameric adaptor-related protein complex 3 (AP-3), which recognizes tyrosine-based sorting signals within the cytoplasmic domains of transmembrane cargo proteins and is involved in the biogenesis of lysosome-related organelles [99]. The AP-3 complex is formed by two large subunits (adaptins) δ3 and β3; one medium-sized subunit μ3; one small-sized subunit σ3 [86]. In AP-3 complex, Mu and Beta subunits were found to be either ubiquitous or neuronal expressed and they are encoded by different genes. AP3M1 and AP3B1 encode for the ubiquitous form of μ3 and β3 subunits, respectively while AP3M2 and AP3B2 encode for the neuronal form of these subunits. In addition, the μ3 subunit, seemed to exist only as a dimer with the ß3 subunit, even when the ß3 subunit was barely detectable [187].

AP3M2 amplicons were observed in the primary tumor and maintained in at least two passages of breast cancer xenograft [121], and listed among the amplicons associated with reduced survival in breast cancer [122].

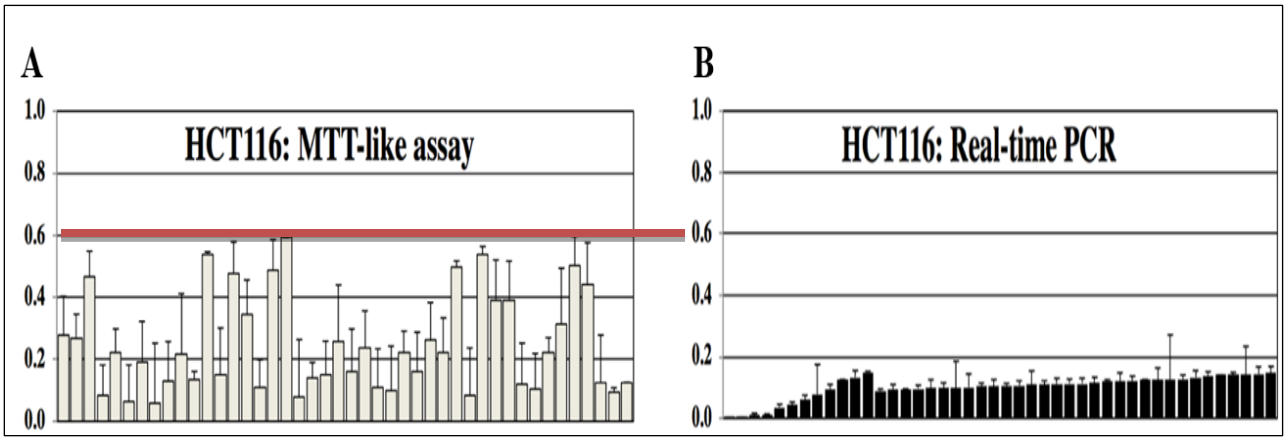


Figure 10. Genes that affect cell viability on HCT-116 cell lines. ***A****. Genes that affect HCT116 cell viability of at least 40%. Y axis represents the ratio between KD/scrambled shRNAs (luminescence).* ***B****. The efficiency of KD was evaluated by real-time PCR. All the values were normalized with GAPDH gene. Y axis represents the ratio between KD/scrambled shRNAs (syber green). The genes are shown on the X axis. The experiments were run in triplicate. Average and standard deviation are shown. (This figure is from our lab preliminary data)*

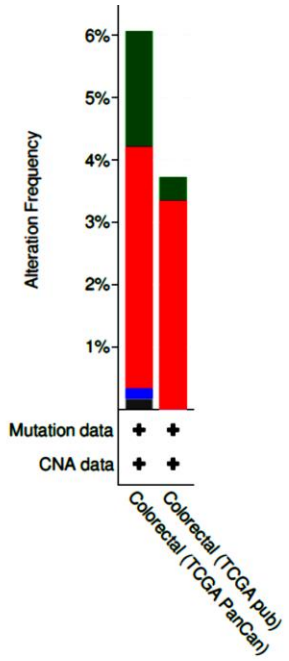
2.3 AP3M2 genetic alterations and their association with CRC survival rate

To further explore the sequence mutations CRC patients, I used the c-BioPortal website, filtering for CRC studies by TCGA, NATURE 2012 and TCGA PanCancer Atlas that covered 870 patients. As shown in **Figure 11,** AP3M2 was altered in approximately 6% of the patients, and several types of alterations were found in CRC including mutations, amplification, deep deletion and others.

Additionally, I queried the survival rate of patients with and without AP3M2 alterations, and it was shown that the overall survival rate decreases up to 50% with AP3M2 mutation; it decreases

from 83 months in AP3M2-UNALTERED group to 43 months in AP3M2-ALTERED group although the data are not significant**.**

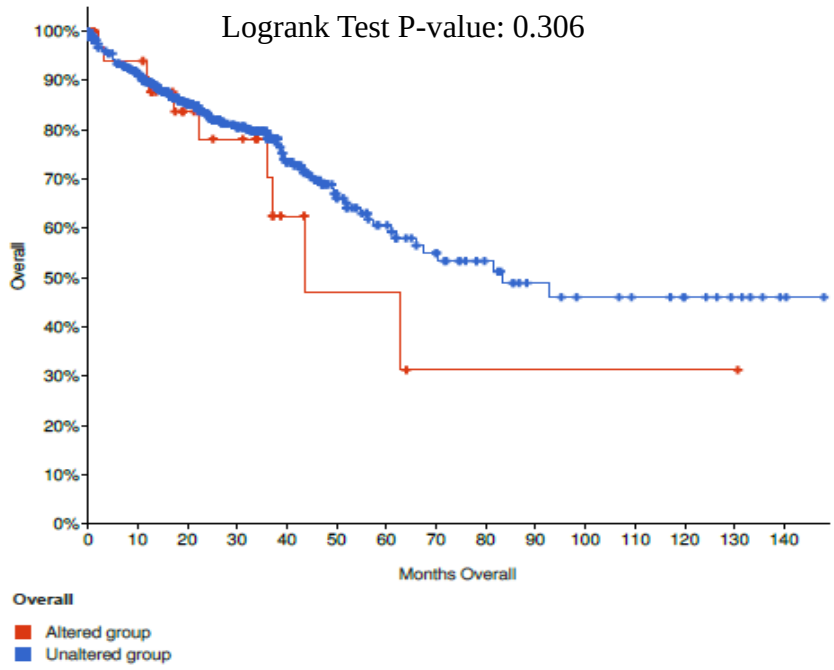
A



B

Alteration	**TCGA PanCancer Atlas** **594 cases**	**TCGA (Nature 2012)** **269 cases**
Mutation	**1.85% (11 cases)**	**0.37% (1 case)**
Amplification	**3.87% (23 cases)**	**3.35% (9 cases)**
Deep Deletion	**0.17% (1 case)**	
Multiple Alterations	**0.17% (1 case)**	

C.



D.

	Number of Cases, Total	Median Months Overall
AP3M2	46	43.76
Unaltered groups	818	83.24

Figure 11: AP3M2 alterations and impact on CRC patients based on TCGA studies (from CbiopPortal, updated 7.7.2020)

A. *AP3M2 alterations in colorectal cancer including copy number alteration (CNA) and mutations based on TCGA studies on 870 patients.* ***B****. Type of alterations and relative frequencies according to TCGA PanCancer Atlas and TCGA Nature 2012.* ***C****. Overall patient survival status AP3M2-altered vs. unaltered cases.* ***D****. Overall patient survival in months comparing cases with AP3M2 alterations vs. non –altered cases.*

2.4 AP3M2 Expression between normal tissues and colon cancer cell lines

AP-3 complexes formed by neuronal AP-3 adaptin isoforms could generate vesicles with different cargo composition. This idea is confirmed by the observation that μ 3A and μ3B subunits present in ubiquitous and neuronal AP-3 complexes recognize different tyrosine-based sorting motifs. The restricted expression in brain tissue of β3B and μ 3B subunits suggest that they assemble exclusively into neuronal AP-3 complex while, β 3A and μ 3A assemble into a ubiquitously expressed complex [99].

To verify the expression of AP3M2, in mice normal tissues, I extracted the proteins from different organs directly after mice sacrifice. I proceeded with western blot using antibody against AP3M2. AP3M2 resulted to be exclusively expressed in the brain and in the testis of mice (**Figure 12**). I proceeded by checking the expression of AP3M2 in cell lines of CRC. Interestingly, AP3M2 was found to be expressed in all the cell lines representing different molecular subtypes of CRC.

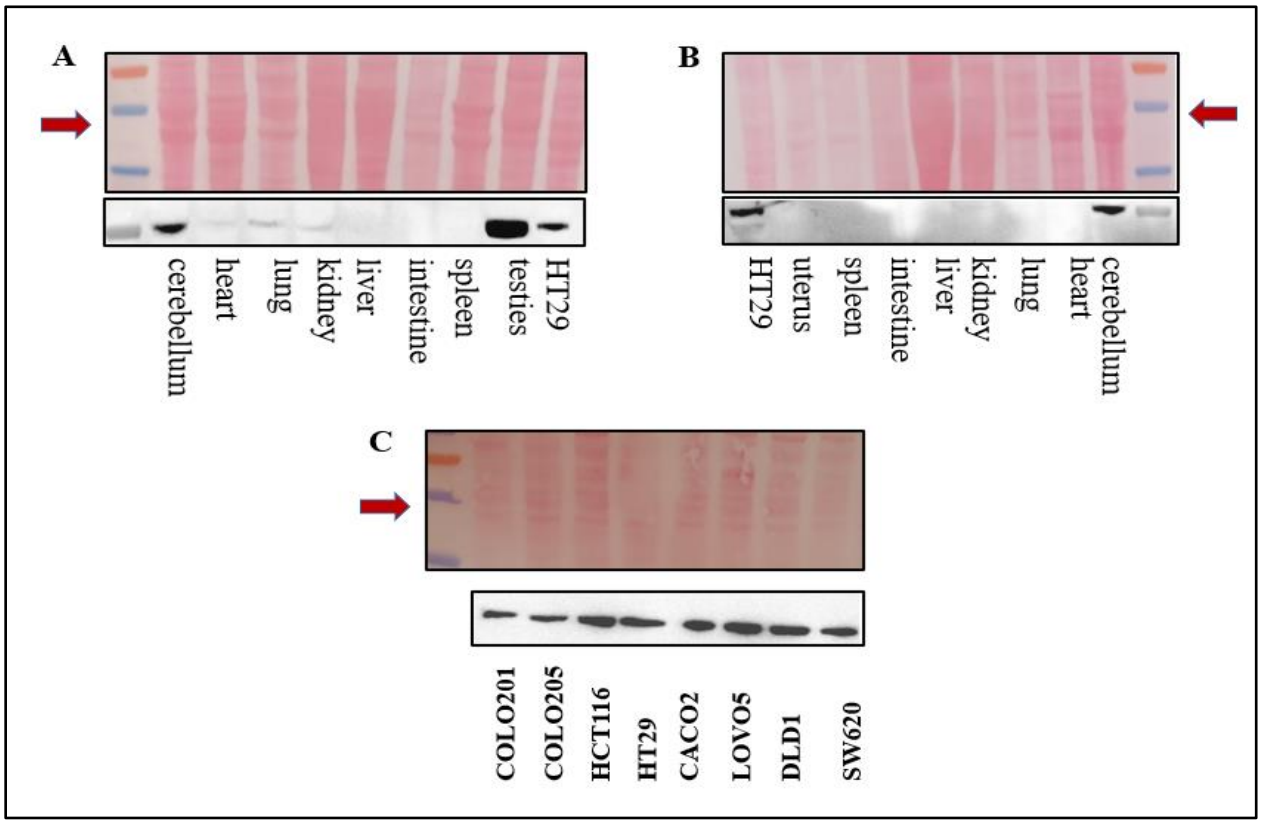
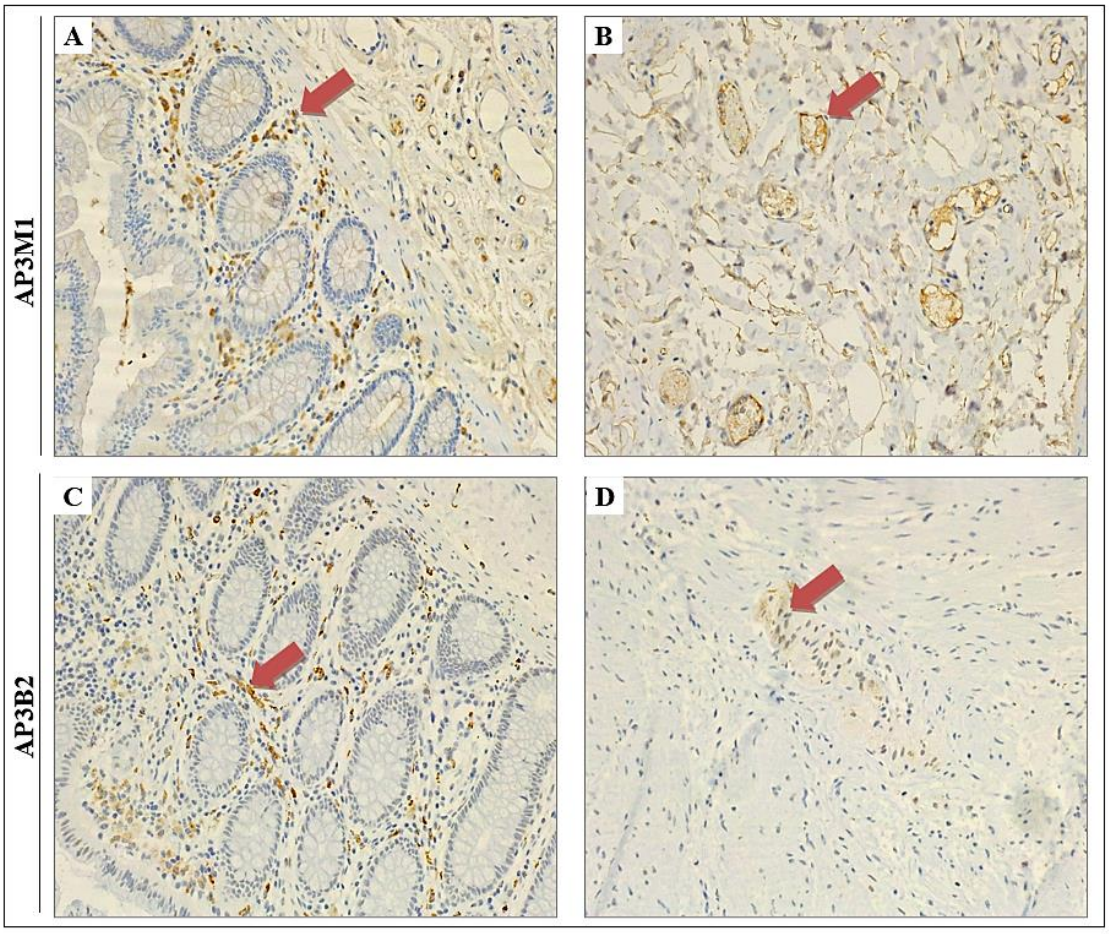


Figure 12: AP3M2 expression in normal mouse tissues and human colon cancer cell lines:

A. *AP3M2 is expressed in the cerebellum and the testis of normal male mouse.* ***B****. AP3M2 is expressed only in the cerebellum of normal female mouse.* ***C****. AP3M2 is expressed in different human colorectal cancer cell lines. The red arrows correspond to the predicted molecular weight of AP3M2.*

In addition, by immunohistochemical analysis, I assessed the localization of AP3M1, AP3M2 and AP3B2 in human colon tissues from CRC patients (**Figure 13**). AP3M1 was not detected in the membrane of epithelial glandular cells (figure 13A) but present in the membrane of endothelial and plasma cells (figure 13A & B). AP3B2 is absent in the glandular epithelial cells (figure C) and present in the myenteric plexus or Auerbach's plexus (figure 13D).

In the normal part of the colon from different patients, AP3M2 was detected in the myenteric plexus or Auerbach's plexus and moderately in the membrane of glandular cells (figure 13E & G). In the tumor part, AP3M2 was abundantly detected.



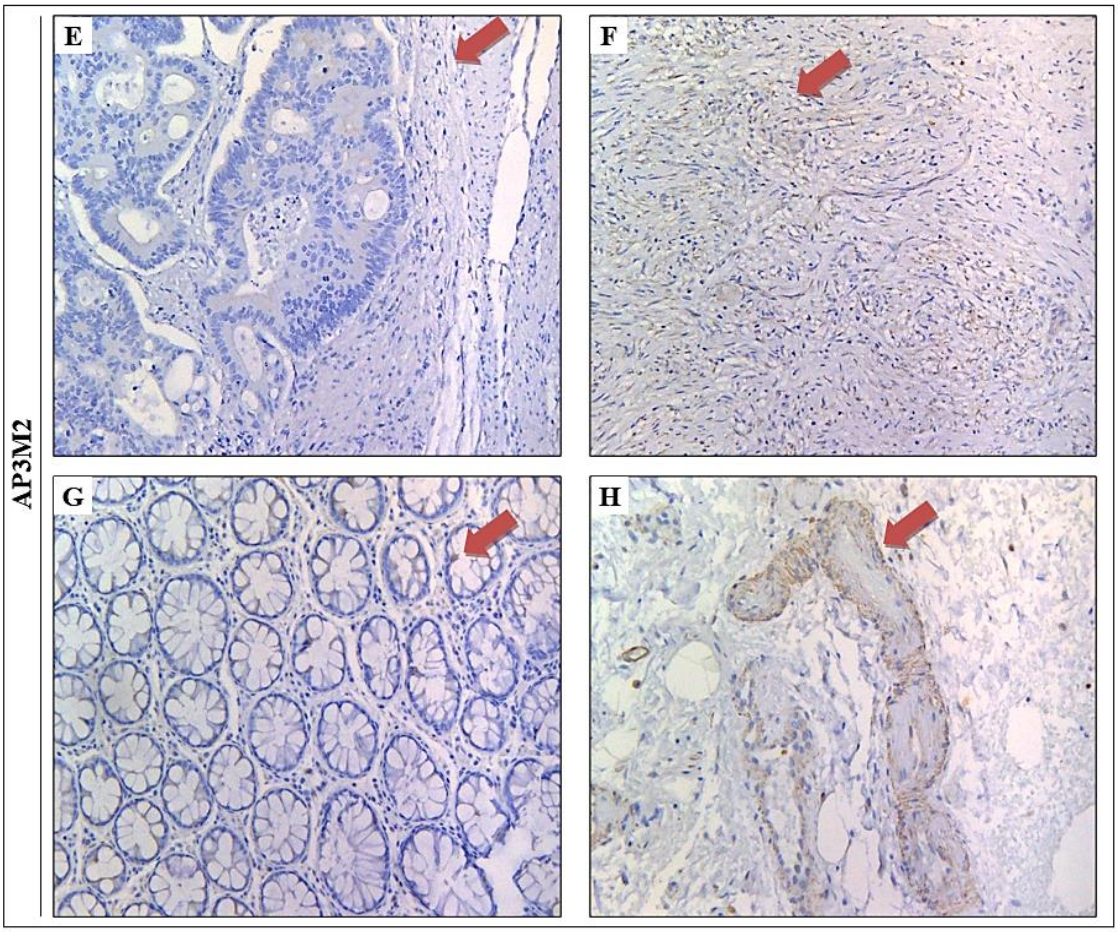


Figure 13: The expression of AP3M2, AP3M1 and AP3B2 in human colon.

Representative immunohistochemistry images using anti-AP3M1, ant-AP3B2 and anti-AP3B2 antibodies.

A. *AP3M1 is absent in the membrane of glandular cells.* ***B****. AP3M1 is detected in the membrane of endothelial cells and plasma cells are AP3M1-positive.* ***C****. AP3B2 is absent in the glandular epithelial cells.* ***D.*** *AP3B2 is present in Auerbach's plexus.* ***E****. AP3M2 is present in Auerbach's plexus.* ***F.*** *AP3M2 is present in the membranes of tumor cells.* ***G****. AP3M2 is poorly detected in the glandular epithelial cells.* ***H.*** *AP3M2 is present in tumor epithelial membrane. The red arrows correspond to the localization of AP3M2. A, B, C, D captures correspond to the normal part of the colon from the same patients; E and F captures correspond to the normal and tumor part respectively of the colon from the same patients; G and H captures' correspond to the normal and tumor parts respectively of the colon from the same patient. Images were taken using 20x objective.*

2.5 AP3M2 affects cell viability of CRC cell lines.

To evaluate the extent of the effect of AP3M2 silencing and to rule out possible cell dependent effect, we tested the effect of AP3M2 knockdown evaluating the variation in terms of cell viability in HCT116, HT29 and CACO2 each one corresponding to a different molecular subtype to minimize the influences derived from a specific cell line [68]. In these experimental settings, to reduce the probability of OTE (off-target effect) we used two shRNA, which paired to a different sequence on AP3M2 messenger RNA. The effect of knockdown on the viability was monitored every 24, 48, 72 and 96 hours after plating. From the analysis we observed a substantial and robust reduction of vitality in all the cells knockdown for AP3M2, (**Figure 14. A, B, C and D**). Moreover, to confirm that AP3M2 was effectively and specifically reduced, we analyzed its protein expression by western blot, detecting a considerable decrease of the protein, indicating the strength and the specificity of shRNA activity (**Figure 14. A', B' and C'**). By the analysis, it was evident that AP3M2 downregulation had a fundamental effect on cell proliferation. In fact, its knockdown caused a decrease of >50% of vitality in all the different cell lines, although the variation of growth rate and timing amongst them, which could have been imputable to the diverse genetic and mutational background of the cell lines (i.e. HCT116 shows a microsatellite instability and HT29 chromosomal instability instead) and experimental variability. The activity and the specificity of AP3M2 silencing was effectively validated and the results showed a remarkable effect on cell viability, even if the differences between the cell lines are considered.

We identified the effect of AP3M2 on viability of colorectal cancer cells, and given its profound consequences when silenced, we speculated on its possible involvement in carcinogenesis as an oncogene. In particular, whereas in CACO2 the shRNA library slightly affected cell viability, in HCT116 and HT29, the genes whose silencing reduces cell viability more than 50% compared to the control.

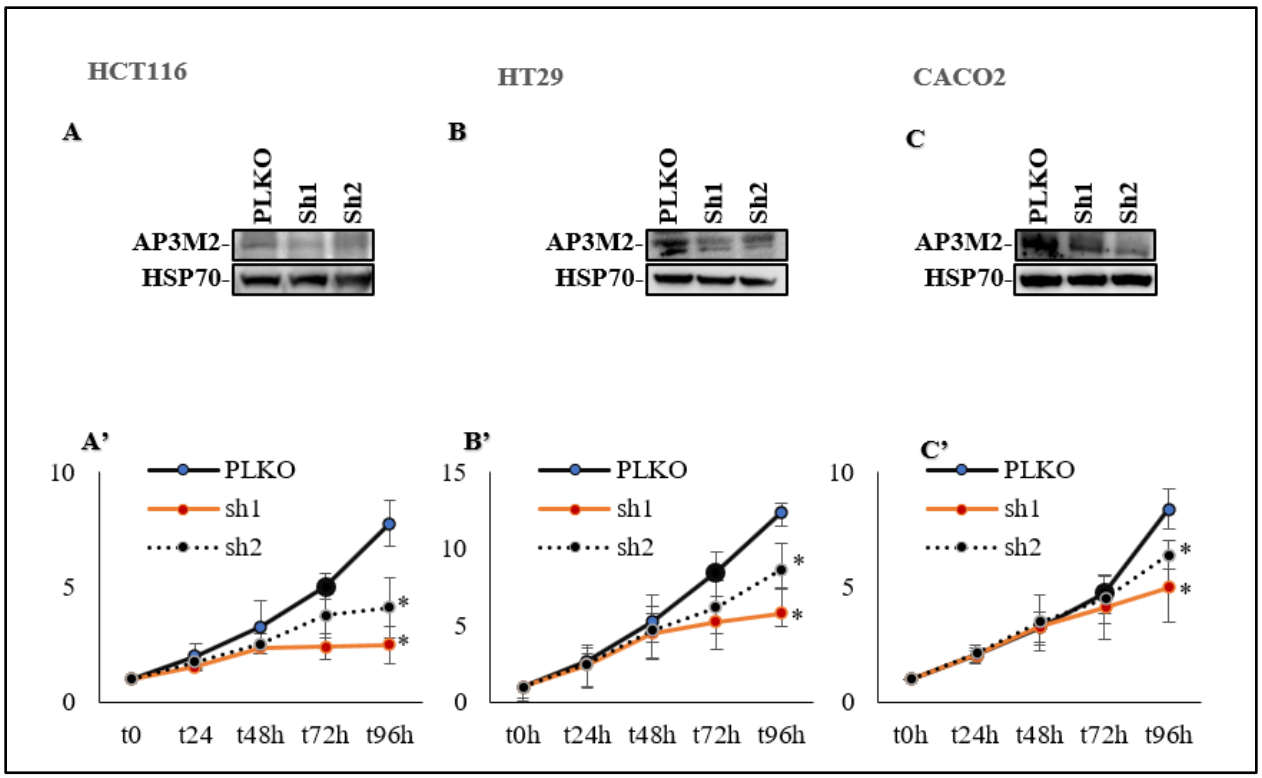


Figure 14: AP3M2 silencing inhibits cell viability.

*Western blot to evaluate AP3M2 expression in HCT116 (**A**), HT29 (**B**) and CACO2 (**C**) after transduction with two different shRNA and the relative control (Sh1 and Sh2). Relative viability of HCT116 (**A'**), HT29 (**B'**) and CACO2 (**C'**) transduced with two different shRNAs. X axis represents the time in hours, Y axis represents the luminescence Tn/T0compared to the control. The cells were seeded at 500 cells/well and the vitality was measured by luminescence over a 96-hours period, every 24 hours, using a microplate reader. The relative cell viability was normalized on the values of cells transduced with empty vector (PLKO) for shRNAs.*

p values were calculated at t96h with two tailed t-test, *, $p < 0.05$

2.6 AP3M2 affects colon cancer cell adherence and clonogenicity

Adherent cells detach from their culture plates during cell death. This characteristic serves as an indirect quantification method of cell death and to determine differences in proliferation upon stimulation with death-inducing agents. To detect maintained adherence of cells, the attached cells are stained with crystal violet dye. Cells undergoing cell death lose their adherence and are

consequently lost from the population of cells, showing lower amount of crystal violet staining in a culture plate [188].

I investigated whether AP3M2 differential expression could affect the clonogenicity of HCT116, HT29 and CACO2 cell lines by evaluating colonies growth. The results indicated that the knockdown of AP3M2 was able to reduce colony formation (**Figure 15. A, B, and C)**.

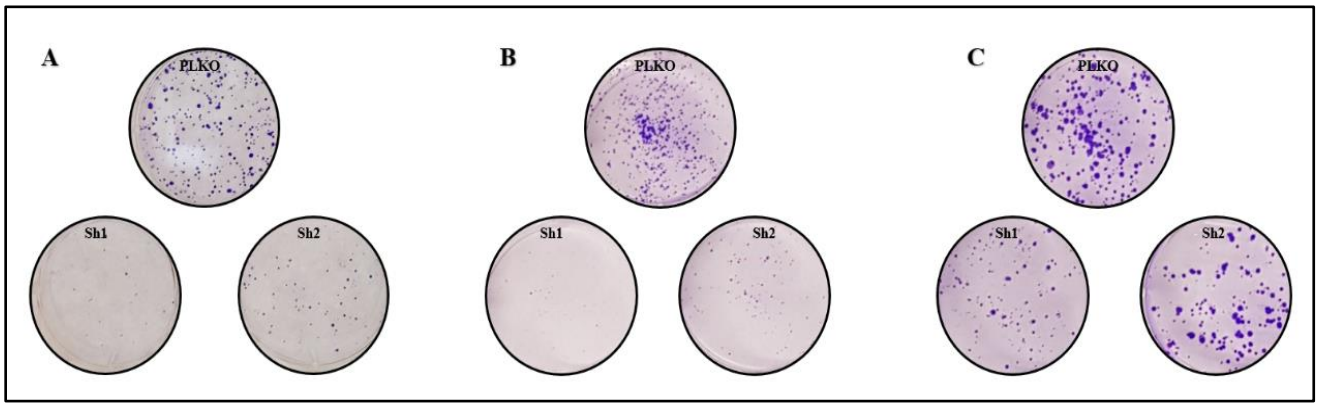


Figure 15**: **Colony formation assay from single anchored cells.

*Cells were seeded at low density (1000 cells per well in 6-well plate) in triplicate. Medium was replaced every 3 days. After 10 days, colonies were fixed with 4% PFA and stained with 1% crystal violet. Digital image showing the crystal violet staining of: **A**. Colonies of HCT116 silenced and control cell on culture plates. **B**. Colonies of HT29 silenced and control cell on culture plates. **C.** Colonies of CACO2 silenced and control cell on culture plates.*

2.7 AP3M2 induces anchorage-independent growth

A key feature of oncogenic behavior is the ability of maintaining cell growth independently from external mechanical factors, such as interaction with a solid substrate, which would induce cell death in normal cells, also called anoikis. In particular, anchorage-independent growth is a hallmark of oncogenic transformation and can be measured by soft agar assay [189]. For this purpose, shAP3M2-transduced HCT116, HT29 and CACO2 cell lines and relative controls, were seeded into a soft agar matrix and cultured for 30 days. Pictures were taken after this period and colonies were counted in term of size of the cell clusters, and the average of 5 captures for each condition were plotted (**Figure 16 A, B, and C**). The analyses showed that AP3M2 played a

pivotal role in cell cluster growth when not anchored to a firm substrate, since where it is silenced, colonies were rare.

These experiments demonstrated that AP3M2 played a fundamental role in supporting cancer progression and in the acquisition of oncogenic features by sustaining anchorage-independent growth of cells.

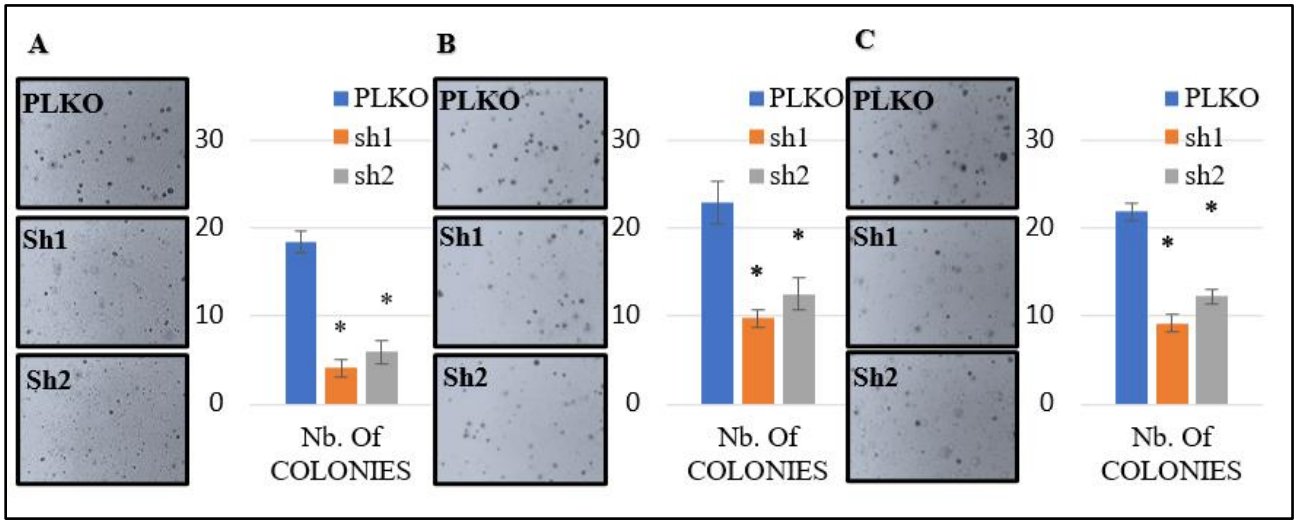


Figure 16. AP3M2 expression prevents anoikis.

The growth assay on soft agar, after AP3M2 knockdown using ShRNA (Sh1 and Sh2) compared to the empty vector (PLKO).

A. *Colonies of HCT116 silenced and control cell on soft agar matrix and the graphical representation of the number of colonies of HCT116 after compared to the control.* ***B.*** *Colonies of HT29 silenced and control cell on soft agar matrix and the graphical representation of the number of colonies of HCT116 after knockdown compared to the control.* ***C.*** *Colonies of CACO2 silenced and control cell on soft agar matrix and the graphical representation of the number of colonies of HCT116 after knockdown compared to the control. X axis represents the different conditions of cells (control, sh1 and sh2), Y axis represents the number of colonies.*

*The cells were embedded in soft-agar matrix, seeded in a multiwell plate (20000 each), and cultured adding the proper medium to prevent the soft agar to dry. After 30 days, colonies were captured using Nikon Eclipse Ti microscope (4X) and images were analyzed using. The average number of colonies was calculated from 5 captures in each experiment. p values were calculated with two tailed t-test, *, $p < 0.05$.*

2.8 AP3M2 expression influences the level of expression of autophagy proteins

The dual role of autophagy as pro-metastatic and anti-metastatic make it important regulator of tumorigenesis [167]. The pro-metastatic role of autophagy promotes cancer cells survival and proliferation in the absence of extracellular matrix (ECM) and their circulation to reach secondary sites [170] and enables ECM-detached cancer cells to bypass anoikis, an anchorage-dependent programmed cell death, and survive [171]. Knockdown of autophagy-related proteins Beclin 1, ATG5, and ATG7 affected migration and invasion of glioblastoma cells [175]. The adaptor protein p62 guides specific substrates to autophagosomes with other cargo proteins and recycled to be used as a measure of autophagic flux [134].

The small GTPase Arf6 has an important role in intracellular vesicular trafficking regulating the recycling of different types of cargo via clathrin-dependent or -independent endocytosis. Arf6 is used for the phagocytosis of pathogens and also in the signaling of Toll-like receptors and in the activation of NADPH oxidases [190].

The KD of AP3M2 induced all ARF6, P62 and ATG7 downregulation in HT29 cell line showing that AP3M2 could be regulating CRC through autophagy (**Figure 17**).

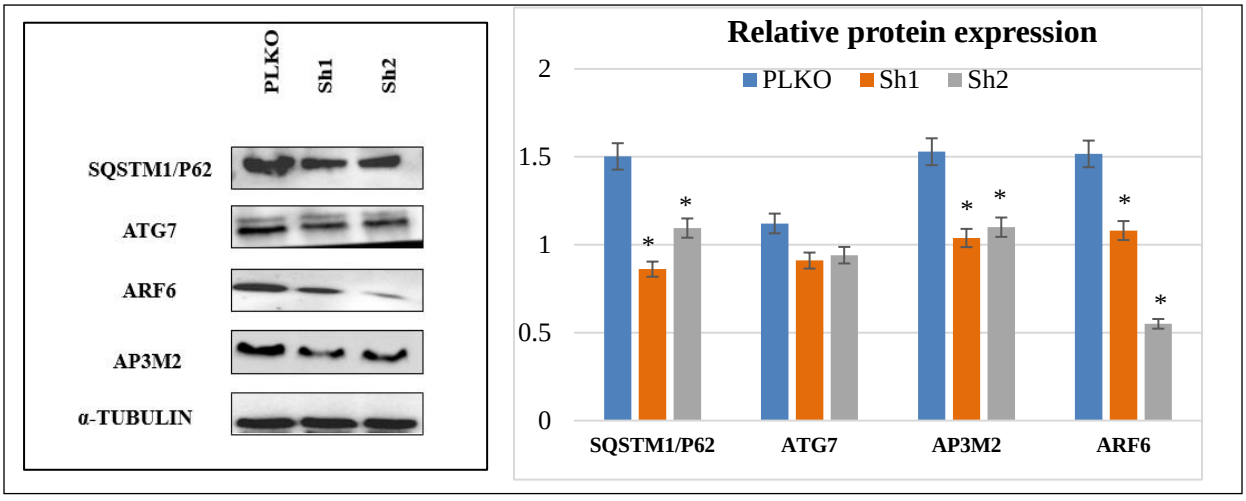


Figure 17: AP3M2 regulates CRC through autophagy.

Expression of P62, ATG7and ARF6 after AP3M2 KD in HT29. Cells were transduced with AP3M2 shRNAs or PLKO and the expression was detected by Western blot. (b) Relative expression and quantification was normalized over α-Tubulin expression. X axis represents the assessed protein and Y axis represents the level of protein expression using ImageJ normalized over α-Tubulin expression. p values were calculated with two tailed t-test, *, $p < 0.05$.

2.9 AP3M2 affects oxygen species production in CRC cell lines

Reactive oxygen species (ROS) play a crucial role in biological processes and constitutive ROS production in normal cells is managed by the regulation between the silver lining of low and high ROS concentration mediated effects. Interestingly, ROS influences the tumor microenvironment as well and is capable to promote cancer angiogenesis, metastasis, and survival at different concentrations. Low concentrations of ROS activate the cancer cell survival signaling cascade involving several pathways that include c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase/extracellular signal-regulated protein kinases 1/2 (MAPK/ERK1/2), p38, and phosphoinositide-3-kinase/ protein kinase B (PI3K/Akt), which in turn activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs).

High concentrations of ROS can induce cancer cell apoptosis. Hence, it critically depends upon the ROS levels, to balance between tumorigenesis or apoptosis [191].

Thus, I hypothesized that AP3M2 may alter the oxidative stress and consequently, AP3M2 depletion would affect ROS production. Therefore, I investigated whether AP3M2 knockdown altered ROS levels using ROS-Glo™ H_2O_2 Assay.

The knockdown of AP3M2 in the three colon cancer cell lines HCT116, HT29 and CACO2 was associated with high levels of ROS compared to the control (**Figure 18. A, B and C**). We can conclude that AP3M2 induces cancer cells survival by maintaining low levels of ROS.

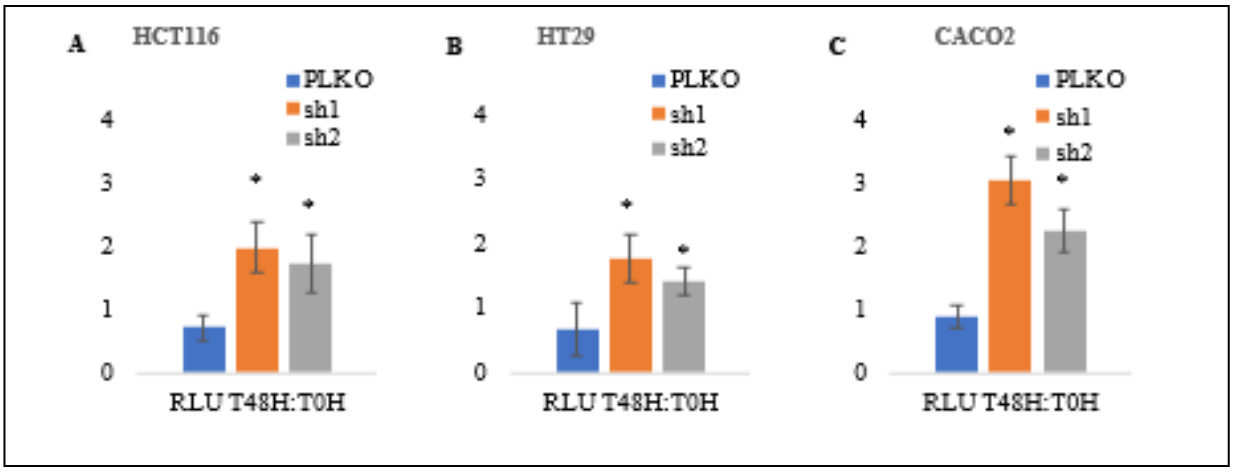


Figure 18: ROS production after KD of AP3M2 in CRC cell lines.

A. *HCT116* ***B.*** *HT29* ***and C.*** *CACO2****.*** *Cells were plated at a density of 10,000 cells/well in a 96-well white cell culture plate, in 100µl medium with 10% FBS.*

Infected cells were plated 48h post-infection. After overnight incubation at 37°C in 5% CO2, 20µl of H2O2 Substrate Dilution Buffer with 125µM H2O2 Substrate was added to wells and the plate was incubated for six hours, then 100µl of ROS-Glo™ Detection Solution was added to the wells. The plate was incubated for 20 minutes at room temperature, and the luminescence was determined with a Tecan M1000 plate reader The same procedure was repeated after 48h. X axis represents the different conditions (control, sh1 and sh2) Y axis represents the relative luminescence (RLU) at T48 normalized to T0. The average RLU and standard deviation of triplicate samples were calculated. p values were calculated with two tailed t-test, *, $p < 0.05$

3. DISCUSSION

The current therapeutic options in CRC have improved, however the survival rate is still debating [192]. The main challenge to be addressed nowadays is the improvement of CRC therapy especially in the case of metastatic tumors [193].

For the aim to investigate new therapeutic opportunities to target CRC we started by carrying out a systematic RNAi screening. Starting from a set of amplified genes in CRC derived from literature, by loss-of-function assay we successfully identified a new potential oncogene: AP3M2. The adaptor related protein complex 3 mu 2 subunit is involved in protein trafficking to lysosomes and specialized organelles. AP3M2 amplicons were observed in the primary tumor and maintained in at least two passages of breast cancer xenograft [121] and associated with reduced survival duration in breast cancer [122]. Additionally, AP3M2 gene was up-regulated in the non-responder patients' groups to FOLFOX therapy [123].

The AP3 complex, which consists of four subunits δ, β3A, μ3A, and σ3, is expressed ubiquitously. Yet two neuronally expressed subunits of the AP-3 complex that are referred to as β3B and μ3B [89]. None of these subunits but μ3B (encoded by the AP3M2 gene) was reported by the TCGA study to be overexpressed or amplified in CRC, highlighting the question regarding the mechanism regulating the oncogenic behavior of this subunit.

Our results align with TCGA data, AP3M2 is expressed in colorectal cancer cell lines belonging to different colorectal cancer subtypes including, HCT11, HT29, LoVo, DLD1, COLO201, COLO205, CACO2 and SW620. In addition, the expression of AP3M2 the tissue coming from patients with CRC was highly detected in the tumor parts.

In the other hand, AP3M2 was exclusively in the brain (male and female) and the testis of normal mouse. In the normal part of the colon tissue from CRC patient, AP3M2 was found to be expressed in the neurons. The other neuron specific subunit AP3B2 was only detected in the neuronal plexus. The ubiquitous isoform of AP3M2, encoded by AP3M1gene was expressed in the endothelial cells.

However, AP3M2 direct involvement in oncogenesis was never reported, we are the first to report its oncogenic potential in colorectal cancer. Starting with in *in vitro* models, AP3M2 was shown to possess the typical features of an oncogene, such as ability to migrate, to induce proliferation and clonogenicity in adherence independent manner. Interestingly, these cancer-

promoting capabilities were detected in different cell lines representing different molecular subtypes of CRC such as CIN/MSS (HT29) and from MSI (HCT116 and Caco2).

Strohecker *et al.,* identified novel autophagy regulators that could have potential therapeutic effect. Investigating the link between the deregulation of autophagy and cancer, AP3M2 associated with protein trafficking machinery, was listed with the genes whose loss was associated with the impairment of p62 elimination [124].

The pro-metastatic role of autophagy promotes cancer cells survival and proliferation and enables ECM-detached cancer cells to bypass anoikis, an anchorage-dependent programmed cell death, and survive [171]. Knockdown of autophagy-related proteins affected migration and invasion of glioblastoma cells [175].

The small GTPase Arf6 has an important role in intracellular vesicular trafficking and is used for the phagocytosis of pathogens and in the signaling of Toll-like receptors and in the activation of NADPH oxidases [190]. The KD of AP3M2 induced autophagy associated proteins ARF6, P62 and ATG7 downregulation in HT29 cell line showing that AP3M2 could be regulating CRC through autophagy.

Reactive oxygen species (ROS) play a crucial role in biological processes and constitutive ROS production in normal cells is managed by the regulation between the silver lining of low and high ROS concentration mediated effects. Interestingly, ROS influences the tumor microenvironment and are capable to promote cancer angiogenesis, metastasis, and survival at different concentrations. Low concentrations of ROS activate the cancer cell survival signaling cascade involving several pathways that include JNK, MAPK/ERK1/2, p38, and PI3K/Akt, which in turn activate the NF-κB, VEGF and MMPs pathways.

High concentrations of ROS can induce cancer cell apoptosis. Hence, it critically depends upon the ROS levels, to balance between tumorigenesis or apoptosis [191]. ROS production capability of the CRC after the Knockdown of AP3M2 showed its role in maintaining low levels of ROS in these cells to favor cancer cells survivor.

Recently, quinazoline and its derivatives were used as a novel class of cancer chemotherapeutic agents with a promising activity against different tumors and especially in 5FU resistant CRC. AP2 is the best-characterized member of the family with a heterotetrameric clathrin adaptor complex involved in vesicle trafficking pathways within the cell. The possibility of targeting a universal host protein exploited by most viruses would be a game-changing strategy with a

broad-spectrum solution and rapid pandemic control such as the current COVID-19. The chemical, N-(p-amylcinnamoyl) anthranilic acid (ACA), was found to interrupt AP2M1-virus interaction and thus exhibit potent antiviral efficacy against a number of viruses both *in vitro* and *in vivo*, including SARS-CoV-2. YxxØ mutation (recognized sorting motifs by different mu subunits, present in the cytosolic tail of different cargo proteins), AP2M1 depletion, or disruption by ACA causes incorrect localization of viral proteins, thereby suppressing viral replication [194]

4. MATERIALS AND METHODS

4.1 Genes and shRNAs selection

Selected genes were amongst the significantly overexpressed ($p<0.05$) focal amplification identified by (Cancer Genome Atlas Network, 2012). The miRNAs were excluded from the selection. We interrogated the Mission® library of shRNA provided by Sigma-Aldrich, which use the lentiviral-based vector PLKO.1-puro. For each gene, several shRNA variants were available, but just 1 was selected at the beginning. The selection procedure preferentially included the shRNA with the following characteristics:

- sequence complementarity to all splice variants of a mRNA
- validated shRNA
- sequence complementarity at the 3' UTR region.

Consequently, to the validation processes, we selected another shRNA against AP3M2 with a different target sequence as following:

Oligo Sequence	Symbol	RefSeq ID
CCGGGCCAGACCTTACACTTTCCTTCTCGAGAAGGAAAGTGTAAGGTCTGGCTTTTTG	AP3M2	NM_006803
CCGGCCTAAGAAGATATAGAGTGTTCTCGAGAACACTCTATATCTTCTTAGGTTTTTG	AP3M2	NM_006803

4.2 shRNA plasmid production

The shRNAs were purchased in bacterial clones in glycerol stock format. To each shRNA corresponds a different bacterial clone. Bacteria were streaked on LB agar plate with ampicillin (100 μg/ml, Sigma), and left to grow overnight at 37° C. The formed single colonies were picked, inoculated in LB medium (ThermoScientific), and cultured overnight at 37° C in an orbital incubator. The bacterial culture was centrifuged, to collect the pellet, at 4500 rpm for 10 min, then the supernatant was discarded.

The plasmid DNA was purified from the bacterial pellet by Plasmid Midi Kit (Roche) following manufacturer's recommendation, and the quantity was assessed by nanodrop (Thermo Fisher

Scientific) and quality with both nanodrop and on 1% agarose gel. The plasmids were stored at -20° C.

4.3 Cell culture

HCT116, HT29, Colo201 and Colo205 cell lines were purchased from the European Collection of Authenticated Cell Cultures (ECACC), DLD1, Lovo, Caco2, SW48 and HEK293T from American Type Culture Collection (ATCC).

Unless otherwise stated, cells were maintained in their appropriate growth medium and plastic support at 37°C and 5% CO2.

HCT116 and HT29 colon cancer cells were grown in McCoy's 5A medium supplemented with 10% FBS and antibiotics. Colo201 and Colo205 colon cancer cells were grown in RPMI medium supplemented with 10% FBS and antibiotics. LoVo colon cancer cells were grown in Ham's F12 medium supplemented with 10% FBS and antibiotics. DLD1 colon cancer cells were grown in RPMI medium supplemented with 10% FBS and antibiotics. CACO2 colon cancer cells were grown in MEM medium supplemented with 20% FBS and antibiotics.

Upon reached the confluence of 85-90%, the cells were detached by trypsin (Sigma). After trypsin inactivation, cells were resuspended in the appropriate medium and centrifuged 5 minutes at 1000 rpm. The resulting cellular pellet was resuspended using fresh medium and the cells counted by Bürker chamber. The cells were seeded accordingly.

4.4 Lentiviral production, transduction and stable cell line production

For the lentivirus production, 7×10^5 HEK293T packaging cells were seeded in each of the wells of a 6 multi-well plate 1 day before transfection. For each well, we diluted 2 μg of Sigma-Aldrich custom library plasmid (i.e. shAP3M2), 0.5 μg of pMD2G, 1 μg of psPAX2 in 100 μl of complete DMEM incubated 20 min at RT, plus 16 μl of transfection agent (FuGENE® HD, Promega). The cells were incubated for 24 h at 37 °C, after which the medium was refreshed. Lentivirus-containing supernatants were collected at 48- and 72-hours post-transfection with 20% of FBS. The two collections of lentiviral particles were pooled, filtered through a 0.45 μm membrane (Sartorius Stedim/PVDF) and stored at −80 °C. Cell lines were transduced with lentivirus supernatants supplemented with 8 μg/ml hexabromide (Sigma).

4.5 Western blot

At the right confluence, the cells were collected and washed in PBS, to remove the left traces of medium. For mice protein extraction, tissues were directly collected in PBS on ice, then homogenized using electric homogenizer. Mice were provided by the lab of "Dr. Monjat, Oncologia Sperimentale 2" lab at CRO.

Cells/ tissues were then lysed in RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories), diluted 1:5 in H2O, on which 2 μl of sample was diluted and placed in a plastic cuvette. The protein concentration was assessed by plotting the result of absorbance of the solution, read at 595 nm, on a titration curve made using BSA. Equal amounts of protein (20-30μg) were separated by 4- 12% SDS– polyacrylamide gel electrophoresis (TruPAGE™ Precast Gels) and transferred to nitrocellulose membrane (GE Healthcare) by miniprotean (Bio-Rad Laboratories) and detected with LiteAblot®TURBO *Enhanced Chemiluminescent* Substrate from *EuroClone* then the results analysed by ImageLab (Bio-Rad Laboratories) and ImageJ (v2.0.0, NIH). The primary antibodies used were: AP3M2 (1:5000, Abcam), ATG7 (1:1000, Cell Signaling), ARF6 (1:500, SantaCruz), SQSTM1/P62 (1:1000, Cell Signaling), HSP70 (1:5000, Sigma), α-Tubulin (1:5000, Sigma), GAPDH (1:5000, Santa Cruz). The secondary antibodies used: anti-mouse (1:5000, Thermo Fisher Scientific), anti-rabbit (1:5000, Thermo Fisher Scientific) and anti-goat (1:5000, Thermo Fisher Scientific).

4.6 Cell viability

Cells (500 x well) were seeded in 96 multiwell transparent microplate (Falcon) and, after experimental timing, cell viability was measured using the CellTiter-Glo® assay system (Promega) according to the manufacturer's instructions. Luminescence was assessed with microplate reader (Tecan M1000 plate reader). The resulting values were normalized on the control, that is cells transduced with PLKO empty vector, in the experiments at 96h end point, whilst in time course experiments, each cell line was normalized on its T0.

4.7 Soft Agar

Cells were plated in 6 multiwell plate, in duplicate, at the concentration of 20000 per well, in 1.5 ml of Soft Agar matrix, composed by DMEM 2X (Sigma), TBP buffer (Sigma), Noble Agar (BD) and FBS (Euroclone), 0.35% final, over a bottom layer of Soft Agar matrix, 0.65% final. Cells were kept in culture and medium was added weekly. Picture of the cells were taken weekly. After proper time (30 days), cells were photographed using a Nikon Eclipse Ti fluorescence microscope and 5 captures were taken for each condition to count the number of colonies and calculate the average.

4.8 Crystal violet

Cells were plated in 6-well plate, in duplicate, at the concentration of 1000 cells per well.
After the proper time (1week), cells were fixed with 4% PFA (Sigma) and stained with Crystal Violet 0.05% (Sigma). The images were acquired after extensive de-staining with ddH2O.

4.9 Intracellular reactive oxygen species (ROS) measurement

We measured H_2O_2 production with the ROS-Glo™ H_2O_2 Assay system (Promega). Cells were seeded into 96-well plates at a density of 2000 cells/well in triplicate. After incubation overnight at 37°C in 5% CO2, the H_2O_2 assay was performed as manufacturer instructions. The H_2O_2 substrate solution was then added at a final concentration of 25µM. The plate was incubated at 37°C in a 5% CO_2 incubator for 6 hours. After the incubation, the assay was performed in 1:1 ratio of ROS-Glo™ detection solution to sample volume. After an additional 20 min incubation at room temperature, luminescence was recorded using Tecan M1000 instrument.

4.10 c-BioPortal database analysis

c-BioPortal (www.cbioportal.org) is an online open access resource for exploring, visualizing and analyzing multidimensional cancer genomics data. In the present study, c-BioPortal was used to access colorectal carcinoma (TCGA,NATURE 2012 and PANCANCER ATLAS) data.

The selected genomic profiles contained mutations, putative copy number alterations from mRNA expression z-Scores (RNASeq V2 RSEM) [84].

4.11 STRING Consortium

Using the online STRING (http://string-db.org/) database [185] ; functional protein association networks. This biological database and web resource for known and predicted PPIs. We developed a network of TCGA proteins with a positive fold change value (overexpressed) in CRC.

4.12 Immunohistochemistry

Immunostaining was performed on paraffin-embedded archival tissue from C.R.O. pathology unit. The paraffin blocks were sliced into 5-µM thick sections, deparaffinized with xylene (Fisher Scientific, Waltham, MA, USA), and rehydrated with decreasing concentrations of ethanol in water. Antigen retrieval was achieved by incubation of the slides for 20 min in the hot (98 ºC) citrate buffer buffer (pH 6.0), followed by20 min of cooling at room temperature and washed 3 times for 5 min each with Tris Buffer Saline-0.025% Triton (TBS-T).

Endogenous peroxidases were quenched by incubating the slides in 3% hydrogen peroxide blocks (Fisher Scientific) for 15 min. The sections were then washed three times with TBS for 5 min each. Endogenous avidin and biotin was blocked using a blocking kit (V-block- Invitrogen) for 5 min at RT. The sections were then washed with TBS for five minutes. Primary antibodies AP3M2 (1:100 Abcam), AP3B2 (1:100 Abcam), AP3M1 (1:100 Abcam), were prepared in 0.5% BSA-TBS and applied O/N at 4° in a humidified chamber.

After rinsing the slides in TBS-T, they were incubated with primary antibody enhancer for 10 min and then with HRP polymer for 15 min at RT. After washing with 3 times TBS-T for 5 minutes each, they were washed with TBS for 5min, then the slides were incubated with DAB plus chromatogen + DAB plus substrate (Invitogem) for the appropriate developing time according to the primary antibody, then immediately washed in distilled water.

The duration of DAB incubation was determined through pilot experiments and was then held constant for all of the slides. After washing in distilled water, the sections were counterstained with haematoxylin, dehydrated through ethanol and xylene, and cover-slipped using a xylene-based mounting medium (Fisher Scientific).

4.13 Statistical Analysis

The p-value was calculated using two-tailed Student's T-test using Microsoft Excel.

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