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Molecular mechanisms underlying the role and functional effects of long non coding RNA H19 in colorectal cancer progression: new insights into its regulatory activity in alternative splicing modulation

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Colorectal cancer (CRC) remains one of the most prevalent and lethal malignancies worldwide [1]. In recent years, alternative splicing (AS) has emerged as a key contributor to the molecular complexity of CRC, influencing crucial processes such as tumor progression, epithelial-mesenchymal transition, and therapy resistance [2], [3]. However, the full landscape of AS regulation in CRC remains incomplete, and novel regulatory elements are still being uncovered.

During my PhD, I focused on investigating the role of long non-coding RNAs (lncRNAs) in the progression of CRC, with a particular emphasis on lncH19, a transcript frequently overexpressed in cancer and known to be involved in tumorigenesis and metastasis [4].

Through a combination of bioinformatic predictions and transcriptomic analysis, I identified 57 transcripts potentially co-regulated by lncH19 and two splicing factors: RBFOX2 and hnRNPM. Among these, RAC1 attracted particular attention due to its alternatively spliced isoform RAC1B, which has been implicated in oncogenic signaling and malignant transformation [5], [6].

The experimental work carried out during my doctoral studies demonstrated that lncH19 physically associates with both RBFOX2 and hnRNPM, and is required for RAC1B expression in CRC cell lines. Loss- and gain-of-function studies in HCT116 and SW620 cells confirmed that modulation of lncH19 levels impacts not only RAC1B, but also downstream effectors such as c-Myc and Cyclin D1, thus supporting a functional role in tumor cell proliferation. These findings were corroborated in CRC patient biopsies, where lncH19, RAC1B, c-Myc and Cyclin D1 were found to be significantly upregulated compared to normal tissues. A positive correlation between lncH19 and RAC1B expression was also observed across patient samples. Taken together, the data reported in this thesis support a model in which lncH19 functions as a molecular scaffold, facilitating the recruitment of splicing factors to specific transcripts, and thereby promoting exon inclusion and isoform switching. In the case of RAC1, this mechanism results in the expression of the pro-tumorigenic isoform RAC1B [7].

This work provides novel insights into the post-transcriptional regulatory functions of lncH19 in CRC, and highlights its potential as a molecular hub in splicing-dependent oncogenic pathways, offering a foundation for future studies aimed at exploring lncRNAs as diagnostic markers or therapeutic targets in colorectal cancer.

CHAPTER 1

Colorectal cancer

1.1 Colorectal cancer : epidemiology, risk factors and prevention

According to Global Cancer Statistics 2020, colorectal cancer (CRC) ranks as the third most commonly diagnosed malignancy and the second leading cause of cancer-related mortality worldwide [1].

The epidemiology of colorectal cancer (CRC) varies considerably across different regions of the world and among populations distinguished by age, sex, and ethnicity. This variability is influenced by a combination of factors, including exposure to environmental and lifestyle risk factors, demographic differences, genetic susceptibility, and the presence of specific mutations, all of which can impact both prognosis and therapeutic response [8] .

Between 2015 and 2019, the average annual incidence rate of CRC was 33% higher in men than in women, with rates of 41.5 and 31.2 per 100,000, respectively. However, this gender gap in incidence appears to depend on both the age at diagnosis and the anatomical site of the tumor [9].

CRC is influenced by a range of risk factors, broadly categorized as modifiable and non-modifiable. Modifiable factors include those linked to lifestyle and behavior, which can be adjusted through preventive measures, while non-modifiable factors refer to inherent characteristics such as age, sex, and genetic predisposition. Recent evidence indicates that a significant proportion of CRC-related mortality—over 50%—is associated with modifiable determinants. This is largely attributed to increasing exposure to environmental and behavioral risks, including physical inactivity and dietary patterns characteristic of a Westernized lifestyle.

Some of **modifiable** risk factors are :

- Obesity and physical inactivity are well-established independent and cumulative risk factors for CRC, significantly impacting both incidence and survival outcomes. Sedentary individuals and those who are overweight are estimated to have a 25–50% higher risk of developing CRC compared to physically active individuals [10]. Notably, gender-specific differences in risk have been observed: obese men face a 50% increased risk of colon cancer and a 20% increased risk of rectal cancer, whereas overweight

women show a 20% and 10% increased risk for colon and rectal cancer, respectively. Moreover, studies suggest that early-life body mass index (BMI) is a stronger predictor of CRC risk in women, while in men, risk is more strongly associated with weight gain later in life [11]. Physical activity appears to mitigate CRC risk through various mechanisms, including reduction in adiposity, suppression of systemic inflammation, enhanced gut motility, and regulation of metabolic hormones [12]. Additionally, the protective effect of physical activity is more pronounced for cancers of the proximal and distal colon, with less consistent associations reported for rectal cancer [13];

- Tobacco use – Extensive evidence has demonstrated a significant association between smoking and increased CRC risk. Tobacco smoke components are known to damage the intestinal mucosa, promoting genetic and epigenetic alterations that may contribute to carcinogenesis [13]. Anatomically, smoking has been specifically linked to a higher incidence of cancer in the proximal colon and rectum, whereas no significant association has been observed with cancers of the distal colon [[13], [14]]. Beyond its role in CRC development, smoking is also correlated with poorer survival outcomes among CRC patients. Consequently, smoking cessation is associated with improved prognosis and a reduced risk of CRC [15];
- Moderate to heavy alcohol consumption – Alcohol consumption is strongly linked to an elevated risk of CRC [16]. Individuals who consume two to three alcoholic beverages per day have approximately a 20% increased risk of developing CRC, while those consuming more than three drinks daily face a risk increase of up to 40%. This association appears to be more pronounced in men, potentially due to sex-related hormonal differences that influence alcohol metabolism [17];
- Diet and drugs - Diet plays a significant role in modulating the risk of CRC onset and progression, independently of obesity status [18]. This influence can be exerted directly through specific dietary components or indirectly via modulation of the gut microbiota and effects on body weight. Diets high in processed foods, red meat, and refined carbohydrates, along with low calcium intake, have been shown to promote inflammation and elevate the risk of CRC, as well as other gastrointestinal malignancies [19], [20]. The relative risk of CRC among frequent consumers of red and processed meat is estimated at 1.22% [21]. In contrast, higher consumption of dietary fiber, leafy green vegetables, folate, and calcium has been associated with a protective effect against CRC development [22]. Moreover, both diet and pharmacological interventions significantly impact gut microbial composition and intestinal inflammation. For instance, the fermentation of complex dietary residues by gut microbiota produces butyrate, a short-chain fatty acid that supports colon health by preserving mucosal integrity and suppressing both inflammation and carcinogenesis [23]. Conversely, antibiotic usage may disrupt microbial balance (dysbiosis), a condition increasingly associated with heightened CRC risk [24], [25]. As such, targeting the gut microbiome represents a promising avenue for CRC prevention and therapeutic strategies [26].

Other risk factors are **non-modifiable**, such as a personal or family history of colorectal polyps, inherited conditions such as Lynch syndrome, a personal history of inflammatory bowel disease, racial and ethnic background, and the presence of type 2 diabetes.

The risk of developing CRC increases significantly after age 50; screening of average-risk individuals aged ≥ 50 years and careful monitoring of higher-risk individuals provides the best opportunity for prevention and early diagnosis of CRC.

Non-modifiable CRC risk factors include:

- Sex, age and ethnicity - In 2020, the incidence of CRC was 44% higher in men than in women, with a particularly marked sex-based disparity observed in rectal cancer compared to colon cancer. Specifically, rectal cancer incidence in men was 75% higher, while colon cancer was 31% higher, relative to women [27]. In the United States, individuals aged over 65 are significantly more likely to be diagnosed with CRC than younger populations, likely reflecting the age threshold at which routine screening is recommended.
- Genetic factors - It is estimated that approximately 35–40% of CRC cases have a hereditary component [28], [29]. These include a positive family history, inherited cancer syndromes such as Lynch syndrome, low-penetrance genetic variants, and other yet unidentified germline genomic alterations [29], [30]. Individuals with a first-degree relative affected by CRC face a two- to fourfold increased risk of developing the disease. Notably, this elevated risk extends beyond first-degree relatives, underscoring the significance of familial aggregation in CRC predisposition [31]. Hereditary CRC syndromes, such as Lynch Syndrome and Familial Adenomatous Polyposis (FAP), significantly increase the risk of CRC. Lynch syndrome is the most common hereditary CRC syndrome, accounting for about 3% of all CRC cases. It is characterized by mutations in mismatch repair (MMR) genes, leading to microsatellite instability and an increased risk of cancer. Individuals with Lynch syndrome have a 50–80% lifetime risk of developing CRC. FAP, on the other hand, is caused by mutations in the APC gene, leading to the formation of numerous adenomatous polyps in the colon and rectum. If untreated, nearly all individuals with FAP will develop CRC, usually before the age of 40. In recent years, the adoption of cancer gene panel testing has been expanding in Japan, providing new opportunities for genetic diagnosis. This comprehensive approach not only aids in identifying hereditary cancer syndromes but also frequently results in secondary findings that lead to a diagnosis of Lynch syndrome. These advancements highlight the growing importance of genetic testing in clinical practice, enabling earlier detection and personalized management strategies for individuals at high risk of CRC [32].
- Diseases and therapies that predispose to increased susceptibility to CRC – Chronic inflammatory conditions are well-established risk factors for CRC, particularly inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease, as well as cystic fibrosis [33], [34]. In addition, individuals who have undergone certain medical treatments or surgical

procedures—such as androgen deprivation therapy, cholecystectomy, or abdominal radiation—also exhibit an increased risk of CRC development [35].

Variations in the incidence and mortality rates of CRC strongly suggest that the disease is influenced by modifiable behavioural factors. As such, effective prevention strategies—such as regular screening, surveillance, and timely access to high-quality care—can significantly reduce the risk of CRC onset. Prevention of CRC can be approached at three distinct levels:

- Primary prevention targets healthy individuals and aims to reduce exposure to established risk factors. This level of prevention typically involves adopting and maintaining healthy lifestyle behaviours, including regular physical activity, a balanced diet, smoking cessation, and moderation in alcohol consumption.
- Secondary prevention focuses on the early detection of disease and is widely regarded as the most effective approach for preventing CRC progression. It includes organized screening programs and surveillance strategies aimed at identifying precancerous lesions or early-stage tumors before they become advanced. The recent implementation of advanced screening methods for early detection, alongside novel treatment strategies aimed at removing precancerous lesions, has significantly impacted CRC incidence and mortality rates [36], [37], [38].
- Tertiary prevention focuses on interventions designed to prevent recurrence and metastatic progression. Current treatment modalities for both primary and metastatic CRC include laparoscopic surgery, radiotherapy, neoadjuvant therapy, biomarker-guided therapies, and chemotherapy [39]. Surgery remains the primary treatment for early-stage CRC; however, it is often insufficient in cases with metastasis, which account for approximately 25% of diagnoses. For these advanced cases, therapeutic approaches extend to radiotherapy, chemotherapy, targeted therapies, and multimodal treatment regimens [40], [41].

CRC remains a significant global health challenge, influenced by genetic, lifestyle, and environmental factors. The rising incidence underscores the need for effective public health strategies. Enhancing screening, early detection, and preventive measures are essential to reducing CRC-related mortality. Continued research and collaboration are critical for developing targeted interventions that can address this growing burden and improve outcomes worldwide [4].

1.2 Colorectal cancer: etiology and pathogenesis

The development of CRC is primarily associated with the abnormal hyperproliferation of epithelial cells within the colon mucosa. CRC can be broadly categorized into two main forms:

- Familial CRC, which includes conditions such as familial adenomatous polyposis (FAP), an autosomal dominant hereditary syndrome predominantly caused by germline mutations in the Adenomatous Polyposis Coli (APC) gene [42], [43]. Individuals affected by FAP typically develop hundreds to thousands of adenomatous polyps throughout the colon and rectum, which, if left untreated, almost invariably progress to colorectal adenocarcinoma. Another major hereditary form is Lynch syndrome, or hereditary nonpolyposis colorectal cancer (HNPCC), resulting from germline mutations in DNA mismatch repair (MMR) genes [44];
- Sporadic CRC, which arises from non-heritable somatic mutations and represents approximately 80% of all CRC cases.

Both genetic predispositions and environmental influences significantly contribute to CRC pathogenesis, underscoring the multifactorial nature of this malignancy.

The healthy colon-epithelium is characterized by continuous self-renewal, a process sustained by a population of intestinal stem cells (ISCs) located at the base of the glandular invaginations of the mucosa, known as crypts. These ISCs are essential for maintaining epithelial homeostasis throughout life.

Over the past decade, significant advances have been made in understanding the molecular signals that regulate ISC maintenance and proliferation. WNT, EGFR/MAPK, and NOTCH signaling pathways have been shown to promote ISC self-renewal and preserve their undifferentiated, proliferative state within the stem cell niche. In contrast, pathways such as BMP and TGF- β act as key regulators of ISC differentiation [45].

The high proliferative rate of ISCs inherently increases their susceptibility to DNA replication errors and the accumulation of mutations. This genomic instability, when combined with extrinsic factors—including lifestyle, dietary habits, and the composition of the intestinal microbiota—can significantly contribute to the initiation of epithelial transformation and colorectal tumorigenesis [46],[47].

CRC is a heterogeneous disease, comprising multiple subtypes defined by distinct molecular profiles and morphological characteristics. One of the hallmark features of CRC is genomic instability, which can develop through at least two major mechanisms.

The most prevalent form, observed in approximately 84% of sporadic CRC cases, is chromosomal instability (CIN). This condition is characterized by alterations in chromosome number and structure, including aneuploidy, deletions, translocations, and other chromosomal rearrangements [48].

CIN has been frequently associated with inactivating mutations or loss of function in the APC tumor suppressor gene, an event considered one of the earliest molecular alterations in the adenoma-to-carcinoma sequence of colorectal tumorigenesis.

The second major subtype, accounting for approximately 13–16% of sporadic CRCs, is characterized by microsatellite instability (MSI), which results from defects in the DNA mismatch repair (MMR) system [49].

In addition, CpG island methylator phenotype (CIMP) represents another key molecular

hallmark, leading to epigenetic instability through promoter hypermethylation and subsequent silencing of tumor suppressor genes, including MLH1, a core component of the MMR machinery [50].

CRC tumors exhibiting CIN typically develop through a sequential accumulation of oncogene activations (e.g., KRAS, PIK3CA) and tumor suppressor genes inactivation (e.g., APC, SMAD4, TP53), mediated by allelic loss and somatic mutations.

These mutational events are closely associated with the progression of tumor characteristics from adenoma to carcinoma. A pivotal initiating event in colorectal tumorigenesis is the hyperactivation of the Wnt signaling pathway, most commonly triggered by mutations in the APC gene. Dysregulation of Wnt signaling is a hallmark of the vast majority of sporadic CRCs, as well as those associated with FAP.

More than 80% of CRCs harbor APC mutations, while an additional 5–10% display mutations or epigenetic alterations in other key components of the Wnt pathway—such as β -catenin (CTNNB1)—leading to sustained pathway activation and contributing to oncogenic transformation [51], [52], [53].

APC functions as a critical negative regulator of the Wnt signaling pathway, being a core component of the complex responsible for targeting β -catenin for proteasomal degradation. When APC is mutationally inactivated, this complex becomes non-functional, resulting in the accumulation of β -catenin in the cytoplasm. The accumulated β -catenin can then translocate to the nucleus, where it acts as a transcriptional co-activator, promoting the expression of oncogenic targets [54].

The progression from normal colonic mucosa to colorectal carcinoma follows a multistep model (Figure 1). As previously mentioned, the initial mutational event often involves the APC gene, whose loss induces dysregulation of the cell cycle and increased cellular proliferation, resulting in mucosal hyperplasia. Subsequent global DNA hypomethylation can promote the transition to early adenomas (class I), characterized by benign but hyperproliferative epithelial growth. The acquisition of activating mutations in the Ras oncogene marks the progression to class II adenomas, where cells exhibit both hyperplasia and initial features of dysplasia, reflecting a loss of cellular differentiation [55]. The advancement to class III adenomas, characterized by more pronounced dysplasia, is often associated with the loss of DCC (Deleted in Colorectal Cancer) function.

The transition from adenoma to carcinoma in situ is commonly linked to mutations or loss of the TP53 tumor suppressor gene, a critical regulator of genomic integrity. Further genetic and epigenetic alterations, including the inactivation of additional tumor suppressors and the activation of oncogenic drivers, ultimately confer invasive and metastatic potential to the tumor.

The liver represents the most frequent site of metastasis in CRC [56]. This tropism is primarily attributed to the anatomical and physiological characteristics of the gastrointestinal tract, whose venous drainage flows directly into the liver via the portal circulation. Once in the hepatic environment, metastatic colonization is driven by a complex interplay between circulating tumor cells and the components of the liver microenvironment, which together influence tumor cell engraftment, survival, and outgrowth [57]. Beyond the liver, CRC metastases can also

occur in the lungs, bones, brain, peritoneum, and other distant organs [58].

The accumulation of genetic mutations in CRC is a gradual process, often requiring several decades to culminate in the development of an invasive carcinoma [46], [59]. CRC exhibits a highly heterogeneous and complex mutational landscape, and the functional significance of many of these alterations remains incompletely understood [60]. This molecular heterogeneity poses significant challenges for the functional characterization of the disease and hinders the identification of universally effective therapeutic strategies.

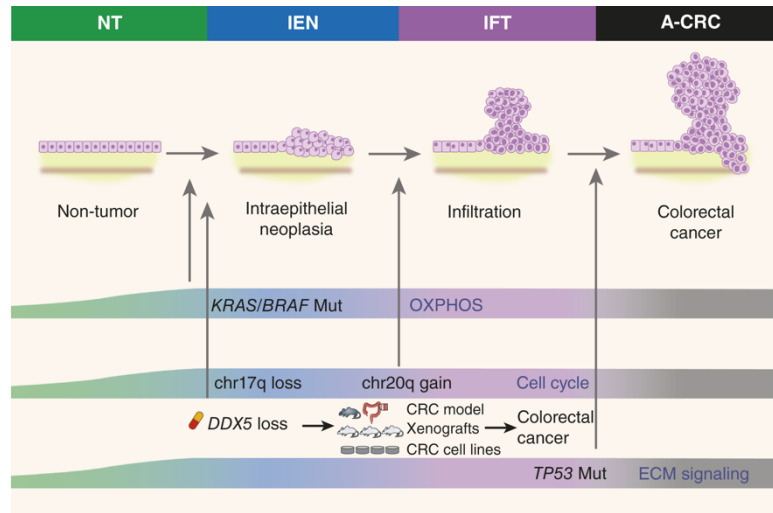


Figure 1. The progression from normal colonic mucosa to colorectal carcinoma through the multistep model. In CRC cells Oxidative Phosphorylation (OXPHOS) is often upregulated to fuel rapid growth and survival [55].

1.3 Colorectal cancer : therapeutic strategies and treatments

Treatment of CRC requires a multidisciplinary approach usually involving a combination of chemotherapy, surgical intervention, radiotherapy, and/or liver directed therapies that are tailored to the disease burden and patient's clinical status [56].

- Surgical resection represents one of the most important relevant approaches for CRC patients with localized tumors, as well as for those with resectable distant metastases. It has long represented the cornerstone of CRC treatment and continues to be the most effective therapeutic option when feasible [61], [62]. The adoption of minimally invasive laparoscopic techniques has further improved postoperative outcomes by enhancing gastrointestinal recovery, reducing hospitalization time, and showing no detrimental effect on long-term survival [63]. Curative-intent surgical resection is associated with a 5-year survival of 30–60% and a 10-year survival of 20–25% [57]. Nearly half of patients undergoing surgical resection for their primary CRC, eventually develop liver metastases [64]. If not treated, liver metastases exhibit an unfavorable prognosis with median overall survival (OS) of 5–20 months [65]. Despite modern surgical techniques and adjuvant systemic therapy, only 20% of patients with distant metastasis achieve long-term remission (which ranges from 25.8–31.4 months when standard

chemotherapy is administered), while 60–70% of patients develop local or distant recurrence [66], [67].

At the time of diagnosis, malignant tumors contain multiple cell populations with diverse biological heterogeneity [68], [69]. The clonal origin and genetic heterogeneity of CRCs and heterogeneity between primary tumors and liver metastases have been recently addressed. It seems that the majority of CRC tumors has a polyclonal origin [70] and that half of liver metastasis is genetically distinct from their primary carcinomas [71], [72], indeed observed accelerated incidence of mutations in metastasis might subsequently result in genetic heterogeneity between primary and metastatic cancers [73], [74].

Considering the extensive molecular and clinical heterogeneity between CRC and distant metastasis, it becomes apparent that an individualized approach based on molecular profiling will be necessary to achieve more promising results [75].

- Radiotherapy uses the action of high-energy radiation (X-rays, gamma rays or charged particles) to eliminate locally present tumor cells through direct and indirect damage (from free radicals produced by radiation), reducing tumor mass and the risk of recurrence.
- Chemotherapy can be employed in neoadjuvant, adjuvant, or palliative settings. As a neoadjuvant treatment, it aims to reduce tumor size prior to surgical intervention, thereby facilitating resection. When administered as adjuvant therapy, it is used following tumor removal to lower the risk of disease recurrence. In advanced or inoperable cases, chemotherapy also plays a crucial role in palliative care, helping to manage symptoms and improve quality of life [76]. Currently, although new drugs have been introduced for CRC patients, such as the monoclonal antibodies cetuximab and panitumumab, according to the main guidelines for the treatment of CRC, the standard chemotherapy is 5-Fluorouracil (5-Fu), widely used in both adjuvant and palliative chemotherapy. Despite this, multiple studies suggest that an increasing number of CRC patients show primary or acquired resistance to 5-Fu, associated with poor prognosis. Development of drug resistance is one of the major challenges in the efficacy of chemotherapy for cancer treatment and is a phenomenon whose underlying mechanisms are not yet fully elucidated. Chemoresistance can arise from various factors, including tumor heterogeneity and molecular or genetic alterations within cancer cells [77]. Mechanistically, resistance may involve impaired drug uptake, often due to modifications in enzymes responsible for drug metabolism—such as increased activity of thymidylate synthase [78] —or the dysregulation of drug efflux transporters associated with multidrug resistance (MDR) [79]. Additional mechanisms include enhanced DNA repair capacity, gene amplification [80], and alterations in key cellular processes such as autophagy, and cell cycle regulation, particularly the suppression of apoptosis in CRC cells. Although better screening, improved diagnostic techniques, and novel treatments have progressively increased the survival for patients diagnosed with CRC since 1970, patients diagnosed with distant-stage disease face unpromising odds with a five-year relative survival rate of only 14%, which is attributed to the development of acquired resistance to chemotherapy [27], [81]. For this reason, it is important to explore the molecular mechanisms involved in the development of

chemoresistance in metastatic CRC, to understand the pathways that contribute to the survival of tumor cells and to develop new therapeutic strategies that address these mechanisms [58], [82], [83] .

- Targeted biologic and immune based therapies have gained traction for many different types of malignancies including CRC. As opposed to cytotoxic chemotherapy, which aims to kill cells as they replicate, targeted therapies are directed at biologic features of cancers, like angiogenesis or immune checkpoints. The goal of targeted therapy is to create a balance between tumor cell death and the off-target effects on healthy cells [84]. In a recent systemic review and meta-analysis, the use of chemotherapy with targeted biologic therapy for unresectable CRC liver metastases was noted to be associated with an increase in overall response rate (ORR) versus chemotherapy alone (68% vs 43%, respectively) [85], [86]. However, ORR does not always translate to improvement in overall survival [54].

In summary, CRC represents a significant global health challenge with variable survival rates depending on the stage at which it is diagnosed. More efforts should be made to understand tumor behaviour and the molecular mechanisms involved in cancer progression [87].

Non-coding RNAs and cancer

2.1 Non-coding RNA : an overview

Recent advances in high-throughput sequencing technologies have revealed that less than 2% of the human transcriptome encodes proteins, while the vast majority consists of non-coding RNAs (ncRNAs). Initially considered transcriptional noise or byproducts of gene expression, ncRNAs are now recognized as crucial regulators of a wide array of cellular processes, including cell differentiation, proliferation, apoptosis, and metabolism. Overall, ncRNAs serve as integrative nodes within complex molecular networks, orchestrating cellular homeostasis and determining cell fate. Their dysregulation has been increasingly associated with the onset and progression of numerous pathological conditions.

NcRNAs are broadly classified based on their length into two main categories: small non-coding RNAs, which are less than 200 nucleotides in length (including microRNAs, small nuclear/nucleolar RNAs, and transfer RNAs), and long non-coding RNAs (lncRNAs), which exceed 200 nucleotides. In addition to these two major groups, circular RNAs (circRNAs) represent a distinct class of ncRNAs characterized by a covalently closed loop structure formed through a 5'–3' phosphodiester bond. This unique circular configuration confers greater stability and resistance to exonucleases, distinguishing circRNAs from their linear counterparts [88], [89], [90].

NcRNAs play key roles in the regulation of gene expression through a variety of mechanisms, both under normal physiological conditions and during disease progression. Dysregulation of their expression has been strongly implicated in the onset and advancement of numerous human cancers [91], [92], [93]. In this context, ncRNAs can act either as oncogenes or tumor suppressors, depending on the molecular environment [94], [95], [96], [97]. Among the various classes of ncRNAs, lncRNAs and microRNAs (miRNAs) are the most extensively investigated in cancer research, with accumulating evidence supporting their context-dependent dual roles in promoting or inhibiting tumorigenesis.

One of the major challenges in elucidating the role of ncRNAs in cancer lies in their functional versatility. A single ncRNA may act as a tumor suppressor in one cancer type while promoting tumor progression in another. Moreover, different transcript variants originating from the same ncRNA gene locus may exert synergistic or even opposing effects within the same molecular pathway [98].

Although computational tools can predict potential targets, particularly in the case of miRNAs, determining whether a given ncRNA functions as an oncogene or tumor suppressor remains complex and context-dependent [89]. Gaining deeper insights into the intricate regulatory networks governed by ncRNAs offers a valuable opportunity to develop more personalized and effective therapeutic strategies. Notably, many ncRNAs can be secreted by tumor cells into biological fluids, either in a free-circulating form, bound to lipoproteins, or encapsulated within extracellular vesicles [99], [100]. This characteristic has sparked growing interest in their potential use as non-invasive biomarkers for cancer diagnosis, as well as for predicting patient prognosis, metastatic potential, and therapeutic response [101].

2.2 Long non coding RNAs

In the past decade, increasing attention has been directed toward lncRNAs and miRNAs due to their pivotal roles as regulators of gene expression across a wide range of biological processes [102]. LncRNAs are transcribed from diverse genomic regions and are generally classified into four main categories based on their positional relationship to nearby protein-coding genes (Figure 2): *sense lncRNAs*, which overlap one or more exons of a protein-coding gene on the same strand; *antisense lncRNAs*, which overlap exonic regions on the opposite strand; *intronic lncRNAs*, transcribed entirely from introns of another gene; and *intergenic lncRNAs*, which are located between two genes and do not overlap coding regions [103], [104].

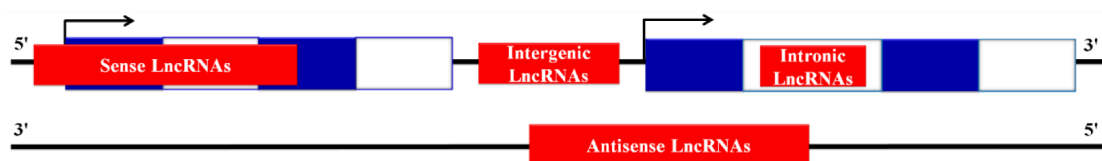


Figure 2. Four classes of lncRNAs. Blue indicates exons and white indicates introns; Black lines represent the coding and non-coding DNA strands. Red indicates lncRNAs, and arrows indicate the direction of transcription. Sense lncRNAs overlap with coding genes on the same strand. Antisense lncRNAs overlap with protein-coding genes on the opposite strand. Intronic lncRNAs occur completely within an intron. Intergenic lncRNAs occur between two genes [105].

Although less than 1% of lncRNAs have been functionally characterized to date, accumulating evidence indicates that these molecules exert regulatory roles at multiple levels of gene expression. They have been implicated in the control of genomic imprinting, transcriptional regulation, RNA interference, alternative splicing, and translational control [106], [107]. In particular, at the nuclear level, lncRNAs regulate gene expression both in cis and in trans through various mechanisms. They can interact with transcriptional co-regulators and

chromatin remodeling complexes, modulate chromatin structure, or hinder the binding of transcription factors to specific genomic regions. Additionally, by binding to RNA-binding proteins, lncRNAs play a key role in the regulation of alternative splicing, further influencing gene expression patterns [108], while cytoplasmic lncRNAs have been shown to influence protein localization and stability, as well as the translation and half-life of target mRNAs [109], [110].

Overall, lncRNAs are now recognized as versatile molecular regulators that act as signals, decoys, guides, or scaffolds, participating in complex regulatory networks through RNA-chromatin, RNA-RNA, and RNA-protein interactions [111],[112].

One of the primary roles of lncRNAs is the modulation of chromatin architecture and transcriptional activity, often through the recruitment of chromatin-modifying complexes that mediate enhancer region methylation. Additionally, lncRNAs can interact directly with mRNAs, influencing their splicing patterns and stability. In some cases, they enhance translation by hybridizing with mature RNAs at the 5' end, thereby increasing translational efficiency.

Similar to miRNAs, lncRNAs exhibit cell- and tissue-specific expression patterns. Dysregulation or aberrant expression of lncRNAs has been associated with a wide range of malignant processes, including uncontrolled proliferation, resistance to apoptosis, enhanced migration and invasion, autophagy, and ultimately, tumorigenesis [102],[113].

In recent years, an increasing number of dysregulated lncRNAs have been implicated in the pathogenesis of various diseases, including cancer [114],[115]. Although some cancer-associated lncRNAs have been functionally well characterized [116],[117], the vast majority remain poorly understood. Notably, the aberrant expression of several lncRNAs in cancer has been correlated with clinicopathological features and patient prognosis, highlighting their potential relevance as biomarkers. Based on this evidence, functional characterization of these molecules could thus pave the way for novel strategies in early cancer detection and the development of targeted therapeutic approaches [118].

LncH19 and X-inactive specific transcript (XIST) are among the first functional lncRNAs identified, both transcribed by RNA polymerase II. XIST plays a central role in X-chromosome inactivation in female zygotes by recruiting the Polycomb Repressive Complex 2 (PRC2), thereby inducing epigenetic silencing of one of the two X chromosomes [119],[120].

2.2.1 LncH19

LncH19 is a well-characterized imprinted oncofetal gene located on human chromosome 11p15.5, which encodes a processed non-coding RNA transcript of approximately 2.3 kb. As an imprinted gene, lncH19 is monoallelically expressed from the maternal allele and shares a common enhancer region with the IGF2 (Insulin-like Growth Factor 2) gene. LncH19 is highly expressed during embryonic development, particularly in extraembryonic and fetal tissues, while its expression is largely silenced postnatally in most somatic tissues [121].

Numerous studies have reported that lncH19 is predominantly localized in the cytoplasm, where it participates in the regulation of protein translation and activity, mediates mRNA degradation,

and functions as a competing endogenous RNA (ceRNA) by sequestering specific miRNAs [122], [123].

However, emerging evidence has also demonstrated its nuclear localization, where lncH19 contributes to chromatin remodeling, epigenetic regulation, and transcriptional control through interactions with chromatin-modifying proteins, either in cis or in trans, and may also influence alternative splicing processes [124], [125].

For instance, a recent study revealed that nuclear lncH19 promotes DNA damage repair and confers resistance to PARP inhibitors by directly interacting with ILF2 [126].

Additionally, lncH19 has been implicated in the regulation of gene methylation: Zhou et al. demonstrated its role in modulating SAHH (S-adenosylhomocysteine hydrolase), ultimately affecting DNA methylation patterns [127].

Recent findings have demonstrated that lncH19 expression can be reactivated in adult tissues under conditions such as tissue regeneration and tumorigenesis, suggesting a potential role for lncH19 in cancer development and progression [121].

The oncogenic functions of lncH19 are largely attributed to its ability to generate microRNA-675 (miR-675), a small non-coding RNA processed from the first exon of the lncH19 transcript [128],[129]. During lncH19 transcription, the primary miRNA precursor of miR-675 is co-transcribed, linking the expression of the lncRNA to that of the miRNA. However, the precise regulatory relationship between lncH19 and miR-675 remains a matter of debate, with some studies suggesting independent roles and others indicating a coordinated function in tumorigenesis [130].

The functional role of lncH19 in cancer remains a subject of ongoing debate, as its activity appears to be highly context-dependent. Whether lncH19 exerts oncogenic or tumor-suppressive effects is influenced by cancer type, tumor stage, and the status of specific molecular signaling pathways. Aberrant lncH19 expression has been documented in several malignancies, including breast and pancreatic cancers, choriocarcinomas, hepatocellular carcinomas, and ovarian cancers, among others. Notably, lncH19 overexpression has been associated with poor prognosis, particularly in high-grade and invasive transitional cell carcinomas [121].

Furthermore, lncH19 has been strongly implicated in cancer metastasis. In colorectal cancer cells, it has been shown to promote epithelial to mesenchymal transition (EMT) by upregulating hnRNPA2B1 expression [131] [4].

According to the literature, lncH19 contributes to tumor development through multiple mechanisms, including acting as a molecular sponge for miRNAs, serving as a miRNA precursor, and functioning as an epigenetic regulator via interaction with EZH2 and the Polycomb repressive complex [132] [133]. In the project that I have carried out in these three years under the supervision of Professor Alice Conigliaro, we aimed to investigate, for the first time, a potential role for lncH19 in the regulation of alternative splicing (AS) [7].

CHAPTER 3

The role of RBP and lncRNAs interactions in alternative splicing process

3.1 Alternative splicing

Gilbert was the first to propose the concept of alternative splicing [134], a mechanism that explains the discrepancy between the number of protein-coding genes (~20,000) and the >90,000 proteins actually translated [135] [136].

More than 95% of human genes have been found to undergo splicing in a developmental, tissue-specific, or signal transduction-dependent manner [137].

Constitutive splicing is the process of intron removal and exon ligation mainly in the order in which they appear. Alternative splicing (AS) is a deviation from this preferred sequence, where certain exons are skipped, resulting in various forms of mature mRNA.

Numerous studies have reiterated the critical and fundamental role of alternative splicing across biological systems [138] and have shown that more evolved species exhibit a higher percentage of alternatively spliced genes, supporting the idea that this mechanism plays a key role in evolutionary processes. AS regulation has an important role in almost every aspect of eukaryotic biological processes, including cell growth, death, pluripotency maintenance, differentiation, development, circadian rhythms, response to external changes, and disease [139], [140].

This process is mediated by a dynamic macromolecular machine, the **spliceosome** [141]. The basal splicing machinery (spliceosome) is comprised of five small nuclear ribonucleoprotein particles (snRNPs), such as U1, U2, U4/U6, and U5 in the case of the major spliceosome, and U11, U12, U4atac/U6atac, and U5 in the case of the minor spliceosome. AS is primarily regulated by approximately 200 RNA-binding proteins (splicing factors) together with a basal spliceosome, through direct recognition of short sequence motifs near exon/intron boundaries [142].

The overall function of alternative splicing is to increase the diversity of mRNAs expressed from genome changing proteins' functional properties encoded by mRNAs. Experimental

analysis of these protein isoforms showed that alternative splicing regulates binding between proteins, between proteins and nucleic acids as well as between proteins and membranes. It regulates their localization, enzymatic properties and their interaction with ligands [143].

Furthermore, AS events can introduce premature termination codons in mature mRNAs, triggering mRNA degradation by the process of nonsense-mediated mRNA decay (NMD) [144] and can also cause mRNA untranslated region (UTR) variation, which affects mRNA translation efficiency, stability, and localization [145], [146], [147].

Taken together, all this information reveals the central role of alternative splicing in regulating gene expression [148].

The exons that end up in the mature mRNA during the process of alternative splicing are entirely defined by the interaction between cis-acting elements and trans-acting factors. Cis-acting elements include **exonic splicing enhancers (ESEs)** and **intronic splicing enhancers (ISEs)** that are bound by positive trans-acting factors, such as SR proteins (serine/arginine-rich family of nuclear phosphoproteins), whereas **exonic splicing silencers (ESSs)** and **intronic splicing silencers (ISSs)** are bound by negative trans-acting factors, such as heterogeneous nuclear ribonucleoproteins (hnRNPs). The collaboration between these elements results in the promotion or inhibition of spliceosome assembly on the splice sites [149], [150] [151].

Depending on the pattern of exon inclusion/skipping, AS events can be categorized into at least six major types (Figure 3): *including cassette exon skipping, mutually exclusive exons, alternative 5' splice site selection, alternative 3' splice site selection, alternative retained intron, and tandem cassette*. There are more complex patterns, but they are much fewer in number than these major types; therefore, most analyses of AS events focus on these six types, particularly cassette exon skipping, which represents the majority of AS events [152].

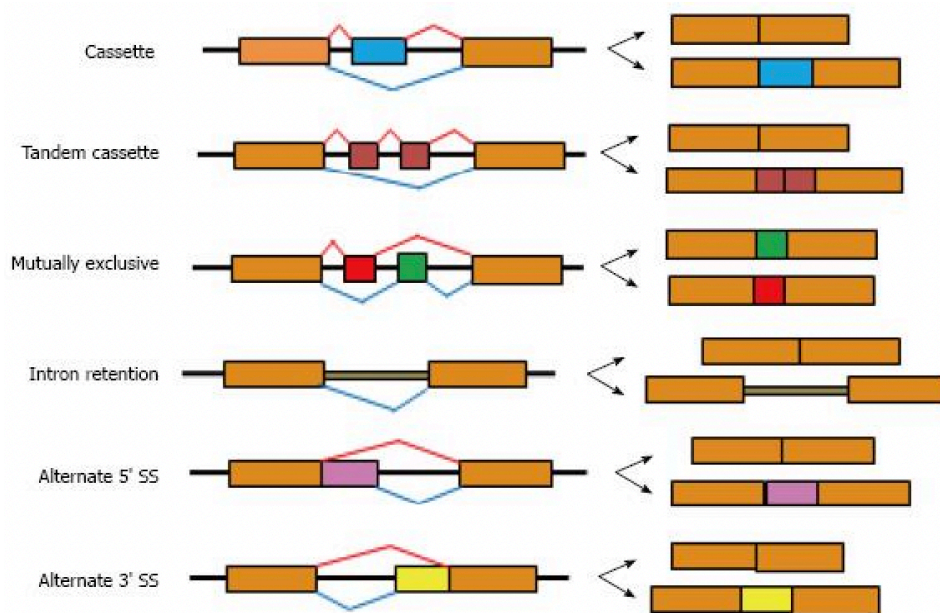


Figure 3. Major alternative splicing events in the metazoan transcriptome. Major types of alternative splicing

events are shown. Brown boxes indicate constitutive exons, while boxes in other colors indicate alternative spliced exons [152].

3.2 The role of alternative splicing in cancer progression

AS is frequently dysregulated in various human diseases, particularly in cancer [153]. High-throughput sequencing technologies have revealed aberrant splicing patterns in tumor tissues compared to their normal counterparts across multiple cancer types [154], [155] and increasing evidence indicates that splicing alterations actively contribute to tumorigenesis, cancer progression, and resistance to therapy [156], [157].

Given these observations, the identification of cancer-specific splicing isoforms is of critical importance for the development of novel diagnostic and prognostic biomarkers ; understanding the mechanisms underlying splicing dysregulation is necessary to shed light on its role in cancer biology but also to offer potential avenues for innovative therapeutic strategies [158].

Cancer progression is primarily driven by the accumulation of genetic alterations and widespread genomic dysregulation. During tumor development, cancer cells progressively acquire key characteristics, including continuous growth signals, evasion of programmed cell death (apoptosis), unlimited replication, angiogenesis , and the ability to metastasize [159].

These malignant features can be further modulated by alterations in AS, which can lead to the production of protein isoforms with altered or even pathological functions [160].

Given the importance of AS, a number of recent studies focused on the role of aberrant splicing in CRC. Aberrant splicing events in CRC were identified using either microarray or RNA-seq techniques in the past [2], [3] with focuses on different aspects.

For instance, by modulating alternative splicing, SRSF6 plays a key role in driving colorectal cancer progression or ,as a histone methyltransferase, SETD2 regulates alternative splicing through epigenetic regulation of RNA processing to inhibit intestinal tumorigenesis. In addition, HNRNPLL has been reported to be a novel metastasis suppressor of CRC influencing CD44 alternative splicing process. PRPF6, a member of the trisnRNP (small ribonucleoprotein) spliceosome complex, induces CRC proliferation through preferential splicing of multiple genes related with growth regulation [98].

Other studies have further highlighted the role of alternative splicing in the regulation of genes related to energy metabolism [161], impacts on protein function [162] and neoantigens [163].

Among the principal contributors to aberrant splicing profiles in cancer, alterations in the expression levels of splicing factors represent a key driver although the molecular mechanisms underlying this expression changes remain incompletely understood.

Although recurrent somatic mutations in genes encoding splicing regulators have been found in several solid tumors [164], growing evidence suggests that oncogenic signaling pathways play a key role in controlling the expression of splicing factors [165].

Indeed, constitutive activation of oncogenic pathways is a hallmark of cancer. In colorectal cancer, for example, oncogenic mutations in Kirsten Rat Sarcoma viral oncogene (KRAS) lead

to activation of the RAS–MAPK signaling cascade. This, in turn, enhances the expression of the splicing factor polypyrimidine tract-binding protein 1 (PTBP1) via transcriptional regulation by ELK1. Elevated PTBP1 levels subsequently drive alternative splicing changes in several tumor-associated transcripts, including the small GTPase RAC1, the adaptor protein NUMB, and the pyruvate kinase isoform PKM [166].

Although significant progress has been made in understanding abnormal alternative splicing in colorectal cancer, the full landscape of splicing events involved in its development and progression remains unclear [98].

3.2.1 AS in CRC progression: Rac1B

Rac1 is a member of the Rho family of small GTPases, which cycle between an inactive GDP-bound and an active GTP-bound state. Once in the active state, Rac1 can interact with downstream effector proteins and control signaling pathways that regulate the actin cytoskeleton and gene transcription.

When in the active GTP-bound conformation, Rac1 interacts with downstream effector proteins and activates signaling cascades.

In tumors, Rac1 activation is known to become deregulated [167], and one recently unraveled mechanism is through increased expression of alternative spliced Rac1b in colorectal or breast tumor samples [168], [169].

It is reported that the mammalian *RAC1* gene contains seven coding exons, which give rise to two alternative transcripts, RAC1 and RAC1b (Figure 4). Typically, exon 3b is skipped, and RAC1 is the predominant transcript produced. However, exon 3b-containing variant RAC1b has been found overexpressed in a subset of colorectal tumors and this promoted tumor cell survival.

RAC1b differs in 57 nucleotides resulting from the in-frame inclusion of alternative exon 3b [170]. The resulting Rac1b protein adopts predominantly the active GTP-bound conformation in cells. This reflects altered regulatory properties because of an increased intrinsic nucleotide exchange activity, a decreased rate of GTP hydrolysis and a failure to interact with Rho-GDI, a major downregulating factor *in vivo* [171], [172].

Furthermore, the variant Rac1b displays unique signaling properties. It does not stimulate several classical Rac signaling pathways, including lamellipodia formation or the activation of protein kinases PAK1 or JNK. However, Rac1b retains the ability to stimulate the NF-κB pathway [173], [174], which initiates an anti-apoptotic transcriptional response and also promotes cell cycle progression by an increase in cyclin D1 expression [175],[176].

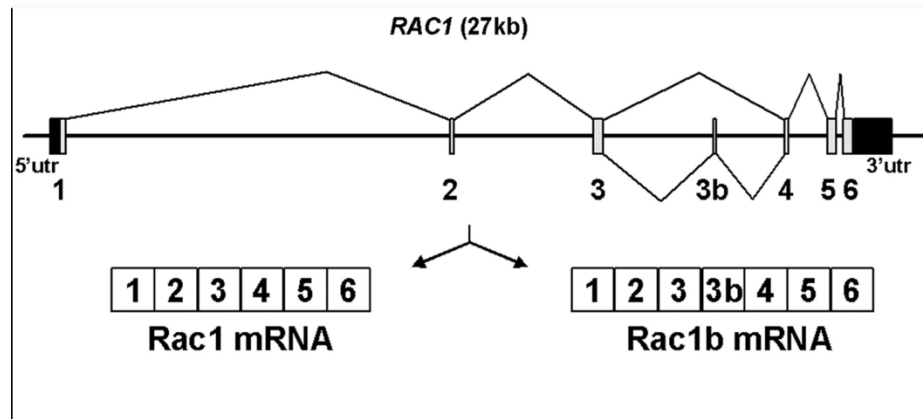


Figure 4. Alternative splicing of the small GTPase Rac1 in colorectal cells. Schematic representation of the human *RAC1* gene with its seven coding exons, including the alternative exon 3b, and of the two alternative transcripts *RAC1* and *RAC1b* [176].

Interestingly, in colorectal cancer cells, inhibition of the PI3K/AKT signaling pathway has been shown to upregulate endogenous levels of the splicing factor SRSF1, thereby promoting the inclusion of the alternative exon 3b in the *RAC1* transcript giving rise to **RAC1B, the pro-tumorigenic splice variant of the small GTPase RAC1** [176]. Subsequent studies identified SRPK1 and GSK3 β as upstream regulators essential for sustaining *RAC1B* splicing in CRC cells [177]. In particular, GSK3 β appears to modulate *RAC1B* expression indirectly by regulating SRPK1 activity, which in turn phosphorylates SRSF1. GSK3 β depletion leads to reduced SRPK1 activity, diminished nuclear accumulation of SRSF1, and consequently, decreased production of *RAC1B*.

Another pivotal oncogenic pathway frequently activated in colorectal cancer is the Wnt signaling cascade. Remarkably, this pathway also influences *RAC1B* alternative splicing. It was demonstrated that the gene encoding the splicing factor SRSF3 (also known as SRp20) is a direct transcriptional target of the β -catenin/TCF4 complex, resulting in elevated SRSF3 expression [178]. In a follow-up study, this increase in SRSF3 levels was found to suppress *RAC1B* formation by promoting the exclusion of exon 3b from the *RAC1* transcript.

Together, these findings highlight the intricate crosstalk between oncogenic signaling pathways and the splicing machinery, underscoring how signaling-mediated modulation of alternative splicing contributes to tumor-associated transcriptomic reprogramming [158].

3.3 LncRNAs as key modulators of alternative splicing

Some studies have found that ncRNAs have lots of unique and interesting functions in the process of AS [179], especially lncRNAs.

A unique function of lncRNAs is that they interact with various molecules and can form a variety of RNA–RNA, RNA–DNA, or RNA–protein complexes through specific RNA functional domains. For this reason, lncRNAs, as important regulatory molecules, play a crucial biological role in the occurrence and development of cancer through various pathways.

AS of human genes is a widespread process, and the generation of lncRNAs is closely linked

to this mechanism. Many lncRNAs arise as splicing products, particularly in the context of tumor development, where their abnormal expression can directly impact downstream pathways and exert specific biological functions. Furthermore, lncRNAs may also participate in the abnormal AS process of certain cancer-related genes through the regulation of precursor messenger RNA (pre-mRNA) and through the association with splicing factors [180].

There are different strategies through which lncRNAs regulate AS:

- lncRNAs can interact with target pre-mRNAs to regulate AS : the formation of RNA–RNA duplexes is one of the functions performed by lncRNAs in AS [181]. A major apoptosis pathway is triggered by the Fas receptor-Fas ligand (FasL) interaction. A lncRNA located in the nucleus, Fas antisense or Saf, interacts with the Fas pre-mRNA and splicing factor human splicing factor 45 (SPF45) to promote the cleavage of exon 6 [182]. The product is soluble Fas that can protect cells from Fas-FasL-induced apoptosis by binding to FasL.
- lncRNAs regulate AS through the encoded peptide : although lncRNAs belong to a class of RNA molecules that don't have coding ability, with the development of bioinformatics and high-volume measurement, small open-reading frames in lncRNAs have been gradually discovered, which can play a biological function by encoding small peptides. Many studies have shown that small peptides encoded by lncRNAs also play an important role in the development of cancer. A previous study identified a small peptide encoded by the lncRNA HOXB-AS3, which was named the 53-amino acid (aa) peptide, and showed that patients with CRC with low levels of 53-aa peptide have a poor prognosis. The 53-amino acid peptide binds competitively to the arginine residue within the RGG motif of the splicing factor hnRNPA1. This interaction prevents hnRNPA1 from binding to PKM pre-mRNA, thereby blocking the exclusion of exon 9. As a result, the production of PKM2—an isoform of pyruvate kinase involved in glycolysis—is reduced, leading to impaired glucose metabolism reprogramming and ultimately inhibiting CRC growth [183].
- lncRNAs regulate AS by interacting with splicing factors : splicing factors play a central role in the regulation of AS by interacting with the pre-RNAs and lncRNAs often play the role of cooperating with or hijacking splicing factors to regulate AS. MALAT1 is a well-known lncRNA associated with various tumors. It localizes to nuclear speckles and has been shown to interact with several SR proteins, including SRSF1, SRSF2, SRSF3, and SRSF5. Through these interactions, MALAT1 regulates the phosphorylation status of SR proteins, thereby influencing their distribution between transcription sites and nuclear speckles, and ultimately modulating their transport and function. [184]. Previous studies have shown that MALAT1 can upregulate SRSF1-mediated series of cancer-promoting splicing events [185]. Another study showed that mutant p53 and ID4 proteins can form complexes with MALAT1 and SRSF1, regulating the AS of VEGFA pre-mRNA. In this way the anti-angiogenic VEGFA isoform is inhibited and angiogenetic process is activated [186]. MALAT1 also hijacks SFPQ from

the splicing factor complex SFPQ/PTBP2 [187].

Deregulation of certain lncRNAs is associated with AS in CRC [7].

3.4 Regulatory functions of RNA-Binding Proteins in alternative splicing

AS is tightly controlled by a complex network of up to a thousand trans-acting splicing factors. These proteins typically interact with pre-mRNAs within the nucleus by recognizing short sequence motifs, thereby orchestrating the regulation of extensive and interconnected splicing networks. Notably, many splicing factors exhibit tissue-, condition-, or disease-specific expression patterns, enabling dynamic and programmable modulation of AS programs [188].

RNA-binding proteins (RBPs) interact with RNA to form ribonucleoprotein complexes, thereby influencing the maturation and fate of their target RNA substrates. These interactions regulate key steps of gene expression, including pre-mRNA splicing and polyadenylation, RNA stability, localization, editing, and translation. A wide range of RBPs are involved in both physiological and pathological processes. Their proper function is essential for human health, as alterations in RBP activity have been implicated in various genetic and acquired disorders, including neurodegenerative diseases, autoimmune conditions. By binding to specific sequences within exons or their flanking intronic regions, RBPs can modulate alternative splicing events, promoting exon inclusion or skipping, as well as the alternative usage of 5' or 3' splice sites.

Since the early 21st century, increasing evidence has highlighted the crucial role of RBPs in the pathogenesis of numerous diseases, including various types of cancer. The advent of high-throughput sequencing technologies has enabled genome-wide investigations of AS and RBPs, significantly advancing our understanding of their functions and regulatory networks.

Notable progress has been made in elucidating the role of AS-related RBPs, which can be summarized in three key aspects:

- RBPs regulate alternative splicing events (ASEs) by **specifically recognizing nucleotide sequences or unique secondary and tertiary RNA structures**. These interactions influence spliceosome assembly and its engagement with pre-mRNA, ultimately modulating splicing outcomes;
- RBPs are centrally involved in the **development and progression of a broad spectrum of diseases**, both oncological and non-oncological, primarily through their contribution to aberrant ASEs and the resulting dysregulation of downstream signaling pathways;
- The regulatory functions of AS and associated RBPs are governed by **complex molecular mechanisms**. These include RNA methylation events mediated by RBPs, and disease-associated mutations that alter splicing accuracy.

Many RBPs have well-defined RNA-binding domains (RBDs) that enable them to interact with

RNA in a sequence- and/or structure-specific manner. The human genome encodes over 1,500 RBPs with characterized RBDs. Among these, two major classes are distinguished based on their opposing roles in exon recognition: serine/arginine-rich splicing factors (SRSFs) and heterogeneous nuclear ribonucleoproteins (HNRNPs). ESEs and ISEs primarily recruit SRSFs, which function as splicing activators, whereas ESSs and ISSs are typically bound by HNRNPs, which act as splicing repressors. Notably, SRSFs and HNRNPs often function in a competitive manner during alternative splice site and exon selection.

Structurally, AS-related RBPs frequently contain conserved RBDs, such as the RNA recognition motif (RRM), K homology (KH) domain, double-stranded RNA-binding domain (dsRBD), cold shock domain (CSD), arginine–glycine–glycine (RGG) box, tyrosine-rich motifs, and several zinc finger (ZnF) domains [189] [190].

3.4.1 RBFOX2-Mediated Alternative Splicing Events in Colorectal Tumorigenesis

One of the most studied AS factors is RBFOX2, the master regulator of tissue-specific AS, implicated in the development of ovarian and breast cancer as well as in EMT [191]. The data in the literature attributed to RBFOX2 a role as an inductor of oncogenic splice-switching that drives an invasive phenotype in cancer.

The RBFOX family also includes RBFOX1 and RBFOX3 [192]. All RBFOX proteins contain a central RNA recognition motif that recognizes a consensus sequence, (U)GCAUG, usually found in the introns that flank target exons. RBFOX proteins usually promote exon skipping when they bind upstream of the alternative exon, but inclusion when they bind downstream of this exon.

RBFOX1 is expressed in the brain, heart, and muscles and RBFOX3 is expressed exclusively in the brain. RBFOX2 is more widely expressed, especially in muscle and mesenchyme/mesoderm. EMT-associated splicing is only partially regulated by RBFOX2 and requires the expression of a large number of RBPs, including ESRPs, CELF, MBNL, hnRNPs, SAM68, and SRSF1. Nevertheless, cell invasion is significantly reduced in RBFOX2-depleted tumor cells, suggesting that this protein enables epithelial cells to gain invasive properties during EMT [193].

The role of RBFOX2 in regulating EMT appears to be linked to its role in cancer development. Over the last few years, it has become clear that this factor recognizes distinct sets of alternatively spliced RNAs in various tissues, and not all of the identified splicing targets of RBFOX2 contain its RNA-binding motif. Indeed, several studies have identified RBFOX2-dependent alternative exons that are not located near a canonical (U)GCAUG motif, as well as the presence of the motif not resulting in the expected splicing outcome.

For example, in several documented cases, the presence of the binding site upstream of the exon results in the inclusion of the exon rather than its exclusion. The origin of this behavior is unclear, but it has been suggested that it may be explained in part by the large number of RBFOX2-interacting proteins that can act as recruiters, chaperones, or co-factors, capable of

modifying the AS activity of RBFOX2. Indeed, it was shown that RBFOX2 is part of a ‘large assembly of splicing regulators’ (LASR), a multimeric complex containing different proteins (Figure 5): hnRNPM, hnRNPH, hnRNPC, Matrin3, NF110/NFAR-2, NF45, and DDX5 [194]. In particular, a study conducted by Zhou et al. indicates that RBFOX2 is recruited to its targeted RNA using different binding modes and protein partners, permitting the regulation of a broad range of splice sites exhibiting different sequence motifs and functional outcomes. Overall, the contribution of these proteins to the selection of RBFOX2 splicing targets and a full characterization of the functional complex responsible for RBFOX2 RNA selectivity are still lacking [188].

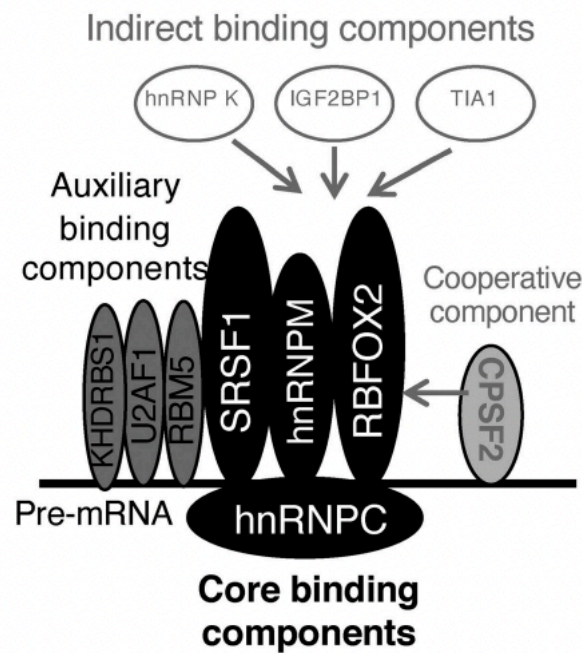


Figure 5. Schematic illustration of RBFOX2 partner proteins and their contribution to RNA binding. Black, gray, light grey and white ellipses indicate major recruiters of RBFOX2, minor recruiters of RBFOX2, cooperative factors that stabilize RBFOX2-RNA interaction by binding to adjacent sequence and factors that do not directly bind to RBFOX2-targeted RNA sequences, respectively [188].

3.5 The Functional Crosstalk Between lncRNAs and RNA-Binding Proteins in alternative splicing regulation

RBPs interact with various classes of RNA molecules, including lncRNAs, through specific structural motifs and binding domains. These interactions play a pivotal role in numerous biological processes and are increasingly recognized as key regulators of tumorigenesis [195].

The reciprocal nature of RBP and lncRNA interactions influences lncRNA biogenesis, post-transcriptional modifications, stability, and functional activity [196]. Recent advancements in RNA–protein interaction mapping techniques, such as cross-linking immunoprecipitation (CLIP) and RNA immunoprecipitation (RIP), have significantly enhanced our ability to investigate these complex regulatory networks.

In the context of cancer, several studies have identified numerous dysregulated lncRNA–protein interactions (LPIs), involving RBPs and their target lncRNAs, which are implicated in tumor initiation and progression [197], [198]. *RBPs modulate lncRNA function through a variety of mechanisms*. For instance, human antigen R (HuR) and serine/arginine-rich splicing factor 1 (SRSF1) **stabilize specific lncRNAs by preventing their degradation**, either by blocking decay signals or shielding them from exonucleases. Conversely, some RBPs have been shown to **promote lncRNA degradation**. Additionally, RBPs can **facilitate the nuclear export of lncRNAs** to the cytoplasm, thereby contributing to their spatial regulation [196].

Moreover, numerous lncRNAs have been shown to interact with a wide range of RBPs, including DNA methyltransferases, transcription factors, and splicing regulators. A notable example is the lncRNA HOTAIR, transcribed from the HOX gene cluster, which recruits the Polycomb Repressive Complex 2 (PRC2) to specific genomic loci, thereby **facilitating epigenetic silencing of target genes** and promoting cancer metastasis [195].

LPIs mediate a broad spectrum of regulatory mechanisms that can influence both lncRNAs and RBPs. These include: (a) modulation of RNA or protein stability, (b) regulation via N6-methyladenosine (m6A) modifications, (c) control of AS events, and (d) determination of subcellular localization of either lncRNAs or RBPs. These mechanisms are inherently complex and highly dynamic. Although our understanding of these multifaceted interactions is expanding, further research is essential to elucidate the underlying mechanisms and their implications in both physiological and pathological conditions [199].

A) Stability of lncRNAs and/or RBPs:

The stability of lncRNAs and RBPs is a key determinant of their functional capacity, directly influencing the efficiency of the regulatory processes in which they are involved. A growing body of evidence indicates a strong correlation between the functional roles of ncRNAs and their molecular stability [200]. Transcript stability is modulated by several factors, including RNA length, secondary structure, subcellular localization, and interactions with RBPs.

One of the earliest lncRNAs implicated in CRC is Overexpressed in Colon Carcinoma-1 (OCC-1), which has been characterized as a tumor suppressor [201]. OCC-1 harbors multiple HuR-binding motifs within its 3' UTR [202], facilitating the recruitment of HuR to the E3 ubiquitin ligase Transducin Repeat-containing Protein 1 (TrCP1). This interaction promotes TrCP1-mediated ubiquitination and subsequent degradation of HuR, leading to the downregulation of HuR target mRNAs involved in tumorigenesis (Figure 6).

RBPs can also contribute to the stabilization or destabilization of lncRNAs. For instance, in glioma cells, the oncogenic splicing factor SRSF1 binds to the lncRNA Nuclear Enriched Abundant Transcript 1 (NEAT1), enhancing its stability. Silencing of SRSF1 accelerates NEAT1 degradation upon transcriptional arrest with actinomycin D, demonstrating a stabilizing effect. The positive feedback between SRSF1 and NEAT1 was shown to promote glioma progression through cell cycle regulation [203].

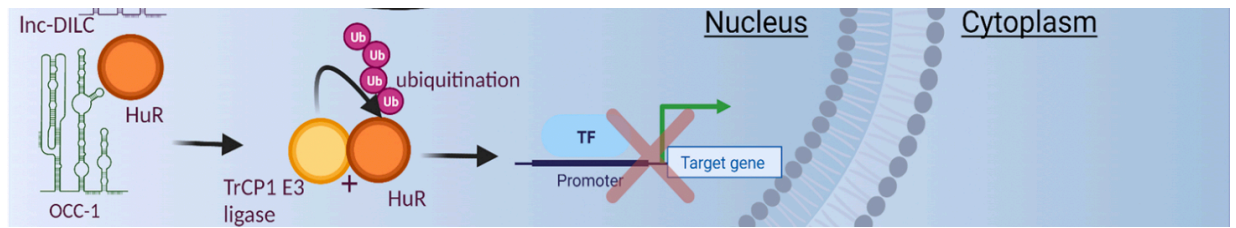


Figure 6. Schematics presentation of LPIs in cancer. OCC-1 directly binds to HuR, stabilizing it and enriching its association with TrCP1 ubiquitin E3 ligase-, leading to increased HuR ubiquitination and degradation, reducing transcription of HuR gene targets [199].

C) AS Regulation by lncRNAs:

Emerging evidence highlights the crucial role of LPI in the regulation of AS. A comprehensive study by Yang et al. functionally annotated the involvement of lncRNAs in AS regulation, identifying 113 lncRNAs that modulate 46 exon skipping events through interactions with 70 distinct RBPs [204].

Among these, Heterogeneous Nuclear Ribonucleoprotein M (hnRNPM), a member of the hnRNP family involved in pre-mRNA processing and mRNA transport, plays a pivotal role. hnRNPM contains three RRM, and mapping analyses have identified nucleotides 206–279 as critical for its interaction with the lncRNA PLANE [205]. Both hnRNPM and PLANE co-localize with the SRSF, a marker of nuclear speckles subnuclear domains that work as sites of splicing factor storage and modification, as well as spliceosome assembly (Figure 8, upper panel) [206].

Another illustrative example involves the interaction between the lncRNA Tropomyosin 1 Antisense RNA (TPM1-AS) and the RBP RNA-binding motif protein 4 (RBM4) in human esophageal cancer cells. This interaction disrupts the binding of RBM4 to TPM1 pre-mRNA, preventing the inclusion of exon 2a. The resulting TPM1 isoform, lacking exon 2a, has been shown to inhibit cell migration and filopodium formation, underscoring TPM1-AS as a potential therapeutic target (Figure 7), [207].

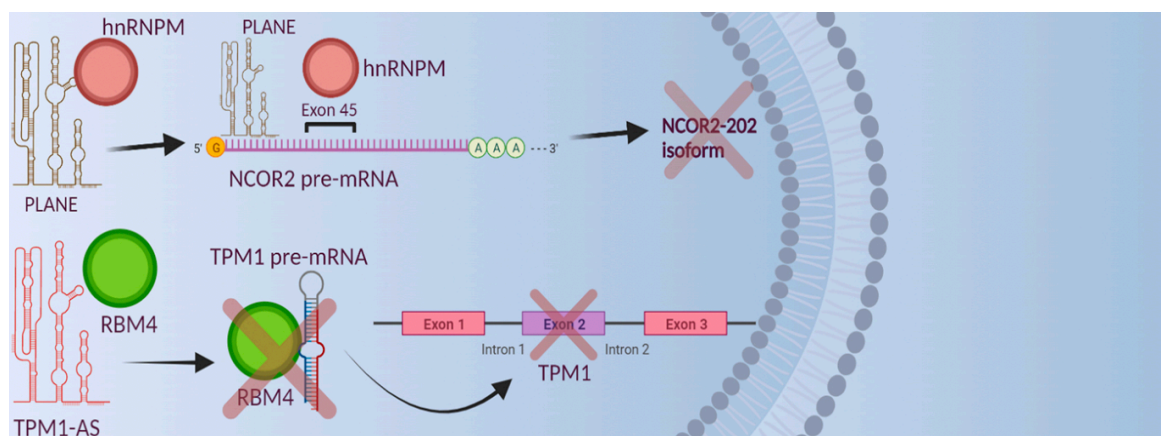


Figure 7. C) LPIs affecting lncRNA metabolism: alternative splicing. PLANE facilitates the binding of hnRNPM with NCOR2 pre-mRNA at intron 45. This interaction is necessary for decreasing hnRNPM-mediated regulation of NCOR2–202 pre-mRNA alternative splicing, as this isoform has tumor suppressor properties (upper panel). Direct binding of TPM1-AS and RBM4 interrupts the binding of RBM4 to TPM1 pre-mRNA, which inhibits the inclusion of endogenous exon 2a in TPM1 (lower panel) [199].

Numerous studies in the literature underscore the critical role of interactions between lncRNAs and RBPs in the regulation of alternative splicing of pro-tumorigenic genes.

CHAPTER 4

OBJECTIVES

As described in the introduction, the aim of this PhD thesis is to understand the molecular mechanisms through which the lncH19, takes part to cancer onset and progression.

Numerous studies have demonstrated that ncRNAs are abnormally expressed in tumors and play a role in important biological processes like cell proliferation, differentiation, and tumor progression. Alterations in AS are now recognized as major contributors to CRC progression, metastasis, and therapy resistance. Among AS regulators, lncRNAs have attracted growing interest due to their ability to interact with RBPs and guide them to specific transcript targets. In particular, my research focused on understanding how the interaction between lncH19 and specific RBPs could influence AS events, thus affecting tumor progression.

Transcriptional analyses from RNA Antisense Precipitation (RAP) showed that lncH19 binds a conspicuous number of mRNAs both in intron and exon sequences. This led me to hypothesize about the direct involvement of the lnc in mRNA maturation and AS.

Since several non-coding RNAs affect the AS process influencing the recruitment of SFs, I investigated the binding between lncH19 and SFs involved in AS with pro-tumoral effects.

My study focused on RBFOX2, since it is among the most studied alternative splicing factors implicated in the development of EMT, cancer progression, and metastatic process.

lncH19-RAP in two different CRC cell lines, SW620 and HCT116, confirmed lncH19 interaction with RBFOX2 and with RBPs that are likely to be recruited by RBFOX2 to splicing sites, hnRNPM and SRSF1.

These data allowed me to hypothesize the existence of a molecular platform that includes lncH19 and SFs. To identify putative targets of this complex, I intersected the list of mRNAs bound by lncH19, as identified by the RAP analysis, with the RBP interactome for hnRNPM and RBFOX2 obtained from eCLIP profiling. The analyses identified 57 transcripts associated with lncH19 and containing binding sites for the investigated SFs.

My investigation focused on RAC1, as it has been widely observed that its AS leads to RAC1B, a constitutive-active Ran GTPases which has been found to stimulate cancer cell proliferation, enhance EMT, and induce drug resistance.

Splice-sensitive PCR showed that lncH19 binds the longer isoform RAC1B, thus enforcing the hypothesis that the lncRNA is involved in AS, probably cooperated by RBFOX2.

To demonstrate the correlation between RBFOX2 and RAC1B expression I transiently silenced two CRC cell lines for RBFOX2. Transcriptional and protein data indicated that RBFOX2

silencing negatively affects RAC1B expression at transcriptional and protein levels, thus confirming, also in CRC, this correlation.

Moreover by gain and loss of function experiments I demonstrated that lncH19 is required for the induction of RAC1 AS with effect on the expression of the constitutively active form. Furthermore I investigated if lncH19 could exert its oncogenic activity also through RAC1B-induced oncogenes' activation. To this aim, I treated lncH19 over-expressing cells with EHT-1864, the inhibitor of Rac-GTPase, able to inhibit also RAC1B activity. Transcriptional data confirmed that lncH19 over-expression promotes the expression of c-Myc and Cyclin D in both cell lines however, treatment with the RAC1B inhibitor leads to complete rescue. Overall, these data revealed a new axis, in the control of CRC gene expression passing through lncH19, RAC1B, and the Wnt target genes c-Myc and Cyclin D.

To further investigate the molecular mechanism endorsing this process I hypothesized that lncH19 might have the role of bringing the splicing complex directly to RAC1 mRNA. RIP analyses showed that the binding of RBFOX2 to RAC1 and RAC1B is strongly reduced in cells silenced for the lncH19 confirming this hypothesis.

To validate this axis in vivo I investigated gene expression in biopsies from 20 patients with CRC. Real-time PCR confirmed that CRC tissues present higher levels of lncH19 compared to respective marginal non-tumor tissue. By selecting the CRC samples with higher levels of lncH19 compared to their health counterpart ($n = 14$) Pearson Correlation analyses revealed a positive correlation between lncH19 and RAC1B. In addition, the same samples presented higher levels of both oncogenes Cyclin D and c-Myc.

The results obtained and included between paragraph 6.1 and 6.4 are published in the Journal of Experimental and Clinical Cancer Research with the title: ***“Regulatory role of lncH19 in RAC1 alternative splicing: implication for RAC1B expression in colorectal cancer”*** [7].

CHAPTER 5

MATERIAL AND METHODS

5.1 Cell culture

Human SW620 and HCT116 colorectal cancer cells (ATCC–LGC Standards S.r.L., Italy) were cultured respectively in RPMI and McCoy's 5 A medium (Euroclone, United Kingdom) supplemented with 10% fetal bovine serum (FBS, Euroclone, United Kingdom), 1% penicillin/streptomycin (10.000 U/mL penicillin and 10 mg/mL streptomycin), and 200 mM L-glutamine (all from Euroclone). Cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C and used at early passages for all the experiments. The culture medium was changed every 2–3 days, and cells were split at 70–80% of confluence. Depending on the experimental setup, treatment with the RAC1 inhibitor EHT1864 (cat n° SC-361175, Santa Cruz Biotechnology), was done as follows: 24 h after seeding in 12wells multiwell plates, cells were treated with 50mM of EHT1864 for 18 h; then the cells were processed for the following experiments.

5.2 Patient's tissue samples

Twenty paired tissues (CRC and adjacent non-tumor) were collected from patients who were diagnosed with CRC at Giglio Hospital (Cefalù, Palermo, Italy). All patients underwent no preoperative therapy before surgical resection and have signed informed consent. The use of the collected samples was approved by the Institutional Ethics Committee of Palermo 1/Fondazione Giglio Cefalù prot 246/2020. The specimens collected from the patients were preserved in RNeasy Lysis Buffer (cat.n°. AM7021 Thermo Fisher® Scientific, United States) for subsequent RNA extraction and gene expression analysis.

5.3 Cell transfection to overexpress lncH19

SW620 and HCT116 cells were seeded respectively at 3×10^4 or 2.5×10^4 per cm^2 . After 24 h, cells were transfected with 1.2 $\mu\text{g}/\text{ml}$ of H19 pFLAG-CMV-2 expression vector (cat. n° E7033, Sigma-Aldrich, USA), or empty pFLAG-CMV-2 as Negative Control (Sigma-Aldrich). For cell transfection, HiPerFect Transfection Reagent (cat. n° 301704, Qiagen, Germany) was used following the manufacturer's standard instructions. Eighteen hours after transfection, the cells were processed for the following experiments.

5.4 Infection with lentiviral vectors to stably silence lncH19

SW620 and HCT116 cells were stably silenced for lncH19 by lentiviral infection with H19 human shRNA lentiviral particles (cat. n° TL318197V, OriGene Technologies, Inc., United States), relative control cells were infected with control shRNA lentiviral particles (cat. n° TR30021V, OriGene Technologies, Inc., United States). The infected cells were selected by cell sorting (BD FACS Aria™ III Sorter, ATeN Center, Italy) and maintained under selective pressure with 1 mg/mL of puromycin (Gibco™ puromycin dihydrochloride, cat. n° A1113802, Thermo Fisher® Scientific). QRT PCR and fluorescence microscopy regularly tested H19 silencing efficiency.

5.5 Cell transfection to silence RBFOX2

SW620 and HCT116 cells were seeded respectively at 3×10^4 or 2.5×10^4 per cm^2 . After 24 h, cells were transfected with two different silencer® Pre-designed siRNA for RBFOX2 (siRNA ID respectively 136602 and 136602 from Life Technologies), or siRNA scramble (scr) as Negative Control. For cell transfection, HiPerFect Transfection Reagent (cat. n° 301704, Qiagen, Germany) was used following the manufacturer's standard instructions. Eighteen hours after transfection, the cells were processed for the following experiments.

5.6 RNA extraction and real-time PCR (qRT-PCR)

Total RNA was isolated from the cells by using a commercially available miRNA purification Kit (NucleoSpin™ miRNA kit, cat. n° 740971.250, Macherey–Nagel, Germany), according to the manufacturer's instructions. The total RNA concentration was detected with the Nano-drop spectrophotometer (Thermo Fisher®) and reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (cat. n° 4368814, Applied Bio-system™, United States). Quantitative real-time polymerase chain reactions (qRT-PCR) were performed by using the SYBR™ Green PCR Master Mix (cat. n° 4309155, Applied Biosystems™), following the manufacturer's instructions in a Step One™ Real-time PCR System Thermal Cycling Block (Applied Biosystems™). The primers' sequences used for gene expression analysis are reported in Table 1. The relative expression of mRNAs was analyzed using the $2^{-\Delta\Delta C_t}$ method with β -actin or 28 S serving as internal reference genes.

Gene	Primer Forward (5'-3')	Primer Reverse (5'-3')
H19	TCGTGCAGACAGGGCGACATC	CCAGCTGCCACGTCCTGTAACC
β-ACTIN	CAAGAGATGGCCACGGCTGCT	TCCTTCTGCATCCTGTGCGCA
28 S	CCGTGCCTTGGAAGCGTCGC	CAGAGGCTGTTACCTTGGAGA
RAC1	AAACCGGTGAATCTGGGCTT	AAGAACACATCTGTTTGCGBA
RAC1B	AAACCGGTGAATCTGGGCTT	ATCGGCAATCGGCTTGCTT
CYCLIN D1	AAAGAATTTGCACCCCGCTG	GACAGACAAAGCGTCCCTCA
cMYC	TACAACACCCGAGCAAGGAC	CTAACGTTGAGGGGCATCGT
rbFOX2	CCAGCTTTCAAGCAGATGTGCC	CAAATGGGCTCCTCTGAAAGCG

Table 1. Sequence of the primers used for gene expression analysis

5.7 RNA antisense precipitation (RAP) to investigate lncH19-protein interaction

SW620 or HCT116 cells were harvested and lysed in a specific polysome lysis buffer (10 mM Tris-HCl, 20 mM KCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40, 5 mM DTT, 5% Glycerol, 40 U/ml RNase inhibitor) on ice for 1.30 h. A cross-linking step was not included to avoid the formation of non-specific interactions. Cell debris was removed by centrifugation at 15.000 x g for 10 min at 4°C and the supernatant, containing the protein lysate, was quantified through the Bradford micro-assay method (cat. n° 1856210 ThermoFisher) using bovine serum albumin (BSA, Sigma-Aldrich) as a standard. For each assay, a total of 500 µg of proteins, were incubated with 100pmol of biotin-labeled H19 probes or control probes (designed from Sigma-Aldrich) to pull down lncH19-protein complexes, for 30 min at room temperature. Streptavidin-labeled magnetic beads were then added to the samples and incubated for 30 min at room temperature to precipitate lncH19-proteins-probe-coated beads. After washing the beads with TENT Buffer (20 mM Tris-HCl pH8.0, 2 mM EDTA pH 8.0, 500 mM NaCl, 1% Triton X-100) the lncH19-protein complex was eluted by incubating the beads with elution buffer (0.5 M NH₄OH, 0.5 mM EDTA) 30 min at 4 °C. The protein complexes bound to the beads were denatured by heating at 70 °C for 10 min and analyzed by Immunoblotting (See Table 2).

Oligo name	Sequences (5'-3')
comH19 var 1-1	GTGCAGCATATTCATTCCA
comH19 var 1-2	GTTTTGTGTCCGGATTCAAAGG
comH19 var 1-3	TGCCCCCTGTGCCTGCTACTAAAT
comH19 var 1-4	TCCTGCCAGACTCCAGATGT
comH19 var 1-5	CTTCCCCAGTTTCCCCGTTACC
comH19 var 1-scr1	GAAAGAGATGCTGACCTATT
comH19 var 1-scr2	GCGATCAACTCCAACGACTAAT
comH19 var 1-scr3	GTGGCATCTACAGCGAAAGAGGT
comH19 var 1-scr4	GAGGCTATGGAGCGACCTAT
comH19 var 1-scr5	GATGGACGAGACGAGGGAGGAGT

Table 2. Sequence of the biotin-labeled H19 probes used for RNA pull-down assays

5.8 RAP to investigate IncH19-RNA interaction

The cells were harvested and lysed in a specific polysome lysis buffer (50 mM Tris-HCl pH 7.0, 10 mM EDTA, 1% SDS, 200U/ml RNase inhibitor, and protease inhibitor cocktail) on ice for 1.30 h. Cell debris was removed by centrifugation at 13.000 x g for 20 min at 4 °C and the supernatant, containing the protein lysate, was quantified as described before. For each reaction 500 µg of total proteins was resuspended in the hybridization buffer (50 mM Tris-HCl pH 7.0, 1 mM EDTA, 1% SDS, 750 mM NaCl, 15% Formamide added extemporaneously) and incubated with 100 picomoles of biotin-labeled H19 probes or control probes (designed from Sigma-Aldrich) 4 h under moderate agitation on a tube rotator at room temperature. After three washes with a specific washing buffer (0.5% SDS, 2X SSC) the samples were incubated with 50 µl streptavidin-magnetic beads overnight on a tube rotator at room temperature. After three washes with the washing buffer, the samples were incubated with proteinase-K (20 mg/ml, cat. n° 1409006, Invitrogen) for 45 min at 50 °C and 10 min at 95 °C. Finally, the RNA complexes bound to the beads were purified using a commercially available miRNA purification Kit (NucleoSpin™ miRNA kit, cat. n° 740971.250, Macherey–Nagel), following the manufacturer's instructions and analyzed through PCR and qRT-PCR.

5.9 RNA immunoprecipitation (RIP)

The cells were processed and lysed by adding a specific polysome lysis buffer (25 mM Tris-HCl pH 7.4, 100 mM KCl, 0.5% Nonidet P-40, 5 mM DTT, 5% 40 U/ml RNase inhibitor and protease inhibitor cocktail) for 30 min on ice. The magnetic Protein G-beads were pre-incubated with 2 µg of antibody against RBFOX2 or hnRNPM (respectively cat. n° sc-271407, sc-20002 both from Santa Cruz Biotechnology) or specific control mouse IgG (cat.n° 62-6520 EMD Millipore Corp., USA) at room temperature. After 30 min the corresponding cell lysates were added, and the samples were incubated overnight at 4 °C. The samples were subjected subsequently to RNA purification and protein extraction. The RIP efficiency was verified by Western blot assay and the enrichment of specific mRNAs was verified by qRT-PCR assay.

5.10 RNA sequencing

Next-generation sequencing experiments were performed by Genomix4life S.R.L. (Baronissi, Salerno, Italy). Indexed libraries were prepared with TruSeq Stranded totalRNA gold Sample Prep Kit (Illumina) according to the manufacturer's instructions. Libraries were quantified using the TapeStation 4200 (Agilent Technologies) and Qubit fluorometer (Invitrogen Co.), then pooled such that each index-tagged sample was present in equimolar amounts, with a final concentration of the pooled samples of 2 nM. The pooled samples were subject to cluster generation and sequencing using an Illumina NextSeq550 System (Illumina) in a 2×75 paired-end format. The raw sequence files generated (.fastq files) underwent quality control analysis using FastQC.

5.11 Bioinformatic analyses

5.11.1 Data preprocessing

Removal of adapters, poly(A) sequences, low-quality nucleotides, and trimming with Cutadapt tool version 2.5, alignment of reads to the reference genome using STAR version 2.7.5c [208], with the standard parameters for paired reads. The reference track was assembly Human obtained from GenCode (HG38 - Release 37 (GRCh38.p13)).

5.11.2 Peak calling

The bioinformatics tool MACS2 was used to predict the binding sites. This tool identifies statistically significantly enriched genomic regions using the MACS2 algorithm (Model-Based Analysis). Bedtools utilities have been used to annotate and characterize the peaks as located in exons or introns of protein-coding, non-coding RNA, and Pseudogenes transcripts.

5.11.3 Prediction of lncRNA-RNA interaction

The Rblast method has been used for the computational prediction of lncRNA-RNA interactions [209]. This method consists of two steps. First, the “database construction” step generates Rblast database files from FASTA formatted RNA sequences file. To this scope, we downloaded all the available to date FASTA formatted RNA unspliced sequences files from Biomart. Second, the “RNA interaction search” step searches for RNA-RNA interaction of a query sequence in the previously computed Rblast database. In this case, we used as a query the lncH19 sequence (i.e. ENST00000428066 transcript). The output is a prediction file providing the following information: Id, Query name, Query Length, Target name, Target Length, Accessibility Energy, Hybridization Energy, Interaction Energy, and BasePair. The overlapping predictions for each gene were grouped, assigning to the union of them the lowest value of the Interaction Energy. The lncH19-RNA computationally predicted interactions have been used to filter the peaks experimentally obtained with the analysis of RNA sequencing data from lncH19 RAP, maintaining only those falling into the predicted binding site with an interaction energy lower than -10 . The genes involved in interaction with Interaction Energy lower than -10 have been saved for further processing.

5.11.4 Binding Affinity prediction

The CatRAPID algorithm [210] has been employed to predict the binding affinity of RBPs (hnRNPM, RBFOX2, and SRSF1) and lncH19. This algorithm leverages secondary structure predictions in conjunction with hydrogen bonding and van der Waals calculations to estimate the binding affinity of protein-RNA pairs. Interaction propensity is a metric that gauges the

probability of interaction between one protein and lncH19 based on the observed tendency of the components of ribonucleo-protein complexes to exhibit specific properties of their physico-chemical profiles.

5.11.5 ENCODE analysis

To investigate the existence of a complex made by lncH19 and RBPs that regulate mRNA splicing, RBFOX2 and hnRNPM peaks (enriched genomic region) derived from enhanced CLIP (eCLIP) data have been collected from the ENCODE portal. The eCLIP peak co-occurrences across RBPs enable the discovery of novel co-interacting complexes. In particular, the ENCSR328LLU experiment used for this analysis belongs to a work that enabled the mapping of targets for 150 RBPs in HepG2, creating a unique resource of RBP interactomes profiled with a standardized methodology [211]. For each RBP, the bed files downloaded from ENCODE portal have been annotated and the genes associated with each peak have been identified. The gene lists obtained from the filtered peaks of lncH19 and SFs peaks obtained from ENCODE have been intersected and represented with the Venn diagram.

5.12 Immunofluorescence assay

Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained for 1 h at room temperature with RAC1b primary antibody (1:100, cat. n° 09-271 EMD Millipore Corp., USA). The secondary antibody used was DyLight 488 (1:500 cat n° 35552 Thermo Fisher Scientific). Samples were counterstained with Hoechst 3342 (1:1000, cat n° H3570 Life Technologies) for 30 min at room temperature to detect nuclei and analyzed by confocal microscopy (Nikon A1) median nuclear planes. Quantification of the RAC1B signal has been performed using NIS-Elements software. We used a confocal microscope to capture the nuclear plane of each field. Using software, we identified the nuclei as Regions of Interest (ROI) based on the Hoechst signal. The software then calculated the intensity of the signal in the green channel (RAC1B) within each ROI, providing a measure of the mean fluorescence intensity (MFI) relative to the nuclear region. We analyzed three different fields per condition. The experiments have been performed in triplicate.

5.13 Western blot assay

SDS-PAGE and Western blotting were performed according to standard protocols. To obtain total protein extracts, SW620 and HCT116 cells were lysed using a lysis buffer (15 mM Tris/HCl pH 7.5, 120 mM NaCl, 25mM KCl, 1 mM EDTA, 0.5% Triton X100, and protease inhibitor cocktail) for 1.30 h on ice. Cell debris was removed by centrifugation at 14,000 x g for 15 min at 4 °C and the supernatant, containing the protein lysate, was quantified through the Bradford assay method (Pierce™Coomassie Plus Assay Kit, cat. n° 23236, Thermo Fisher

Scientific, United States) using bovine serum albumin (BSA, cat. n° A2153, Sigma-Aldrich, United States) as a standard.

To obtain nuclear and cytoplasmic protein fraction was used the Nuclear Extract Kit (Active Motif, Belgium) following the manufacturer's instructions. Briefly, cells were first washed with PBS/phosphatase inhibitors, the supernatant aspirated, and fresh ice-cold PBS/phosphatase inhibitors added. The cells were removed by gently scraping and the cell suspension was centrifuged for 5 min at 200 x g at 4 °C. Then the supernatant was discarded, and the cell pellets were resuspended in 1X hypotonic buffer and incubated 15 min on ice. After this incubation specific detergent was added and the samples were vortexed 10 s at the highest setting. After centrifugation for 30 s at 14,000 x g at 4 °C, the supernatant (cytoplasmic fraction) was transferred into a pre-chilled microcentrifuge tube and stored at -80°C until ready to use. The pellet was resuspended in 50 µl Complete Lysis Buffer by pipetting up and down and incubated for 30 min on ice on a rocking platform set at 150 rpm. After 30 s of vortex at the highest setting the samples were centrifuged for 10 min at 14,000 x g at 4°C. Finally, the supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge and stored at -80°C for the following experiments. A total of 20 µg of proteins obtained from standard cell lysates or from nuclear and cytoplasmic fraction, or the specific amount of protein samples derived from RIP or RNA pull-down assay (see the specific section in material and methods paragraph), was separated using Bolt Bis-Tris gel 4–12% (cat. n° NP0326BOX, ThermoFisher Scientific) and transferred on nitrocellulose membranes (cat. n° 1060000, Amersham™ PROTRAN™ GE Health-care; Chicago, IL, USA). The correctness of loading samples was evaluated by the staining of the membranes with 0.1% red Ponceau in 5% acetic acid. The membranes were then blocked for 1.30 h at 4 °C in 5% BSA solution (5% BSA, 20 mM Tris, 140 mM NaCl, 0.1% Tween-20) and incubated overnight at 4 °C with the following primary antibodies: anti-RAC1b (1:1000, cat. n° 09-271 EMD Millipore Corp., USA), anti-hnRNPM1-M4 (1:500, cat. n° sc-20002, Santa Cruz Biotechnology), anti-RBM9/rbFOX2 (1:1000, cat. n° A300-864 A Bethyl Fortis, Montgomery, Texas), anti-SRSF1 (1:500, cat. n° 324500, Invitrogen), anti-b-actin and anti-Laminin B1 (both from Santa Cruz Biotechnology 1:1000, respectively cat. n° sc-81178, sc-365962). After washing with Tris-buffered saline-Tween-20 (TBS-T, 20 mM Tris, 140 mM NaCl, 0.1% Tween-20) three times at room temperature, the membranes were incubated for 1 h at 4 °C with appropriate secondary antibody HRP-conjugated goat anti-rabbit IgG (1:10.000, cat. n° 31460, Invitrogen™, Thermo Fisher® Scientific, United States) and anti-mouse IgG (1:10.000, cat. n° 7076, Cell Signaling Technology, United States). The chemiluminescent signal was revealed using a chemiluminescence solution (ECL™ Prime Western Blotting System, cat. n° GERPN2232, Cytiva, Germany) and detected using the ChemiDoc acquisition instrument (Bio-Rad, United States). The obtained images were analyzed with the Image Lab software (Bio-Rad).

5.14 Cell cycle analysis

SW620 and HCT116 cells were seeded respectively at 3×10^4 or 2.5×10^4 per cm^2 . After 24 h, cells were transfected with H19-pFLAG-CMV-2 expression vector or empty pFLAG-CMV-2 as Negative Control. After 18 h of transfection, the cells were treated with 50 µM of RAC1B

inhibitor EHT-1864 (Santa Cruz Biotechnology, cat.n° sc-361175) for 4 h. Cells were harvested and washed twice in cold PBS and centrifuged. The pellet was carefully fixed in 70% cold ethanol while vortexing to avoid cell clumping and stored at -20 °C for at least 24 h. Cells were then pelleted and washed with cold PBS and resuspended in 1 ml of cold cell cycle buffer (30 µg/mL propidium iodide, Sigma-Aldrich; 100 µg/mL DNase-free RNase A, Sigma-Aldrich; in PBS, pH7.4) [212]. Samples were incubated at least for 1 h at 4°C in the dark before acquisition. Acquisition was performed on a FACS Aria III (Becton-Dickinson, Milan, Italy) equipped with a 488 nm laser in the PE channel (585 nm), using DIVA v.8 software (BD). Cells were gated on the SSC/FCS dot-plot to exclude debris and further gated on the SSC-W/SSC-H and then on the FSC-W/FSC-H dot-plot to exclude doublets, and the gate was applied on the PE-A histogram. Data were analyzed and deconvoluted using ModFit LT v6.0 (Verity Software, MA, USA).

5.15 Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) of independent biological replicates $n \geq 3$. The normal data distribution was assessed by the Shapiro-Wilk test. When data followed a normal distribution, the statistical significance of differences was analyzed using one-sample t-test, to compare the mean to a hypothetical mean, or a two-tailed Student's t-test to compare the group; otherwise, a non-parametric method (respectively Wilcoxon or Mann-Whitney test). A p-value ≤ 0.05 was considered significant. Pearson correlation analysis was used to correlate the expression of couples of genes in tumor samples expressing higher levels of lncH19 compared to normal counterparts ($n = 14$). Statistical analyses were performed using GraphPad Prism 10 software (GraphPad Software, United States). p-values were indicated in the graphs.

CHAPTER 6

RESULTS

6.1 The long non-coding RNA H19 interacts with mRNA precursors and splicing factors

To better understand the molecular mechanisms by which lncH19 contributes to CRC progression, we performed RNA sequencing from lncH19 RAP in SW620 cells.

The table in Figure 8A shows that in CRC cells, lncH19 binds a substantial number of mRNAs, including both intronic and exonic regions. This observation led us to hypothesize a direct role for lncH19 in mRNA maturation and AS.

It is known that several ncRNAs influence AS by acting as natural antisense transcripts. Through RNA–RNA base pairing with pre-mRNAs, they can modulate splice site selection and the recruitment of SFs [213], [214].

To validate our hypothesis, we investigated the binding between lncH19 and SFs involved in alternative splicing with pro-tumoral effects. Our study focused on RBFOX2 since it is among the most studied alternative splicing factors implicated in the development of EMT, cancer progression, and metastatic process [191],[193]. To investigate the binding between lncH19 and RBFOX2 we proceeded with lncH19 RAP in two different CRC cell lines SW620 and HCT116. The western blot in Fig. 9B is the first evidence that, in CRC cell lines, lncH19 interacts with RBFOX2. We examined also the bond between lncH19 and RNABPs that are likely to be recruited by RBFOX2 to splicing sites, namely hnRNPM, SRSF1, and hnRNPC [188]. The western blot from lncH19-RAP, allowed us to detect the presence of hnRNPM and SRSF1, with the latter in smaller amounts than the former (Figure 8B).

However, no signal was detected for hnRNPC. Moreover, by the use of the CatRAPID algorithm [210] we estimate the binding affinity between the identified SFs and lncH19. The results shown in Figure 8D further confirm a higher binding affinity with hnRNPM and RBFOX2 and a lower affinity with SRSF1.

The RIP assays further confirmed the binding between the lncH19 and the RBPs RBFOX2 and hnRNPM while no enrichment in lncH19 was obtained by SRSF1 immunoprecipitation (Figure 8C and data not shown).

Together, these results suggest the presence of a molecular platform composed of lncH19 and specific SFs.

To identify putative targets of this complex, we have intersected the list of mRNAs bound by lncH19, as identified by the RAP analysis, with the RBP interactome for hnRNPM and RBFOX2 obtained from eCLIP profiling [211] (Figure 8D). The analyses identified 57 transcripts associated with lncH19 and containing binding sites for the investigated SFs, listed in Supplementary File 2. Our investigation focused on RAC1, as it has been widely observed that its AS leads to RAC1B, a constitutive-active Ran GTPases which has been found to stimulate cancer cell proliferation, enhance EMT, and induce drug resistance [215].

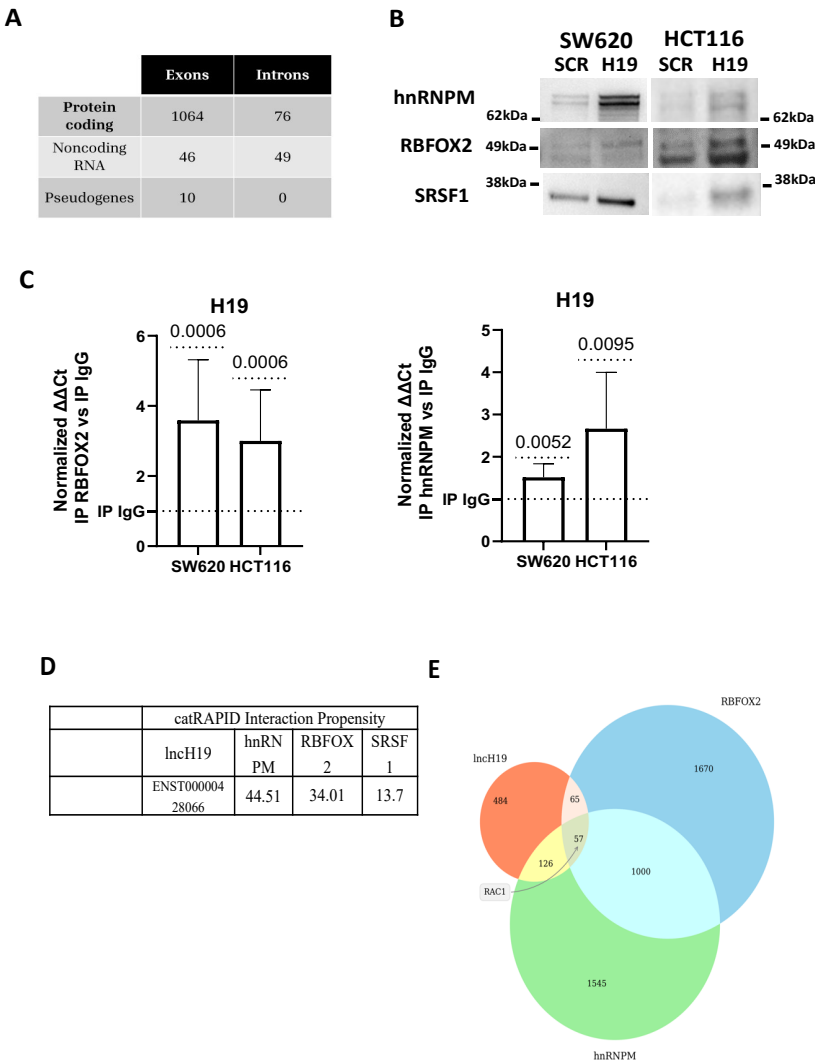


Figure 8. lncH19 interactors network. **(A)** Summary of sequences obtained by RNASeq from lncH19 Antisense Precipitation. **(B)** Western blot for the indicated proteins from lncH19 Antisense Precipitation in CRC cell lines (SW620 and HCT116). One representative experiment of three is shown. **(C)** RIP assay with anti-Fox2 (left) and anti-hnRNPM (right) antibodies to assess the binding of the RNABPs to lncH19 RNA in CRC cell lines (SW620

and HCT116); IgG was used as control. LncH19 levels were determined by qRT-PCR, normalized with input and presented as fold enrichment in RBFOX2 or hnRNPM relative to IgG. Statistical analyses were performed using normality test and t-test, p-value is shown in the graphs. **(D)** Binding affinity prediction between lncH19 and the indicated Splicing Factors by the use of catRAPID algorithm **(E)** Venn Diagram of lists of genes whose mRNA interacts with lncH19 (interactions are determined by the analysis of our RAP/RNA sequencing data and in-silico predictions), RBFOX2, and hnRNPM (interactions are determined by the analysis of eCLIP data from ENCODE portal)

6.2 Both RBFOX2 and lncH19 control RAC1B expression in CRC cell lines

We first performed RIP assays to assess whether RBFOX2 (Figure 9A) and hnRNPM (Figure 9B) are able to bind RAC1 mRNA in CRC cells. Both RBPs showed clear enrichment for RAC1, confirming their interaction. In parallel, RAP assays for lncH19, followed by qRT-PCR, validated the RNA-Seq findings, confirming that lncH19 also interacts with RAC1 mRNA (Figure 9C).

Of note, the alternatively spliced RAC1B isoform includes an additional 57 nucleotides corresponding to exon 3b, leading to the insertion of 19 extra amino acids in the protein product. Splice-sensitive PCR (Figure 9D) revealed that lncH19 specifically binds the longer RAC1B isoform, further supporting its role in modulating AS—likely in cooperation with RBFOX2.

This interaction was further confirmed by real-time PCR analysis (Figure 9E). Recent evidence correlated the activity of RBFOX2 in promoting EMT signature to RAC1B expression [216].

Moreover, it has been shown that RBFOX2 silencing but not hnRNPM silencing affects RAC1 splicing [188]. We transiently silenced two CRC cell lines for RBFOX2 (Figure 10A,E). Transcriptional and protein data indicated that RBFOX2 silencing negatively affects RAC1B expression at transcriptional (Figure 10B, F) and protein levels (Figure 10C, G), thus confirming, also in CRC, the correlation between RBFOX2 and RAC1B expression. Notably, RBFOX2 silencing did not affect lncH19 expression (Figure 10D, H). Since the main aim of this study is to investigate if lncH19 takes part in RAC1 alternative splicing, we stably silenced CRC cell lines for the lncRNA (Figure 10 I-M). Noteworthy, in H19-silenced cells RAC1B is significantly downregulated compared to wild-type cells both at transcriptional and protein level (Figure 10 I-M). The levels of RAC1 were not affected in lncH19-silenced cells enforcing the hypothesis that lncH19 plays a role in RAC1B splicing, rather than in RAC1 mRNA stabilization. Neither RBFOX2 nor hnRNPM protein levels were altered by lncH19 silencing.

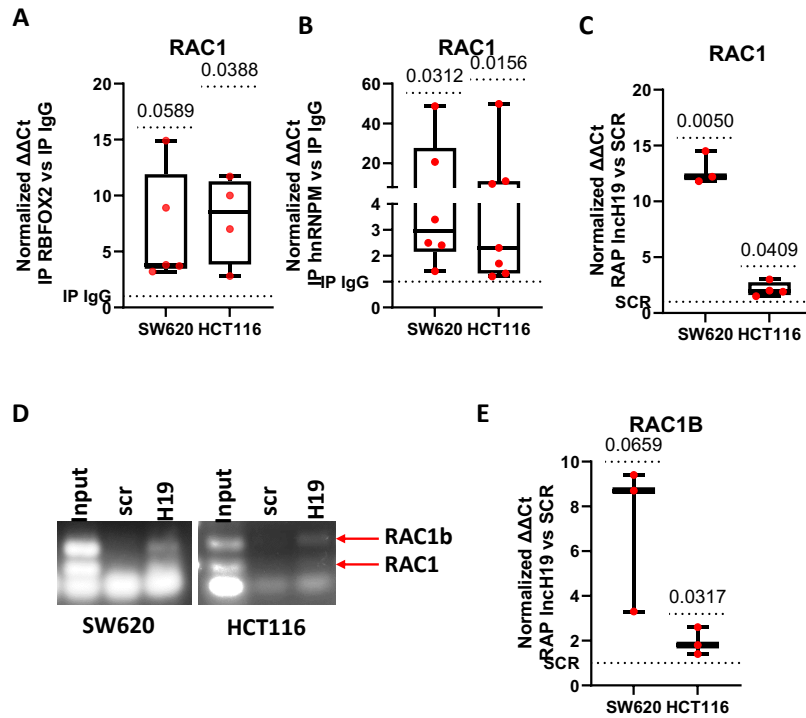


Figure 9. RBFOX2, hnRNPM and lncH19 bind RAC1 mRNA. **(A-B)** RIP assay with anti-Fox2 and anti-hnRNPM antibodies to assess the binding of the RNABPs to RAC1 RNA in HCT-116 and SW620 cells, IgG was used as control. RAC1 levels were determined by qRT-PCR normalized with input and presented as fold enrichment in RBFOX2 or hnRNPM relative to IgG. (Normality test and subsequent t-test **(A)** or Wilcox test **(B)**). **(C)** RNA pull-down with biotin-labeled lncH19 oligonucleotides (lncH19 RAP) in CRC cell lines (SW620 and HCT116) to analyze the interaction between lncH19 and RAC1 mRNA. RAC1 levels were determined by qRT-PCR and presented as fold enrichment in lncH19 samples respect to RNA pull-down obtained with scrambled oligonucleotides (Normality test and t-test). **(D)** Agarose electrophoresis of splice-sensitive PCR RAC1-RAC1B from lncH19 RAP in CRC cell lines (SW620 and HCT116). One representative experiment of three is shown. **(E)** Quantitative analysis of RAC1B levels determined by qRT-PCR and presented as fold enrichment in lncH19 samples relative to input. Statistical analyses were performed using one sample t-test, the p-value is shown in the graphs

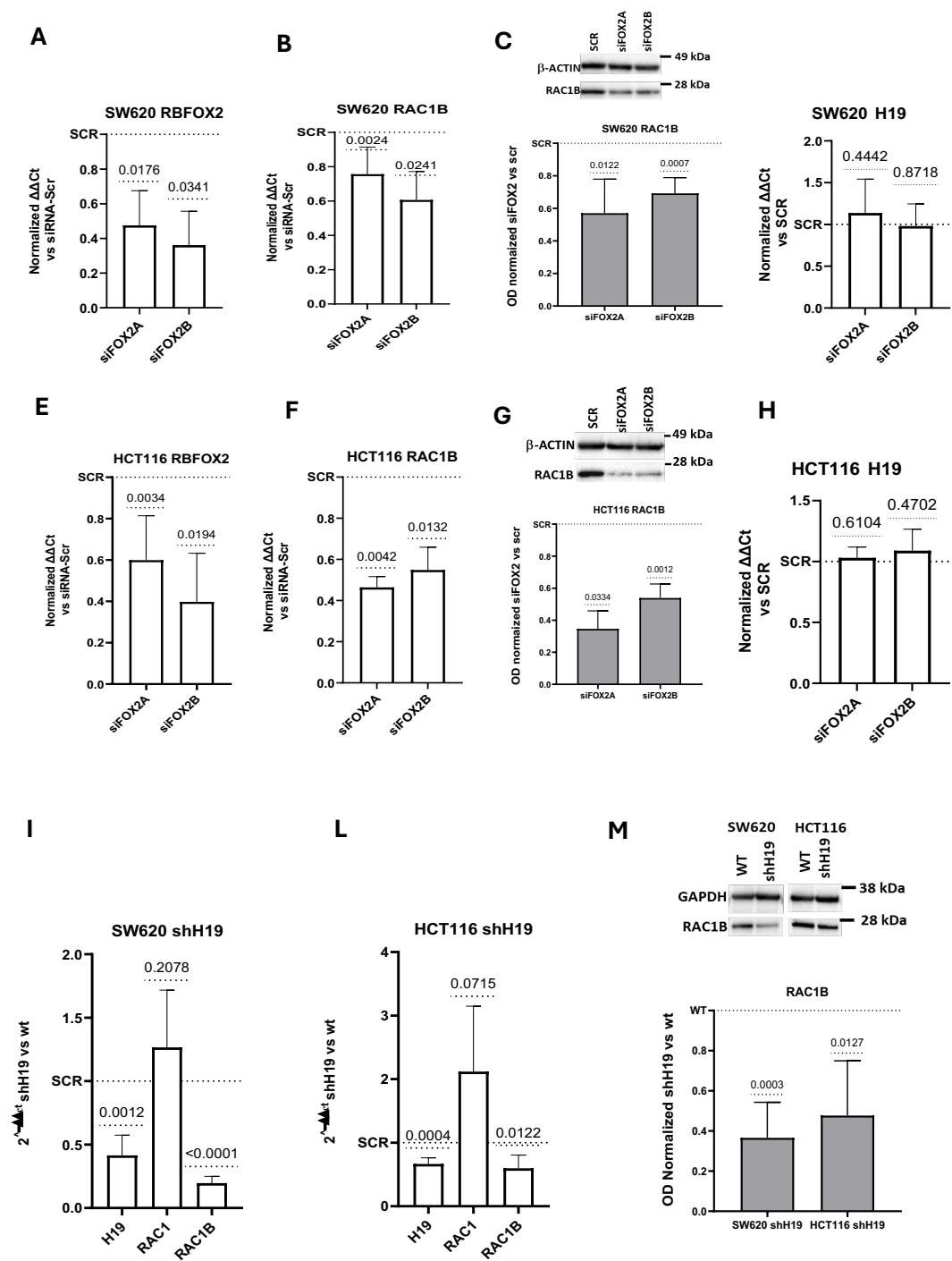


Figure 10. RBOX2 is involved in RAC1 alternative splicing in CRC cells. (A-B-D, E-F-H) qRT-PCR for the indicated mRNA in CRC cells, silenced for RBOX2 with two different siRNA. Graphs show $2^{-\Delta\Delta Ct}$ calculated in silenced cells respect to relative controls (Normality test and t-test). (C, F) Western Blot and densitometric analyses for RAC1B in SW620 and HCT116 silenced for RBOX2 and relative controls. For densitometric analysis data are represented as normalized OD. (I-L) qRT-PCR of the indicated genes in SW620 and HCT116 silenced for H19, the graphs represent the $2^{-\Delta\Delta Ct}$ of the indicated calculated respect the expression in control cells. (M) Western Blot of RAC1b protein levels in CRC cells (SW620 and HCT116) in H19 silenced cells and relative control cells. Statistical analyses were performed using normality test and t-test, p-value is shown in the graphs

6.3 LncH19 enhances oncogenes' expression through RAC1B

We over-expressed lncH19 in CRC cell lines SW620 and HCT116 (Figure 11A) to investigate its effects on RAC1B expression. Transcriptional and protein analyses revealed that lncH19 promotes RAC1B expression (Figure 11B, C) moreover, immunofluorescence analyses for RAC1B showed a significant increase in the nuclear localization of the protein (Figure 11D). Data confirmed by western blot from nuclear extracts (Figure 11E). In vivo and in vitro studies have demonstrated that RAC1B is overexpressed in CRC and high RAC1B expression correlates with high WNT activity and poor prognosis [217],[218].

Specifically, in HCT116 cells, chromatin immunoprecipitation assays showed that nuclear RAC1B is recruited to the promoters of Wnt target genes c-Myc and Cyclin D1, acting as a co-activator in β -catenin/TCF-mediated transcription, thereby enhancing the expression of these oncogenes [219].

Based on this, we investigated whether lncH19 could promote oncogenic activity through RAC1B-induced oncogenes' activation. To test this hypothesis, we treated lncH19-overexpressing cells with EHT-1864, a Rac-GTPase inhibitor able to inhibit also RAC1B activity [220].

Transcriptional data (Figure 11 F,G) confirmed that lncH19 over-expression promotes the expression of c-Myc and Cyclin D in both cell lines however, treatment with the RAC1B inhibitor leads to complete rescue. These data are supported by cell cycle analyses, which show a slight shift of the cell cycle in favor of the G2/M phase 24 h after lncH19 over-expression. Moreover, this shift is impeded by treatment with EHT-1864 (Supplementary File 5). Overall, our data revealed a new axis, in the control of CRC gene expression passing through lncH19, RAC1B, and the Wnt target genes c-Myc and Cyclin D. Finally, to validate this axis in vivo we investigated gene expression in biopsies from 20 patients with CRC. Real-time PCR (Figure 12 A) confirmed that CRC tissues present higher levels of lncH19 compared to respective marginal non-tumor tissue. No significative differences have been revealed in the comparison of RAC1 and RAC1B levels (Figure 12 B, C). However, by selecting the CRC samples with higher levels of lncH19 compared to their health counterpart (n=14) Pearson Correlation analyses revealed a positive correlation between lncH19 and RAC1B (Figure 12 D). In addition, the same samples present higher levels of both oncogenes Cyclin D and c-Myc (Figure 12 E, F).

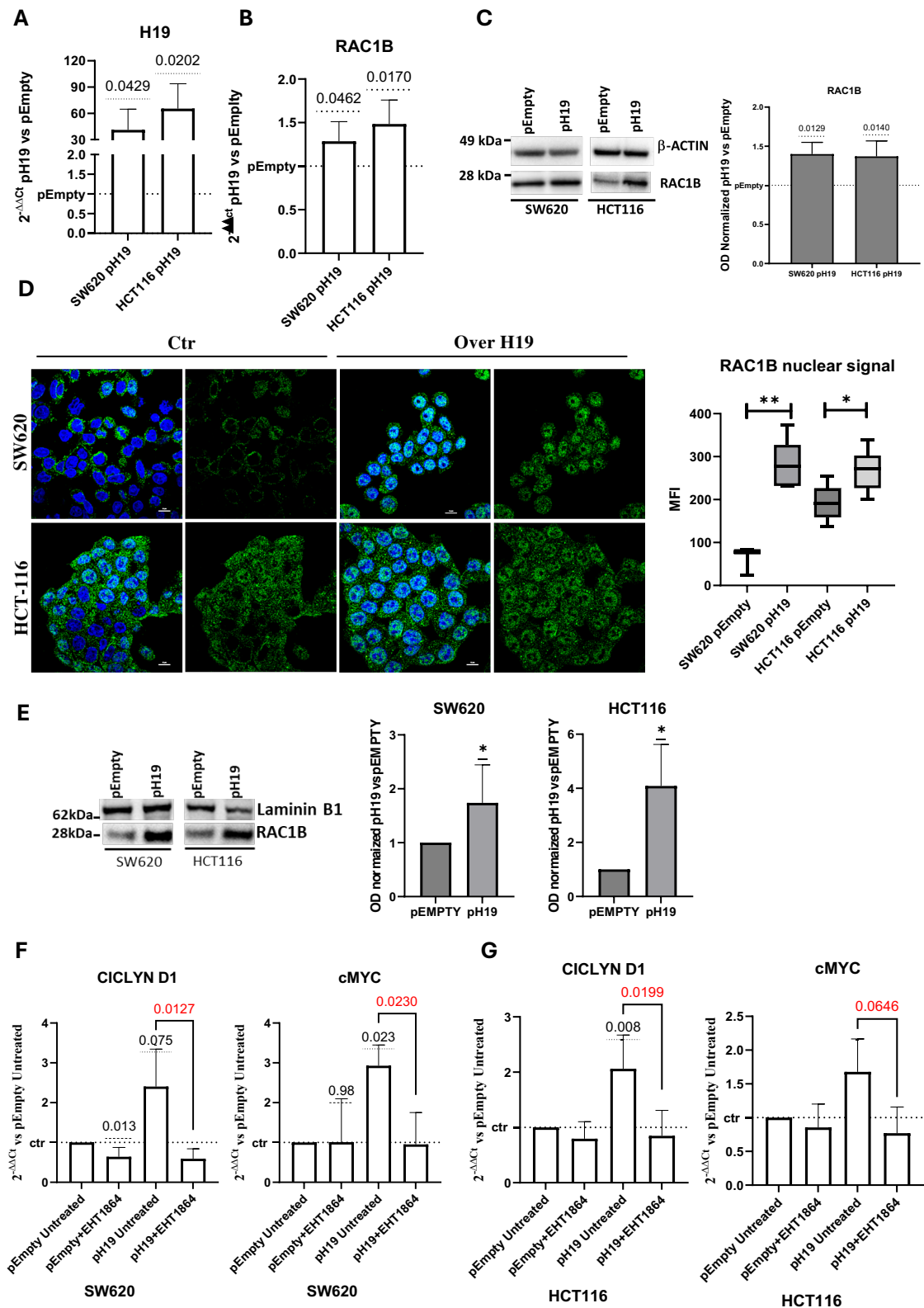


Figure 11. Overexpression of lncH19 upregulates RAC1B expression. (A) qRT-PCR to analyze H19 mRNA levels in CRC cells (SW620 and HCT116) transfected with lncH19 or empty vector. (B) qRT-PCR to analyze RAC1B mRNA levels in CRC cells (SW620 and HCT116) transfected with lncH19 or empty vector. (C) Western Blot for RAC1B in CRC cells (SW620 and HCT116), transfected with lncH19 or empty vector. lncH19 overexpression promotes RAC1B nuclear localization. (D) Representative confocal microscopy images of anti-RAC1b immunofluorescence showed nuclear localization of RAC1b in lncH19-overexpressing CRC cells, SW620 (upper panel) and HCT116 (lower panel). The analysis of the RAC1B nuclear signal is reported on the

histogram. (E) Western blot analysis and densitometric analyses for RAC1B in nuclear protein fractions of CRC cells (SW620 and HCT116) overexpressing H19 cells and relative control cells. E-F) Overexpression of lncH19 upregulate CyclinD and c-Myc expression through RAC1B activity qRT-PCR for the indicated genes in SW620 (F) and HCT116 (G) transfected with pH19 or empty vector and treated or not with RAC1B inhibitor. Statistical analyses were performed using: normality test, one sample t-test to compare different conditions to pEmpty untreated cells, and Two-tails unpaired t-test to compare two groups *; p-value is shown in the graphs

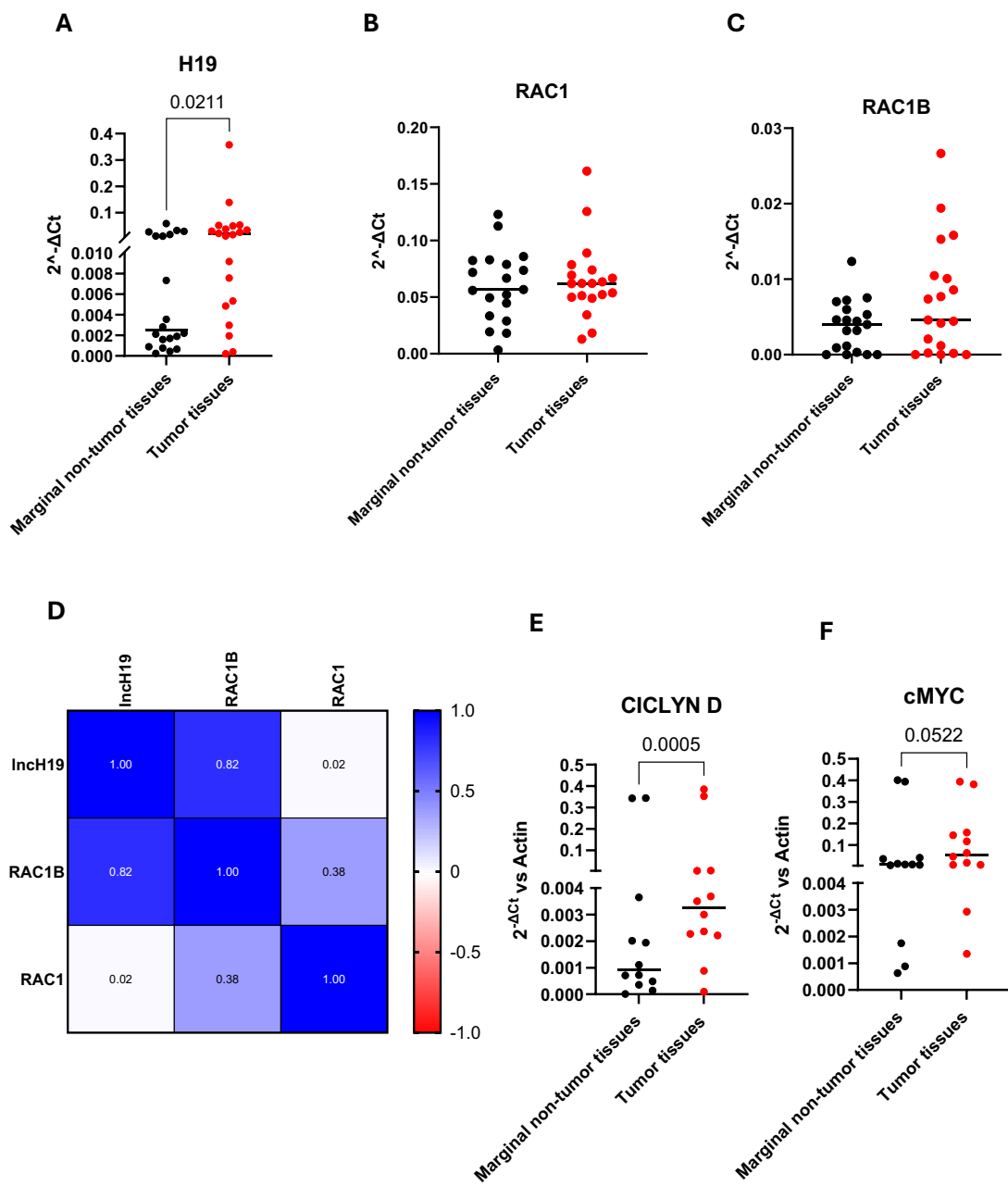


Figure 12. CRC tissues present higher levels of lncH19 compared to respective marginal non-tumor. (A-C) Gene expression levels for the indicated genes were examined by qRT-PCR in tumor and paired marginal non-tumor samples ($n = 20$). (D) Pearson correlation between lncH19, RAC1, and RAC1B expression analyzed in 14

colorectal cancer samples with lncH19 overexpressed compared to marginal non tumor tissue. (E-F) qRT-PCR for the indicated genes in colorectal cancer samples with lncH19 levels. All statistical analyses were performed using two-tail paired t-test, p-value is shown in the graphs

6.4 LncH19 brings the splicing factors RBFOX2 and hnRNPM to RAC1 mRNA

The data presented here demonstrated that lncH19 directly influenced the AS of RAC1, promoting the inclusion of exon 3B. To further explore the molecular mechanism underlying this process, we initially hypothesized that lncH19 might facilitate the assembly of RBPs (RBFOX2, hnRNPM, and SRSF1) into the splicing complex. However, co-immunoprecipitation (co-IP) experiments showed that lncH19 silencing did not disrupt the interaction between the splicing factors (Supplementary file 3).

We then hypothesized that lncH19 might have the role of bringing the splicing complex directly to RAC1 mRNA. RIP analyses confirmed this hypothesis. As shown in Figure 13 the binding of RBFOX2 to RAC1 and RAC1B is strongly reduced in cells silenced for the lncH19 (Figure 13 A, C). The same concerns the binding of hnRNPM (Figure 13 B, D).

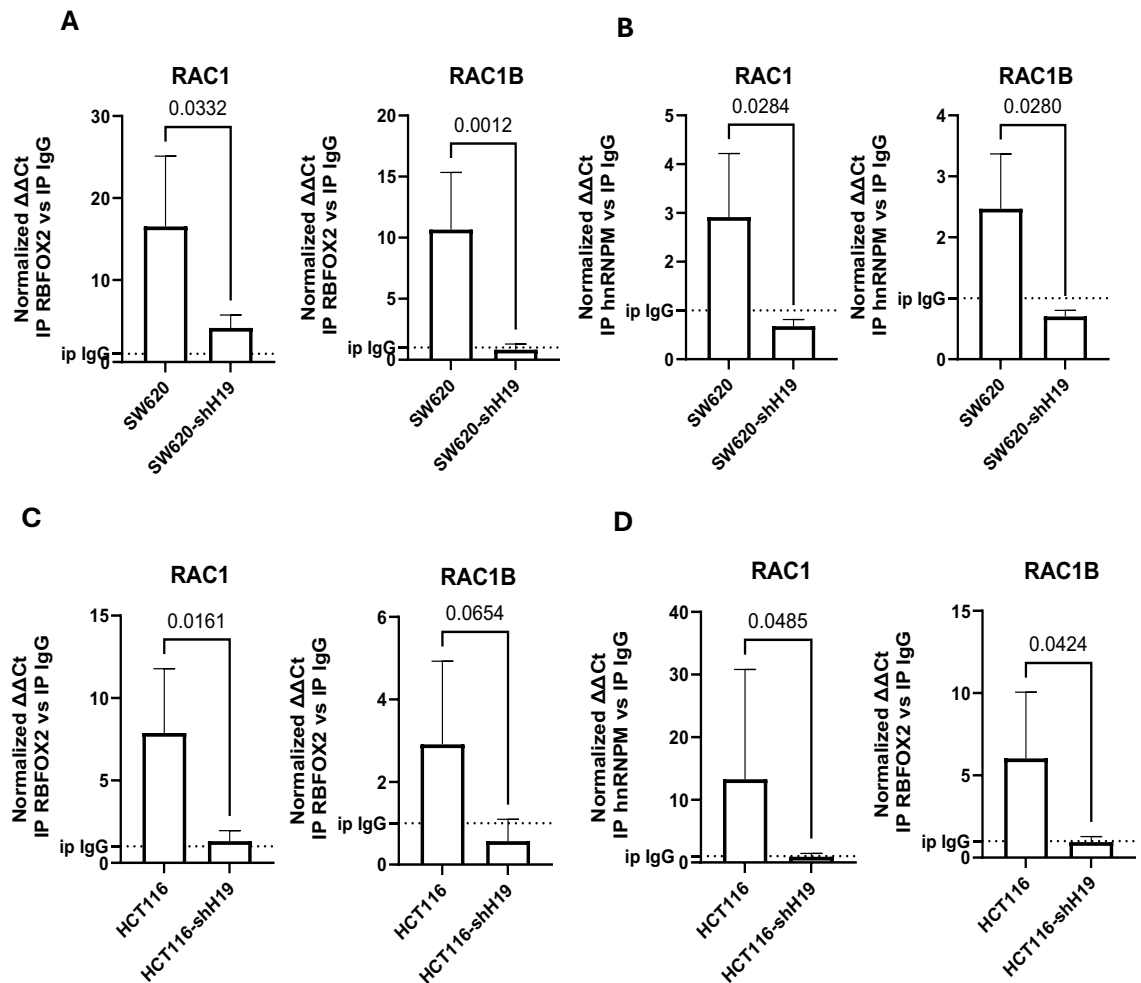


Figure 13. *LncH19 is required to drive splicing factors on RAC1 mRNA. (A-D) QRT-PCR for RAC and RAC1b from RNA-immunoprecipitation (RIP) with anti-Fox2 (A, C) or anti-hnRNPM (B, D) antibodies in SW620 and HCT116 cells, RAC1 and RAC1B levels presented as fold enrichment in RBFOX2 or hnRNPM IP relative to IgG IP. Statistical analyses were performed using two-tail unpaired t-test to compare the binding between wt and shH19 silenced cells, p-value is shown in the graphs.*

Supplementary Information

Below are the link to the electronic supplementary materials:

[Supplementary Material 1.csv](#)

[Supplementary Material 2.txt](#)

[Supplementary Material 3.pptx](#)

[Supplementary Material 4.tif](#)

[Supplementary Material 5.pdf](#)

RESEARCH VISITING

From May to October 2024, I carried out part of my research activities at the Malopolskie Centrum Biotechnologii of the Jagiellonian University in Krakow, Poland, under the supervision of Dr. Marta Pabis. During this period, I focused on expanding the study of the interaction between the lncRNA H19 and the SF RBFOX2, which is a major topic of my PhD project on the control of AS events involved in the progression of CRC. Specifically, I investigated which region of the lncH19 interacts with RBFOX2. To address this, I learned new technical skills, such as bacterial cloning methods and in vitro transcription (IVT) to produce full-length and fragment-specific RNAs.

In order to produce and purify recombinant protein from bacteria, I cloned the entire RBFOX2 coding sequence along with its RRM domain into an expression vector.

Finally, to evaluate the binding between RBFOX2 and the different lncH19 fragments, I performed Electrophoretic Mobility Shift Assays (EMSAs). The knowledge I acquired during this research stay enhances the analyses I conducted for my PhD and lays the groundwork for further investigations into the relationships between others lncRNAs and RBPs involved in tumor progression.

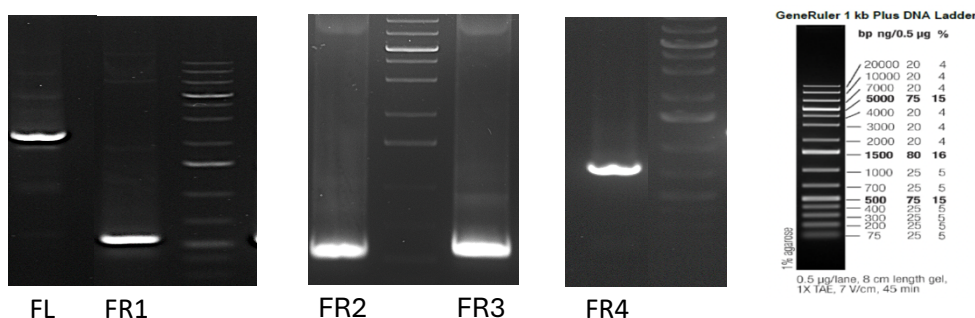
6.5 In Vitro Transcription of Full-Length and Fragmented lncRNA H19

To explore the specific region involved in the interaction between the lncH19 and the SF RBFOX2, a series of in vitro transcription (IVT) experiments were conducted to produce RNAs corresponding to the full-length (FL) H19 transcript and its four overlapping fragments. As a first step, specific primers were designed to amplify the sequences and each forward primer

FR3	FR4
2. FROM THE START TO REVERSE 1 H19_Fw_1_T7: TTCTAATACGACTCACTATAGGAGGGTGAGGAGGGGGTG H19_Rev_515_1: AGCGCTCGATCCCTTAACACT 1' AGGGTGAGGAGGGGGTGGGATGGGTGGGGGTAAACGGGGGAAACTGGGGAATGGGGAACCGAGGGGCA ACCACGGGGAAGATGGGTGCTGGAGGAGAGCTTGTGGGACGACGAGGACACCTTGGACATCTTGGATCTTG GCAGAGTGATGACGGGTGAGGGGGCTAGCTCAGGACGAGGGCTGTGGGGCTCTGAGGCAGTGAAGAGTG TGGATAGGGGCCACGGCATCTTGGACAGGAGGACATCACTGGGAGATGGGTCTGAGCTAGGGCT GGAAAGGAGGGGAGCCAGGACTCATCCCTCGGTACTTTTGTTACAGAGCATGCGACGTGGTGGACGA GGGAGCTGTGGTGGAGAGGTTTATGTCACGAAGCTGGGACACGAGAGTGTAGCTGGACAGCAGCGGACG TAGAGACCCCATCTCCGGGAGCGAGTGGATGCTTCCCTCCCGACGGCTCGCTTCCCGACGCTTCTGAAG AAGAGGGTGAGGAGGGGGTG 515' Length of the amplifier: 515 nucleotides	3. FROM REVERSE 1 TO REVERSE 3 H19_Fw_516_T7: TTCTAATACGACTCACTATAGGGGCGGGGAGAAGACA H19_Rev_1060: ATAACGCGTCAACCAAGTCA 516' GGCGGGGAGAAGACACCCTCCGACGAGAGGGCAGGATGGG CGGAGAGTGTAGCAAGGTGACATCTTCTGGGGGAGCCGAGACTGCGCAAGCTGGGGGGTTATGGG CCCGTCTCAGAGCGAAGAAAGCAAGAGGAGGAGGAGGAGCAGGGGTGCGCAGCTAGGGTCCAGCAG TGGGTGTGATACCCGAGCGCTGGGTGACAGAGGACATGCGAGGAGGACGAGCAGAGGGGCTCCGACT GGGACTACCCACCGCATCTTTATGATGAGAGGACAGGGGAGGTCTGGCAGCGCTTCTCAGGCTCT ATGCGACGCTCCGAGGCTGGAGGCGGAGGAAAGCAGCAGTGTCTGGGAGTTGACGAGGAGCTCAC AGGAGGGGGAAGCGGCAAGGGAGGGGGCCCCGGGACATTGGCCAGCAGAGAGGCTGCAAGGGCTCGG CTCGGGCGCGCGTCCACAGGACATCGCGCCAGGGTCTGTGTGGGAGAGGGCCACACTGACATCTGCG TGACGCTGTAT 1060' Length of the amplifier: 546 nucleotides
FR3	FR4
4. FROM REVERSE 3 TO REVERSE 5 H19_Fw_1061_T7: TTCTAATACGACTCACTATAGGCCTCAGCGCTCAGCCCC H19_Rev_1594: ATGGTGCTACCCAGCTCAA 1061' GCTCAGCGCTCAGCCCCGGGGCTGGCTTGGCAGACAGTACAGCATCCAGGGGAGTC AAGGCGATGGCGGACACGACGATGCGGGGGGGCGGAGTGAATGACTCTCAGAGGGAGAGA TGGTCAAGCAGGAGGTGAGAGGACGAGCGGGCGGCGAGCGAGGACACTGGCTCCAGACCCCTGGCCA AGGCGGGCTCGGGGGCGGCGACGAGCAGGGGATCGTGCTCCTCAGCGTCTGGGCTGGAGACGAGGCGAAG TCTCAGCTGGGTGGAGCTGGCCACGACTGCCGAAGGCGCAAGACGCGAGTGGCGGTGACGCTGACAG CAGGACATGACATGGTCCGGTGTGACGCGGAGGACAGAGAGGCGGCTCGGCGCTTCTGAACACTTAGT GCTGGTGGGGCTGGGACAGCGGGCTGTGTTTCTTACTCTGCTCAGCGAGGTGGCAGCATATGGTG CCTCTGGGCTCCGAGGCCACCACTAGAGAAATGGTGCTACCCAGCTCAA 1594' Length of the amplifier: 531 nucleotides	5. FROM REVERSE 5 TO THE END H19_Fw_1595_T7: TTCTAATACGACTCACTATAGGGCGCTGGGCCCTTGAATCC H19_Rev_2310: GTAAACGCTGTTTATTGATGATGAGTC 1595' GCGCTGGGCCCTTGAAT CGCGGACAAACAAACCTCTAGCTTGGAAATGAATATGCTGCACCTTACAACCACTGCACATCTGTC GAATCGCGCTCTGGAGGTGTGAGCTAGAGGAAACCAAGCACTCATAGCCCAACATCAAAAGCACCA CAGCGAGGCCCGGAGGACGAGGACGAGGACCTGACATCTTCATGAGGACCCCTCTGGGGG TGACCACAGCAGCACCACATCATCCGAGAGCTGAGCTCTCCAGCGGGATGAGCGGCTCCCAACG CTCTTCTCTTTTCATCCTCTGTTCTGTTTCTGAGGCTTCTGCTGCTTCTCTTTTCTG TGAAGGCTCCAGGACTCTGTTGCCCTCTCTGAAATTAATTTGCACTAGCATCTTGG TGAGATTGTGAGACGGCTCTGAGTCTGACTAGCAGTGTGGCTGAGTGTGAGCCACTTGGCGAAC GTCAGAGGCGGCGGCGGCTCTCATCTGGGCGGGGTGACGGGCGGGCTGTGTCGCGAGGAGC TGCCCTCGCTGACTGAGGAGCTCGGACGACATCAGGAGGAGCTCTCAAGCATTTCCATTACG CTCGGCTCTGCGCCCTCCCAACAGGGCTCTCAGCAGAGCGGCTGAGCTCATCATCAATAAACAC AGCAA 2315' Length of the amplifier: 721 nucleotides

Following primer design and optimization of amplification conditions via gradient PCR, the expected products were obtained. DNA bands corresponding to the desired amplicons were extracted from the agarose gel, purified, and precipitated using ethanol and sodium acetate. The MEGAscript™ T7 High Yield Transcription Kit (Cat. no. AM1334, Invitrogen) was utilized according to manufacturer's Instructions to perform IVT reactions from purified DNA. Transcription was carried out overnight at 37°C. The following day, DNA contaminants were removed by DNase treatment, and RNA products were precipitated using a lithium chloride solution.

Quantification of the resulting RNA showed high purity and yield, typically reaching concentrations of approximately 1000 ng/μl. Denaturing formaldehyde agarose gel electrophoresis was used to separate the products in order to verify the size and integrity of the transcribed RNA. This verified that the full-length H19 transcript and its particular regions had been successfully synthesized.



Formaldehyde agarose gel for H19 FL and fragments IVT confirmation

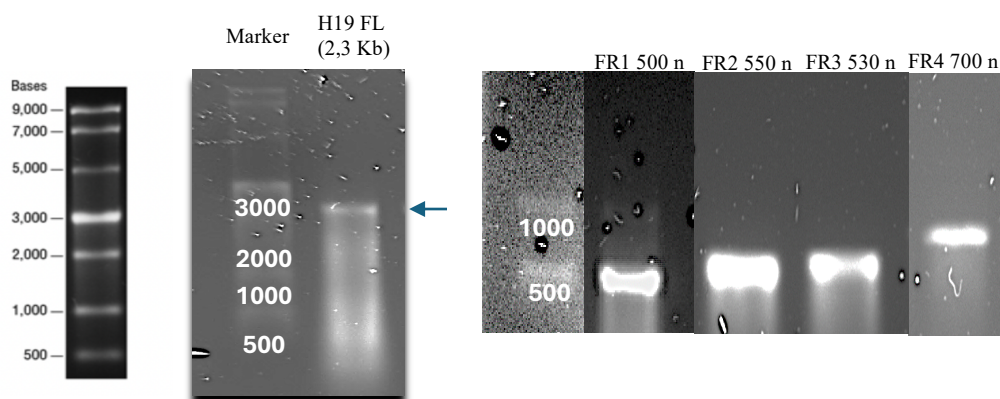


Figure 15. Amplification of H19 FL and its four fragments after gradient PCR and confirmation of RNA after IVT through formaldehyde agarose gel

6.6 Recombinant RBFOX2 protein purification with affinity chromatography and AKTA pure system

Following the in vitro synthesis of the RNA molecules of interest, the subsequent step focused on the production and purification of the recombinant proteins required for the study. Specifically, I aimed to obtain both the full-length RBFOX2 protein (RBFOX2 FL) and its RRM domain, which represents the functional region responsible for RNA binding.

To this end, the coding sequences corresponding to RBFOX2 FL and RBFOX2 RRM were subcloned into the pETM11 bacterial expression vector. This vector enables high-level protein expression in *Escherichia coli* and allows to attach an N-terminal polyhistidine (His₆) tag for affinity purification. Furthermore, a TEV protease cleavage site flanks the His-tag, enabling tag removal following purification if required.

PCR was used to first amplify the two RBFOX2 sequences from plasmids containing the respective inserts.

The DNA products were separated by agarose gel electrophoresis, and extracted from the gel after successful amplification. To produce compatible cohesive ends, the pETM11 vector and the amplified inserts were both digested using the proper restriction enzymes. The digested products were verified on gel to confirm the expected fragment sizes and subsequently purified for the ligation reaction.

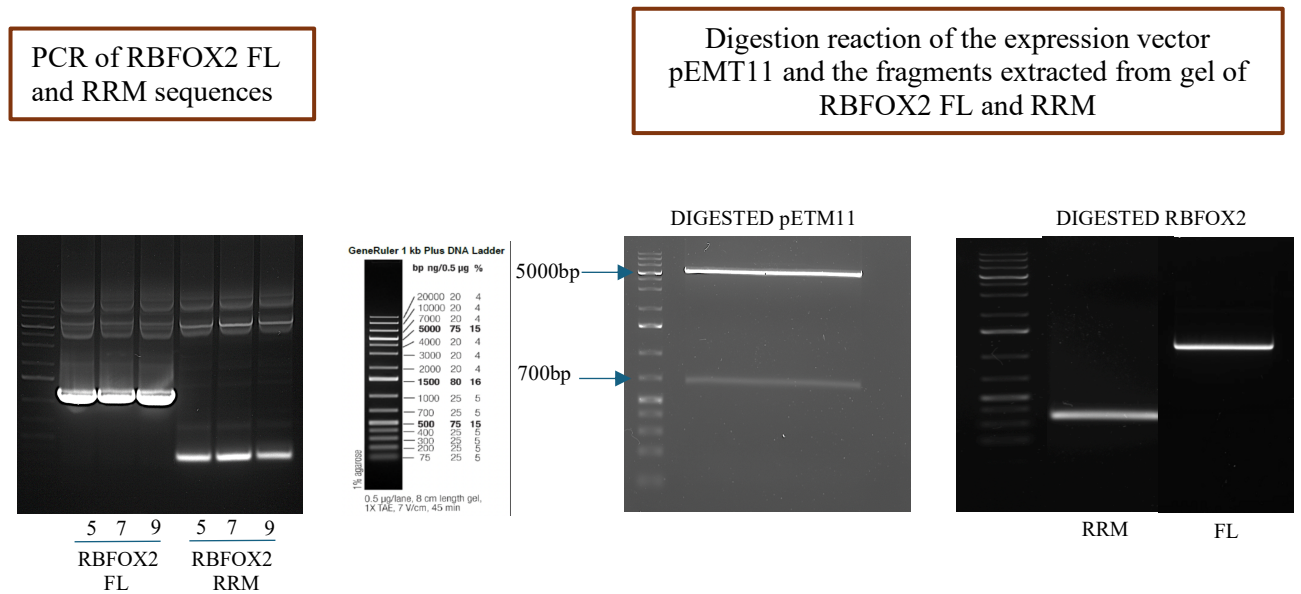


Figure 16. Gradient PCR of RBFOX2 FL and RRM sequences and Digestion reaction of the expression vector pEMT11 and the fragments extracted from gel of RBFOX2 FL and RRM

Following ligation, *E. coli* BL21 competent cells (Cat. no. EC0114, Thermoscientific) were transformed with the recombinant plasmids. The pEMT11 vector carries a kanamycin resistance gene, enabling selection of successfully transformed bacteria on LB agar plates supplemented with kanamycin.

Only bacteria harboring the plasmid—either empty or containing the desired insert—were able to grow on the selective medium. Thus, a number of colonies were chosen randomly from various areas of the plate, inoculated into kanamycin-containing medium, and cultivated overnight in order to verify the presence of the RBFOX2 inserts. Plasmid DNA was then extracted from each culture and subjected to restriction enzyme digestion to assess the presence and integrity of the insert.

Agarose gel electrophoresis was used to analyze the digestion products. The digestion produced two bands, one of which represented the plasmid and the other, smaller, the RBFOX2 insert, as can be seen in the representative gel image. Both the full-length and RRM constructs underwent this cloning validation, which verified that the sequences had been successfully inserted into the expression vector.

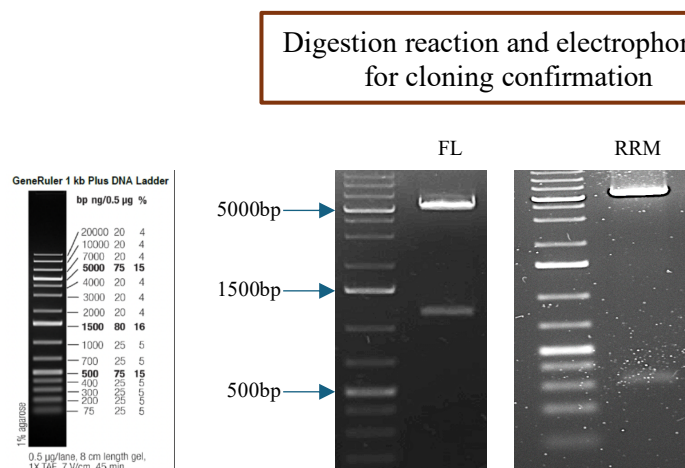


Figure 17. Digestion reaction and electrophoresis for cloning confirmation. The higher band is the pETM11 vector, the lower is the specific insert, RBFOX2 FL and RRM, respectively

After the confirmation of cloning, I proceeded with the transformation of *E. coli* BL21 cells using the validated pETM11 constructs containing either the FL RBFOX2 or its RRM domain. For protein production, selected colonies were first inoculated into a small volume of LB medium supplemented with kanamycin and cultivated overnight. Later, 500 mL of new selective medium was inoculated with these pre-cultures. Optical density (OD600) was measured at regular intervals to track bacterial growth until it reached an OD of approximately 0.8, which is the ideal value for inducing protein expression.

At this point, protein expression was induced by adding IPTG (Cat. no. 5810-5GM, sigmaaldrich), a non-hydrolyzable analogue of allolactose that activates the lac operon-controlled expression system. Although direct inoculation from colonies into large volumes is possible, the pre-culture step significantly accelerates the growth phase and improves the timing of induction. After 20 hours of expression at proper conditions, bacterial cultures were centrifuged at $5000 \times g$ for 20 minutes.

To evaluate the induction efficiency, whole-cell lysates were collected both before and after IPTG addition. SDS-PAGE analysis of these samples allow the visualization of the overexpressed recombinant proteins, expected to appear as intense bands at the corresponding molecular weights.

Despite my initial goal of purifying both the FL RBFOX2 and its RRM domain, technical difficulties during the early purification steps prevented me from obtaining the FL protein under the tested conditions. As a result, I moved forward with the RRM domain's purification and characterization, which was accomplished with success.

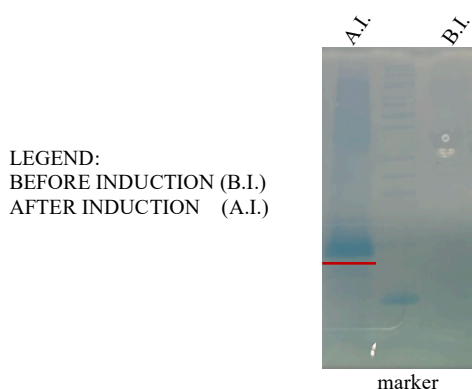


Figure 18. SDS-PAGE analysis to confirm the correct induction of bacteria with IPTG. The band underlined in red in the A.I. lane represents the high amount of protein produced by bacteria after induction (the same band is not present before induction)

The purification process began with the resuspension of the bacterial pellet in lysis buffer, freshly supplemented with DNase I and protease inhibitors to prevent nucleic acid contamination and proteolytic degradation. Efficient lysis of the bacterial cells was achieved through sonication, followed by centrifugation at 18,000 rpm for 40 minutes at 4°C. Purification was then carried out using the supernatant, which contained soluble proteins, including the recombinant RRM domain of RBFOX2.

Affinity chromatography with Ni-NTA agarose resin (Cat. no. R901-15, Invitrogen) was used for the initial purification step. Ni-NTA Agarose is a nickel-charged affinity resin, that can be used to purify recombinant proteins containing a polyhistidine (6xHis) sequence.

Water and a low-salt binding buffer were used to equilibrate the Ni-NTA resin before incubation. Following the resin's incubation with the lysate, the His-tagged protein was able to bind selectively via the interaction of nickel ions and histidine residues. This method exploits the strong affinity between the imidazole ring of histidine and divalent metal cations, with nickel being the most commonly used due to its optimal binding characteristics.

The target protein was eluted using a buffer containing imidazole, which competes with histidine for binding to the nickel ions, after non-specifically bound proteins were eliminated through a series of washes. To track the recombinant protein's enrichment and assess the purification efficiency, flow-through, washes, and elution fractions were collected and analyzed by SDS-PAGE at each purification step.

TEV protease was added to the eluted protein after affinity purification in order to cleave the N-terminal His-tag. To encourage effective dialysis, the sample was moved into a dialysis cassette and incubated overnight at 4°C under constant agitation using a magnetic stirrer. In addition to establishing ideal conditions for TEV protease activity, this step allowed simultaneously removal of imidazole and other small molecules from the elution buffer. The dialysis buffer was chosen to preserve the proper ionic strength and pH, guaranteeing effective and precise His-tag cleavage without sacrificing protein stability.

On the second day, the aim was to separate the cleaved His-tag and the TEV protease from the target protein. This was accomplished by reapplying the dialyzed sample to a Ni-NTA affinity column under the same conditions as the initial purification. The target protein did not bind to the resin because it was no longer carrying the His-tag; instead, it was recovered in the flow-through and early wash fractions. SDS-PAGE analysis verified that the first collected fractions actually contained the majority of the recombinant protein (Figure 19).

The flow-through and relevant wash fractions were then pooled and placed into a new dialysis cassette for a second overnight dialysis step. At this stage, the dialysis buffer was the same as that used to equilibrate the size exclusion chromatography column for the subsequent purification step.

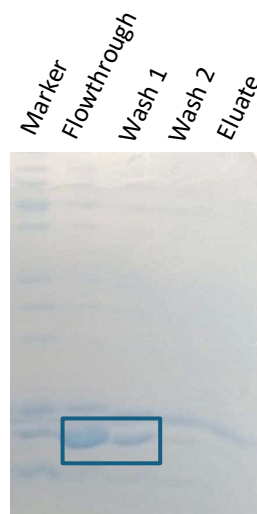


Figure 19. The more intense band in the first two lanes after the marker is the target protein. The SDS page shows all the samples collected : Flowthrough, Washes and Eluate

In a second purification step heparin affinity chromatography was used to further enhance the recombinant protein's purity and get rid of any possible nucleic acid contaminants. Heparin is frequently used to purify proteins with nucleic acid-binding capabilities because of its highly negatively charged structure, which mimics the polyanionic nature of nucleic acids. This matrix binds proteins rich in positively charged amino acids—such as lysine and arginine residues—or proteins that naturally interact with DNA or RNA through electrostatic interactions. This step is appropriate for selective enrichment and the removal of remaining contaminants since RBFOX2 is predicted to exhibit affinity for heparin due to its RNA-binding domains.

So, the eluate from the previous step was loaded onto the heparin column, and the bound proteins were eluted using a linear salt gradient.

At the end of the run, a chromatogram was generated to visualize the elution profile, indicating the distribution and relative abundance of proteins across the collected fractions.

The chromatogram is a graph showing the intensity of the detected signal (y-axis) versus the elution time (x-axis) during a chromatographic separation.

The x-axis indicates how long a compound exits the column while the y-axis indicates how much of that compound is present.

Each peak corresponds to a separated substance; a narrow, symmetrical peak indicates a good separation.

This graphical output is fundamental for identifying and selecting the fractions likely to contain the protein of interest.

The protein of interest was identified by a clear peak in the chromatographic profile.

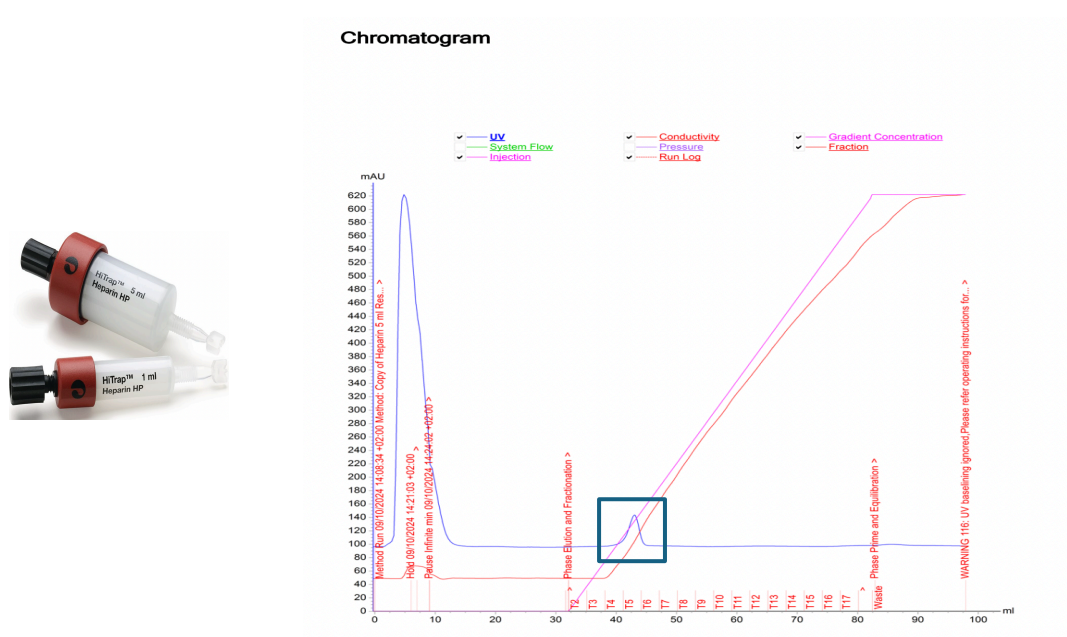


Figure 20. Chromatogram from heparin column step. The protein of interest was identified by a clear peak in the chromatographic profile.

After that, the fractions corresponding to the peak obtained from the run in the heparin column underwent a final purification step through size-exclusion chromatography (SEC) using a high-resolution column attached to the AKTA Pure system, a sophisticated, fully automated chromatography platform commonly used for protein and biomolecule purification. Key parameters like flow rate, pressure, gradient composition, and elution volume can be finely controlled and monitored in real time thanks to this system's high precision and reproducibility. Because of its adaptability to the unique properties of the target protein, it can be used with a variety of columns and resins.

SEC separates proteins according to their molecular size and hydrodynamic radius, in contrast to affinity-based techniques. A porous resin made of beads with an internal channel network is packed inside the column. Contaminants and smaller proteins enter the beads and elute later,

while larger protein complexes are prevented from entering the pores and elute earlier. This approach relies only on the physical characteristics of the proteins rather than any particular interactions. In general, SEC represents a critical step to ensure the removal of aggregates or degradation products.

A chromatogram was generated to visualize the elution profile and an aliquot from each collected fraction was subjected to SDS-PAGE analysis in order to confirm the protein's identity and purity. The electrophoretic analysis showed a single, distinct band at the expected molecular weight confirming the successful purification of the recombinant RBFOX2 RRM domain in a highly pure form.

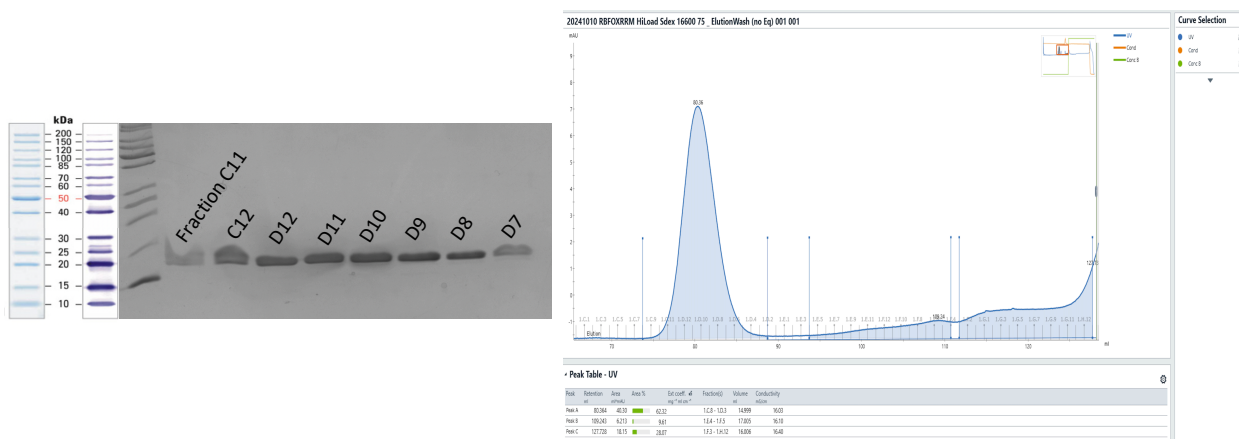


Figure 21. The SDS page represents the RRM domain in the shown fractions from SEC

6.7 Electrophoretic Mobility Shift Assays (EMSA) for the analysis of RBFOX2–H19 RNAs Interactions

To investigate the interaction between RBFOX2 and specific regions of the lncH19, EMSA were performed using the purified recombinant RRM domain of RBFOX2 and the in vitro transcribed FL H19 and its four distinct RNA fragments. Based on the different electrophoretic mobility of free RNA and RNA–protein complexes, EMSA is a commonly used method for identifying protein-RNA interactions.

Following previously published protocols, each binding reaction was performed by incubating 250 nM of RNA with increasing concentrations of RBFOX2 RRM protein, ranging from 500 nM (2:1 protein:RNA molar ratio) to 6000 nM (24:1). To enable complex formation, the RNA and proteins were incubated in an appropriate binding buffer for one hour at room temperature.

After incubation, the samples were loaded onto a non-denaturing polyacrylamide gel and subjected to electrophoresis. To evaluate their migration in the absence of protein, individual

RNA fragments were run as controls alongside each reaction. The interaction between RBFOX2 RRM and particular regions of the lncH19 transcript was confirmed by visualizing the formation of RNA–protein complexes based on their slower mobility in comparison to the free RNAs.

Several experimental parameters were tested to optimize the EMSA conditions. The final configuration that produced the most repeatable and comprehensible result comprised: protein concentrations of 250 nM (1:1 RNA:protein), 1000 nM (1:4), 2000 nM (1:8), and 6000 nM (1:24); a 3.5% native polyacrylamide gel; a loading buffer containing 50% sucrose; and a running buffer at pH 8. To reduce RNA degradation and preserve complex stability, electrophoresis was performed at 80 V for two hours in a cold room (4°C).

Fragment 1 (FR1), in particular, showed a distinct electrophoretic mobility shift that increased with protein concentration and was suggestive of complex formation.

These observations support the hypothesis that the RBFOX2 interaction with lncH19 is mediated through specific structural or sequence elements present in certain regions of the lncRNA, however, these results should be regarded as very preliminary and need to be further validated and confirmed using other experimental systems.

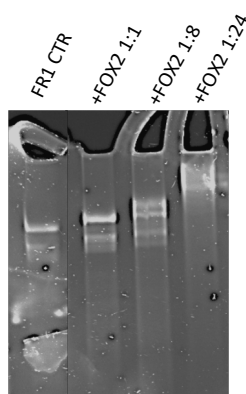


Figure 22. EMSA with FR1 of lncH19. The electrophoretic mobility shift increased with protein concentration with the best result observed with the 1:24 ratio

CHAPTER 7

DISCUSSION AND CONCLUSIONS

CRC represents one of the leading causes of cancer-related deaths worldwide, and its clinical management is often complicated by therapy resistance and tumor heterogeneity. Understanding the molecular mechanisms underlying the progression of CRC, especially those involving post-transcriptional regulation, has received more attention in recent years. AS has emerged as a crucial mechanism contributing to cancer complexity by generating multiple transcript isoforms with unique biological characteristics. Aberrations in AS have been associated with key cancer traits including invasion, EMT, and treatment resistance.

ncRNAs, and in particular lncRNAs, have been increasingly recognized as critical modulators of gene expression that function at multiple levels, including splicing regulation. One of the first discovered and most studied lncRNAs in cancer biology is lncH19. Although it has been linked to a number of carcinogenic processes, its role in the regulation of alternative splicing remained poorly understood.

The data here described revealed another piece of the complex mechanisms through which lncH19 controls the tumor transformation process and the progression of CRC. Like for other lncRNAs, we demonstrated that lncH19 can work as a trans-acting element and control AS. Already in 2010 was described the retention in speckles of the lncRNA MALAT1 where it regulates AS by modulating the levels of active SR proteins [221]. Other lncRNAs control AS in an indirect way, as for the linc01232; it has been shown to interact with the splicing factor hnRNPA2/B1 and stabilize it by preventing ubiquitination and degradation of hnRNPA2/B1 [222]. Building on our experimental and bioinformatic analyses, we propose a model in which lncH19 binds to the 3'UTR of the RAC1 transcript. Additionally, our findings suggest that lncH19 interacts with the SFs RBFOX2 and hnRNPM, bringing them to their respective binding sites that flank exon 3b. As a result, lncH19 impedes the exclusion of exon 3b (Figure 24).

In a study investigating AS of RAC1 in CRC, Gonçalves and colleagues [176] reported that the splicing factor SRSF1 promotes the retention of exon3b, thereby facilitating RAC1B isoform expression. Our research adds further insight into the mechanism that promotes the maturation of RAC1B. Specifically, we demonstrated that SRSF1 operates within a splicing complex that includes hnRNPM and RBFOX2, and that the recruitment of this complex to RAC1 pre-mRNA is mediated by the lncH19.

It appears from our experimental data that the lncRNA binds RBFOX2 and hnRNPM. However the precise structural and functional configuration of the lncH19–splicing factors interaction network remains to be elucidated.

Importantly, the supposed correlation between lncH19 and RAC1B was confirmed by in vivo analyses: tumor samples with elevated lncH19 expression also exhibited higher levels of RAC1B, suggesting a positive correlation between the lncH19 and RAC1B and reinforcing the relevance of our in vitro findings.

It is to be noted that we identified 57 transcripts that not only contain complementary sequences for lncH19, but also display binding sites for the splicing factors RBFOX2 and hnRNPM (Supplementary file 2). Although additional studies will be necessary to validate the involvement of lncH19 in the regulation of each of these targets, our findings suggest that the mechanism proposed for RAC1B splicing may be part of a broader regulatory network. This could help explain the wide-ranging, pleiotropic effects observed upon lncH19 overexpression in CRC cells.

Future research will focus on prioritizing among these 57 candidates those transcripts whose AS is functionally linked to oncogenic transformation or tumor progression. It remains to be clarified whether the model we identified in CRC operates similarly in other tumor types. However, we have developed a strategy to explore potential similarities and identify other targets in tumors that exhibit overexpression of this lncRNA.

The data here presented provided new insights into the mechanisms of action of lncH19, but also highlighted a previously unknown axis through which lncH19 exerts its pro-tumoral activity in CRC. We and others have already associated the expression of lncH19 with an enhanced Wnt pathway activation in CRC models.

In particular, Ding and collaborators demonstrated that lncH19 enhances β -catenin target gene activation by sponging the miR-29b-3p. In our study lncH19 intragenic miRNA (miR-675-5p), promotes β -catenin nuclear localization, controlling Glycogen Synthase Kinase 3 β activity [223]. Moreover, it has also been demonstrated that lncH19 interacting with macroH2A histone variants regulates transcription of cyclin-dependent kinase 8, with a consequent effect on β -catenin activity [224]. Studies conducted in CRC have already shown [225] that RAC1B migrates into the nucleus where it associates with the β -catenin-TCF complex promoting the expression of c-Myc and Cyclin D. Here we demonstrated that through the maturation of RAC1B, lncH19 exerts control on the β -catenin target genes. The over-expression of lncH19 leads to the transcriptional up-regulation of c-Myc and Cyclin D, however, the use of a RAC1 inhibitor is sufficient to bring the expression of these genes back to their basal level.

These results offer the first evidence of a new mechanism through which lncH19 functions as a molecular scaffold, directing SFs to particular transcripts to regulate the splicing patterns in CRC cells. Moreover, the strong correlation between lncH19 and RAC1B expression observed in CRC patient biopsies reinforces the clinical relevance of this axis and suggests its potential as a biomarker or therapeutic target.

The integrated analysis of transcriptomic and proteomic data not only clarified the mechanistic aspects of lncH19 function but also prepared the basis for future investigations into other ncRNA–RBP complexes.

In conclusion, this work advances our knowledge of how lncRNAs coordinate important regulatory networks that affect the development of cancer. The results contribute to the growing body of evidence that recognizes lncRNAs as central players in the post-transcriptional regulation of gene expression, offering potential for new RNA-based diagnostic tests and therapeutic treatments for CRC.

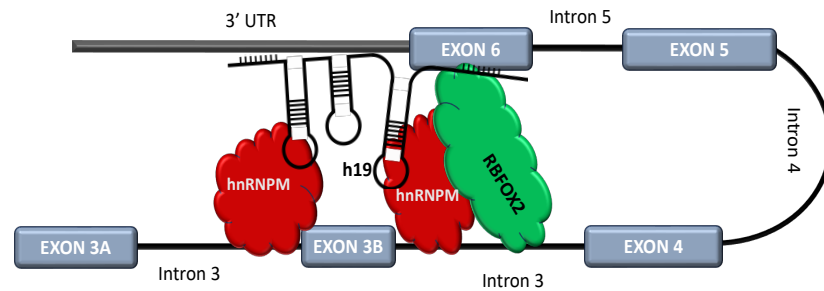


Figure 23. Schematic representation of the proposed model. Representation of binding sites position of the complex lncH19-hnRNPM-RBFOX2 with RAC1 unspliced mRNA

CHAPTER 8

SCIENTIFIC PRODUCTS

8.1 Scientific publications in journals relevant to the topic

- **Cordaro A**, Barreca MM, Zichittella C, Loria M, Anello D, Arena G, Sciaraffa N, Coronello C, Pizzolanti G, Alessandro R, Conigliaro A.
Regulatory role of lncH19 in RAC1 alternative splicing: implication for RAC1B expression in colorectal cancer. *J Exp Clin Cancer Res.* 2024 Aug, doi: 10.1186/s13046-024-03139-z. PMID: 39098911.
- Zichittella C, Barreca MM, **Cordaro A**, Corrado C, Alessandro R, Conigliaro A.
Mir-675-5p supports hypoxia-induced drug resistance in colorectal cancer cells. *BMC Cancer.* 2022 May ; doi: 10.1186/s12885-022-09666-2. PMID: 35596172.

8.2 List of publications or products not related to the project

- Raimondi L, De Luca A, Gallo A, Perna F, Cuscino N, **Cordaro A** , Costa V, Bellavia D, Faldini C, Scilabra SD, Giavaresi G, Toscano A.
Investigating the Differential Circulating microRNA Expression in Adolescent Females with Severe Idiopathic Scoliosis: A Proof-of-Concept Observational Clinical Study.
Int J Mol Sci. 2024 Jan ; doi: 10.3390/ijms25010570. PMID: 38203740
- Giavaresi G, Bellavia D, De Luca A, Costa V, Raimondi L, **Cordaro A**, Sartori M, Terrando S, Toscano A, Pignatti G, Fini M.
Magnesium Alloys in Orthopedics: A Systematic Review on Approaches, Coatings and Strategies to Improve Biocompatibility, Osteogenic Properties and Osteointegration Capabilities.
Int J Mol Sci. 2023 Dec ; doi: 10.3390/ijms25010282. PMID: 38203453
- De Luca A, Ruggiero R, **Cordaro A**, Marrelli B, Raimondi L, Costa V, Bellavia D, Aiello E, Pavarini M, Piccininni A, Tatullo M, Boanini E, Paduano F, Giavaresi G.
Towards Accurate Biocompatibility: Rethinking Cytotoxicity Evaluation for

Biodegradable Magnesium Alloys in Biomedical Applications.

J Funct Biomater. 2024 Dec ; doi: 10.3390/jfb15120382. PMID: 39728182

- Costa V, De Fine M, Raimondi L, Bellavia D, **Cordaro A**, Carina V, Alessandro R, Pignatti G, Fini M, Giavaresi G, De Luca A.
Timing Expression of miR203a-3p during OA Disease: Preliminary In Vitro Evidence.
Int J Mol Sci. 2023 Feb ; doi: 10.3390/ijms24054316. PMID: 36901745
- Bellavia D, Costa V, De Luca A, **Cordaro A**, Fini M, Giavaresi G, Caradonna F, Raimondi L.
The Binomial "Inflammation-Epigenetics" in Breast Cancer Progression and Bone Metastasis: IL-1 β Actions Are Influenced by TET Inhibitor in MCF-7 Cell Line.
Int J Mol Sci. 2022 Dec ; doi: 10.3390/ijms232315422. PMID: 36499741
- De Luca A, Presentato A, Alduina R, Raimondi L, Bellavia D, Costa V, Cavazza L, **Cordaro A**, Pulsatelli L, Cusanno A.
Hydrothermal Surface Treatment of Mg AZ31 SPF Alloy: Immune Cell Biocompatibility and Antibacterial Potential for Orthopedic Applications
Metals 2025 Dec ; doi: 10.3390/met15121328 PMID: 36499741

8.3 Abstracts, posters and oral presentation presented at scientific congresses

- The Biennial Congress of the Italian Association of Cell Biology and Differentiation 2023– Paestum (SA), ITALY, **poster** presentation: **Cordaro A**, Barreca MM, Loria M, Zichittella C, Fontana S, Alessandro R, Conigliaro A.
The long non-coding RNA H19 regulates alternative splicing in colorectal cancer (September 2023)
- European Molecular Biology Laboratory Symposium 2023- Heidelberg, GERMANY, **poster** presentation: **Cordaro A**, Barreca MM, Loria M, Zichittella C, Alessandro R, Conigliaro A. *Intragenic miR-675: molecular mechanisms for miRNA maturation*. (October 2023)

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