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# The polyamine/hypusine signaling promotes colorectal cancer cell growth by regulating c-Myc translation

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# ABSTRACT

Colorectal cancer is the most frequent malignancy of the gastroenteric system and it is the third most common cancer among males and the second among women. The development of colorectal cancer is determined by progressive genetic and epigenetic alterations which, starting from the formation of adenomatous polyps, lead to the formation of malignant lesions. The mutations include pathways regulatory genes involved in development, proliferation and survival of cancer cells, such as APC/beta catenin, Ras-BRAF/MAPK, p53, pI3K/AKT and SMAD/TGFbeta. In most cases, the aberrant activation of these pathways converges in the overexpression of the oncogene c-Myc. Therefore, inhibition of this oncogene could represent an effective anti-cancer strategy in many types of colorectal cancer. Previous studies have shown that the hypusination, post-translation modification that activates the elongation factor EIF5A, plays an important role in tumour progression. This modification is mediated by two enzymes: DHPS and DOHH that use spermidine as a substrate, an over-expressed polyamine in many solid tumours.

The aim of this project was to understand the role of hypusination pathway in CRC and the effect of its pharmacological and genetical targeting.

We have shown how inhibition of the EIF5A hypusination, induced by treatment with GC7 or by gene ablation of DHPS in colorectal cancer cells (HCT116, HT29, SW480 and LoVo), has a strong anti-proliferative effect on CRC cells *in vitro* and *in vivo*. By analysing the activation status of major pathways involved in tumour onset and progression, we have shown that the DHPS-EIF5A1 axis regulates c-Myc protein levels. The regulation does not alter the mRNA levels or the stability of c-Myc but its translation. Through multi/single site mutagenesis experiments we identified the region of c-Myc involved in EIF5A-mediated regulation.

In conclusion, this work shows that the oncogene c-Myc is a translation target of the elongation factor EIF5A; the inhibition of the hypusination causes the blocking of the translation of this oncogene and a decrease in colorectal cancer cells proliferation *in vitro* and *in vivo*. Such evidence could be the basis for new therapeutic approaches in colorectal tumours based on the blocking of c-Myc translation by DHPS pharmacological or genetical inhibition.

# INTRODUCTION

## COLORECTAL CANCER

### Incidence and development

Colorectal cancer accounts for 44% of all cancer cases in men and 50% in women, constituting one of the main causes of death along with other types of cancer (lungs and bronchi, prostate and breast respectively, for men and women). The decline in colorectal cancer incidence since the mid-1980s has been attributed to changing risk factors and introducing screening [1]. The incidence of colorectal cancer and the mortality rate fell by about 3% in both men and women from 2003 to 2012. Unfortunately in recent years there has been a significant new increase in incidence [2].

Colorectal cancer begins as a benign adenomatous polyp that develops in an advanced adenoma with high degree of dysplasia and eventually progresses to becoming invasive cancer [3]. Invasive cancer develops through four different stages: in stages I and II the cancer cells are confined within the walls of the colon and the disease can be cured through surgical resection; if untreated, the cells reach the lymph node region (stage III) and in this case the disease is treatable for 70% of patients with a combination of surgery and adjuvants chemotherapy [4] [5] [6]. In stage IV cells metastasize to distant sites [6] [7] and the disease is generally incurable [5] [6] although recent advances in chemotherapy have increased survival time [8].

## Colon structure

The cells from which colorectal cancer originates are located at the base of the so-called Lieberkuhn crypt (Figure 1). The crypt represents the fundamental structural unit of the colon and is composed of stem cells, transient amplification cells, terminally differentiated chalice cells, enterocytes and endocrine cells [9] [10]; at the bottom undifferentiated cells reside, while those terminally differentiated lie near the top of the crypt; thus exists a “development hierarchy ” from the bottom (undifferentiated) to the top (differentiated). The process by which cells perform this continuous cycle from undifferentiated to differentiated, moving through crypt regions, is controlled by colon stem cells and surrounding microenvironment [10] [11]. The epithelium of the colon is renewed approximately every 5 days [9]. Within the Lieberkuhn crypt mainly three different pathways act (Sonic Hedgehog, WNT/β catenin, TGFβ/BMP and Notch) that are deregulated in most colorectal tumors. These pathways are interconnected and share some common targets, such as c-Myc.

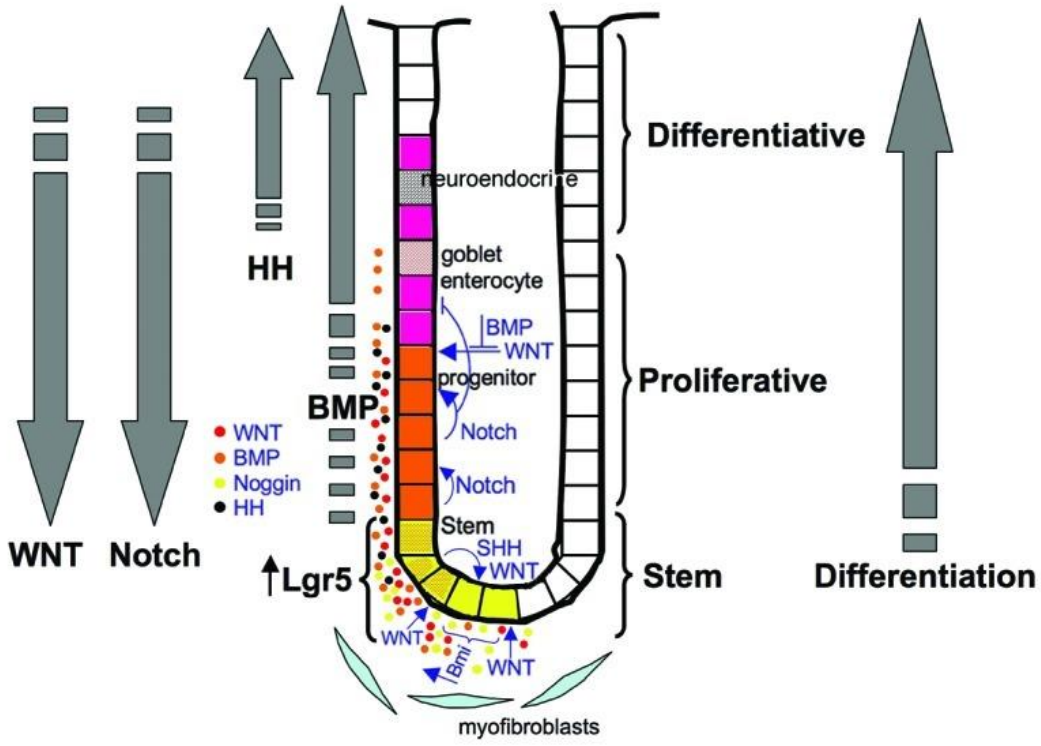


Figure 1: Signaling in the colon crypt**:** *[9]*

# MOLECULAR BASIS OF COLORECTAL CANCER

## Alteration of molecular pathway

The aberrant activation of the Wnt pathway is the most important cause of colorectal cancer [12] [13] [3] (Figure 2). In the Wnt pathway the oncoprotein beta-catenin binds its nuclear partner TCF/LEF creating a complex transcription that regulates genes involved in cellular activation [12] [13] [3]. The degradation complex of the beta-catenin is useful to control and balance its levels: one of the components of this complex is APC which inhibits its nuclear localization [8]. The most common mutation in colorectal cancer inactivates the gene encoding for the APC protein, in the absence of which Wnt signaling is inappropriately and constitutionally activated [8]. The mutation of APC in the germ line determines the familiar

adenomatous polyposis or the syndrome of hereditary predisposition to colorectal cancer (FAP) (Figure 4); in the carriers of this mutation the risk of developing the disease, from the age of 40, is approximately 100% [13] [3] [14]. The somatic mutations and inactivation of both alleles of APC are present in many sporadic adenomas and colorectal tumours [13] [3]. In a small subgroup of wild-type APC tumours, it is possible to find mutations affecting the beta-catenin making it insensible to its degradation complex; also in this case the Wnt pathway is constitutively active [13] [3] [15].

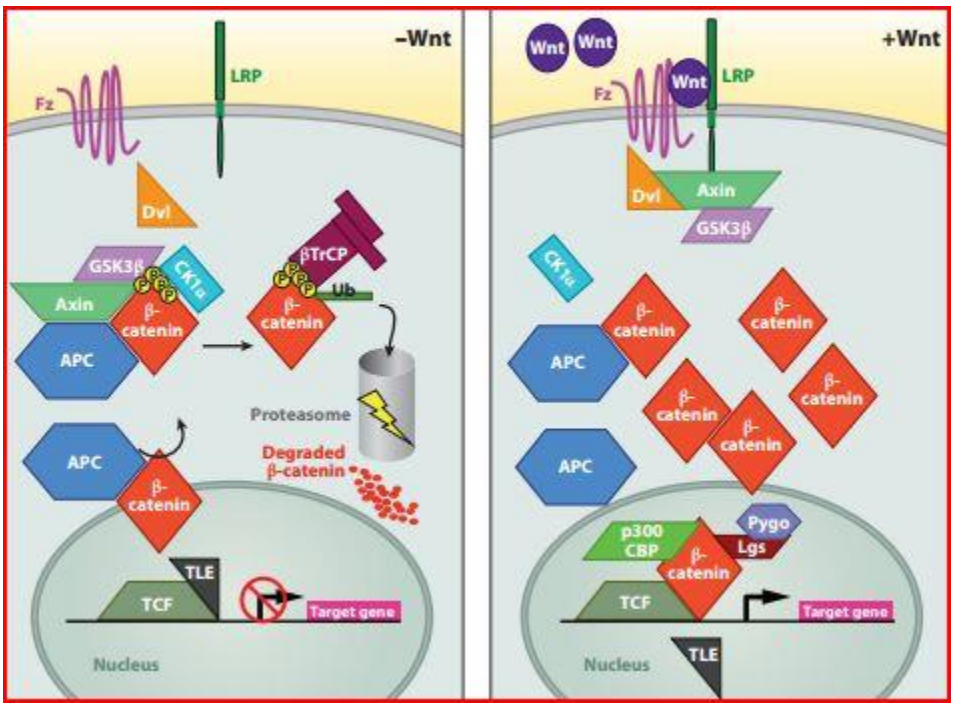



Figure 2: Wnt pathway activation/suppression [16]

The inactivation of p53 pathway by TP53 mutation is the second genetic step in colorectal cancer. In most tumours, both alleles of TP53 are inactivated [8] . P53 wild-type mediate the stop of the cell cycle and it is a death checkpoint that can be triggered by different cellular stress [17]. Inactivation of TP53 often coincides with transition from adenoma to invasive carcinoma [18] (Figure 3).

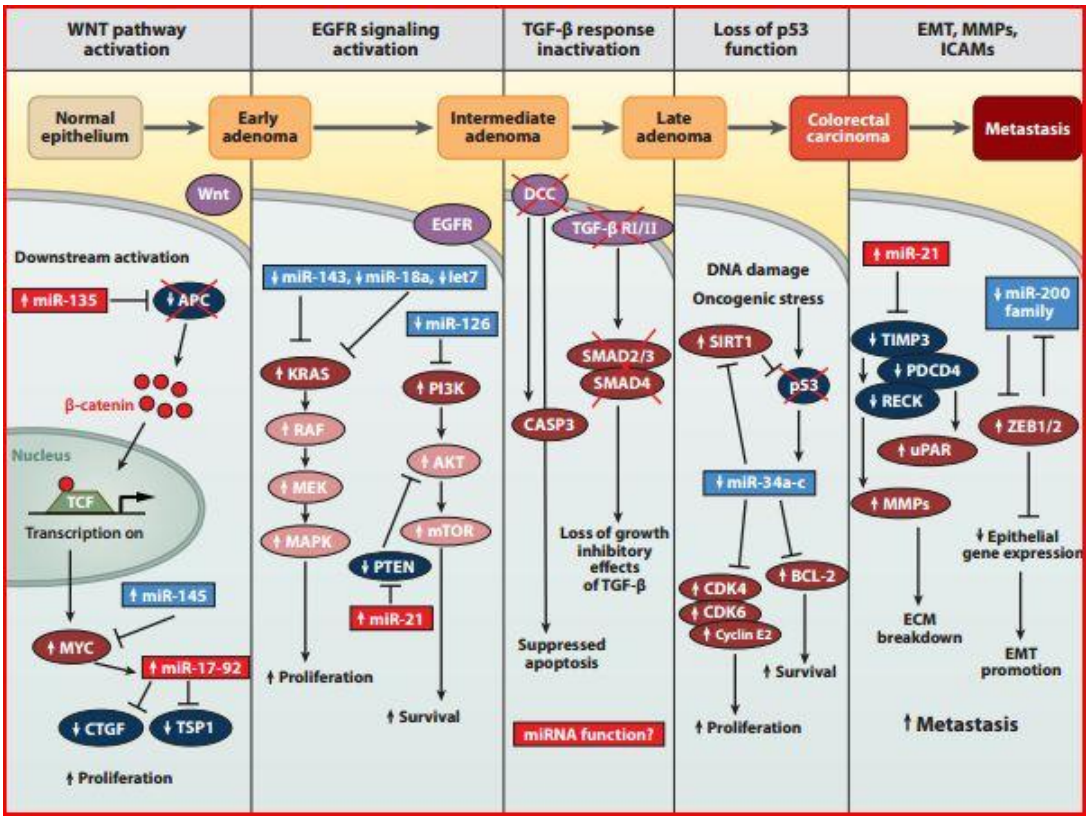



Figure 3: CRC progression from adenoma to invasive tumor [16]

The third step for colorectal tumour progression is the inactivating mutations of the TGFβ/BMP pathway [19]. In general, this signaling regulates the proliferation and differentiation of a wide range of cellular types. In a tumour context it behaves ambivalently: by inhibiting cell proliferation and promoting apoptosis in the early stages of the disease while, in later stages, promotes invasion, metastasis, angiogenesis and suppresses immune response [20]. Specifically for colorectal cancer, mutations in this pathway may be related to the TGFBR2 receptor (inactivation mutations in the somatic line) [19] [21] [8] [22] [23] or may affect pathway downstream components such as SMAD4 or its transcriptional factors partners SMAD2 and SMAD3 [24] [19] [25] [21] [26] [22] [27] [23]. The mutations inactivating the TGFβ/BMP pathway coincide with the transition from high-grade adenoma to dysplasia, or carcinoma [28] (Figure 3). Some oncogenes play a key role in colorectal cancer progression; for example, mutations of oncogenes such as RAS and BRAF, which activate

MAPK pathways, are found in 37% and 13% of colorectal cancers, respectively [29] [30] [31] [32] [33].

One third of colorectal tumors are characterized by somatic mutations activating PI3KCA, which encodes for the PI3K catalytic subunit [34]. PI3K signaling regulates cell growth, proliferation, differentiation, apoptosis, metabolism and cellular motility [20]. Less common genetic alterations, which may replace those in PI3KCA, affect other components of PI3K signaling such as PTEN, IRS2, AKT and PAK4 [35] [8]. In a subset of colorectal tumors the importance of signaling through the EGFR receptor (it promotes cell proliferation, invasion, metastasis, confers resistance to apoptosis and induces angiogenesis) has been demonstrated [20]. The EGFR receptor is bound by the soluble EGF protein (epidermal growth factor) and mediates its action by activating the MAPK and PI3K cascades [36] [8] . Another important element promoting the growth and metastasis of colorectal cancer is the vascular endothelial growth factor VEGF. This is produced as a result of tissue damage or during normal tissue growth, promoting the formation of new blood vessels (angiogenesis).

## Loss of genomic stability

Among the molecular causes of colorectal cancer is the loss of genomic stability which facilitates the acquisition of multiple mutations associated with the tumor itself. In the context of this disease, genomic instability is manifested in various forms [37] [38] [8]: the most common type is chromosomal instability which, generally, causes changes in the number of copies and structure of chromosomes [38]; it is caused by mutations that inactivate genes that would normally be responsible for maintaining chromosomal stability during replication [39]. Chromosome instability is a very efficient mechanism in causing the loss of a wild-type copy of a tumor suppressor gene such as APC, P53 and SMAD4 [12] [40] [3]. Unlike other

types of cancer, colorectal cancer generally does not imply an increase in the number of copies of genes [25] or their rearrangement [8].

In a subgroup of colorectal cancer patients inactivation of genes, required for the repair of mismatches in DNA, may occur; these modifications may be inherited, such as in hereditary nonpolyposis colorectal cancer (HNPCC), or acquired; in the latter case there is silencing, associated with aberrant methylation, of the genes coding for mismatch repair proteins [8] (Figure 4). In patients with HNPCC, the defective germ line in mismatch repair genes, mainly MLH1 and MSH2, gives a risk of contracting cancer of about 80%, with manifestations of this disease from an average age of 45 years [41] [42] [43] [44] [14] [45]. The loss of mismatch repair function in patients with HNPCC is due not only to mutations in repair genes in the germ line, but also to inactivation of parental allele in the somatic line [41]; this situation dramatically accelerates the development of cancer in patients with HNPCC: some tumors appear even within 36 months after a negative colonoscopy result [46]. For this reason, annual colonoscopies are recommended for HNPCC mutation carriers [46] [14]. The somatic inactivation of mismatches repair genes occurs in approximately 15% of patients with not familiar colorectal cancer; in these patients the biallelic silencing of the MLH1 gene promoter region, due to methylation events, inactivate DNA repair function [47] [48] [49].

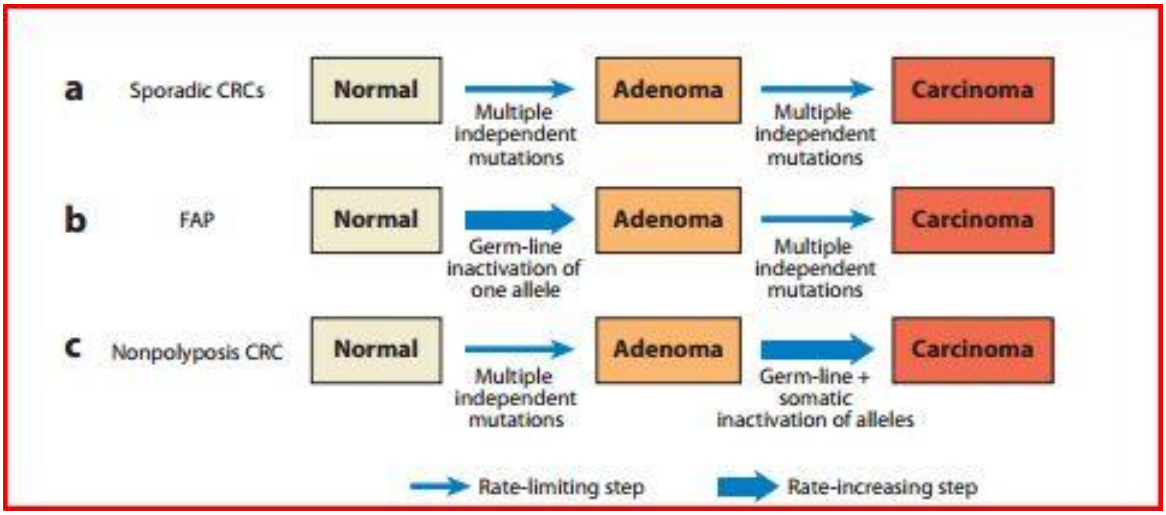



Figure 4: different types of CRC [16].

The loss of mismatch repair function is easily recognised due to the associated phenomenon of microsatellite instability, where the inability to repair DNA within repeated sequences, changes the size of mononucleotide or binucleotide repetitions (microsatellites) that are within the genome [8]. Tumors characterized by inability to repair mismatches occur primarily in the proximal colon and, in sporadic cases, are associated with older age and female sex [14]. In cases where the mismatches repair system is compromised, tumor suppressor genes such as those coding TGFβ, its receptor (TGFBR2) and others, which have functional regions that contain mononucleotide or binucleotide repetitions, can be inactivated.

Another genetic condition that may lead to the onset of colorectal cancer involves inactivation in the germ line of the *mutY* homologous gene (*MUTYH* or *MYH*), which function is the repair of excision of bases modification [50] [51]. The MYH protein's role is to removing from the DNA the 8-oxoguanin produced by the oxidative damage to guanine [50] [52] [51]. People who are carriers of *MYH* gene inactivation on both alleles in the germ line develop a polyposic phenotype and almost 100% of these cases evolve into colorectal cancer from the age of 60 years [51]. *MYH* inactivation in the somatic line has not been detected [8].

Epigenetic silencing of genes, mainly mediated by aberrant DNA methylation, is another mechanism of gene inactivation in patients with colorectal cancer [53] [54]. A methylated form of cytosine, in which a methyl group is bound to carbon 5 (5-methylcytosine), constitutes 1/5 of the DNA bases and is introduced by methyltransferases modifying the cytosines of dinucleotide CpG [53]. In the normal genome, cytosine methylation occurs in areas of repeated DNA outside the exons, excluding CpG islands in the promoter regions of half of all genes [53]. On the other hand, in colorectal cancer, there is a modest but global reduction of cytosine-methylated and an increase of the same phenomenon of methylation within the CpG islands in promoters [53] and this can lead to aberrant silencing of the

involved genes [53]. In cases of sporadic colorectal cancer with microsatellites instability, somatic epigenetic silencing may block the expression of *MLH1* [53] [8].

# C-MYC: CHARACTERISTICS AND ROLE IN THE CRC

## Protein Structure and functions

The gene encoding for the c-Myc protein is found on chromosome 8 and consists of three exons [55] [56]. There are two variants of this protein, which are produced from two different start of translation sites: a 64KDa polypeptide, translated from a canonical codon AUG, and another longer polypeptide (67KDa) produced from a not canonical site CUG, placed 15 codons upstream respect to AUG [56] [57]; there is also an internal site of beginning of the translation that causes a polypeptide of 45KDa [56] [58]. Analysis of the protein c-Myc have highlighted the presence of a domain of transactivation within the N-terminal region and a dimerization domain at the C-terminal; the latter is classified as a helix-loop-helix leucine zipper (HLH-LZ) [56]. Near the dimerization site there is a domain rich in basic amino acids that serves to contact specific DNA sequences [59] [60] [61] [62] [63] [64] [65] [66]. c-Myc can bind to the DNA of its target genes by forming a heterodimer with its molecular partner Max, by means of the HLH-LZ domain [56] (Figure 5).

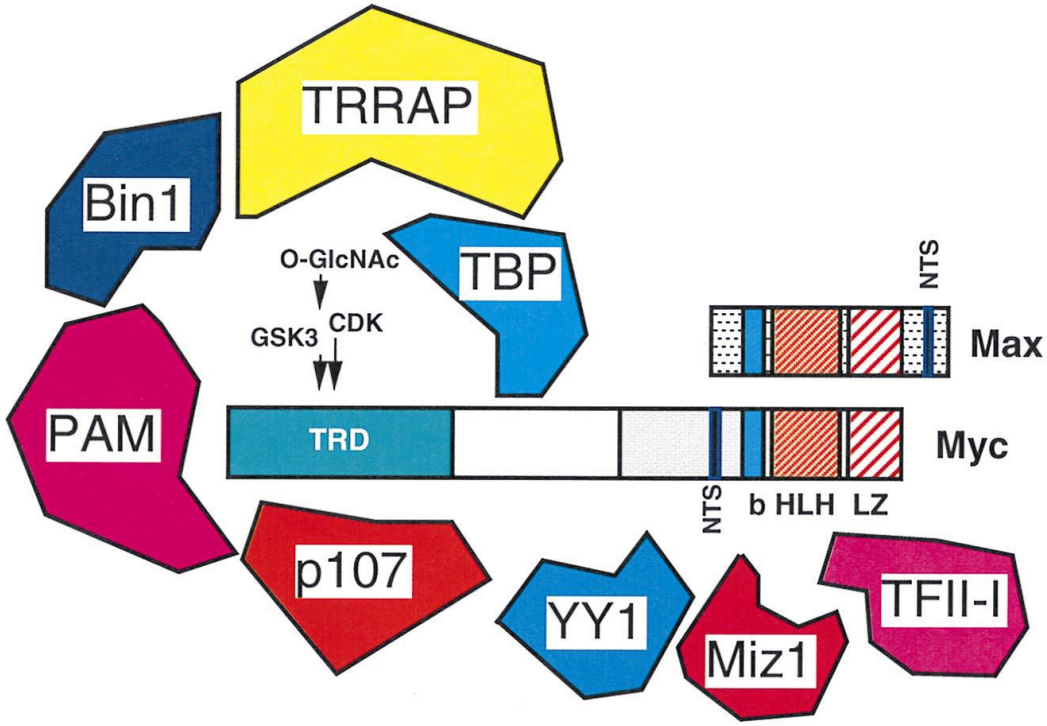



Figure 5: c-Myc associates with Max via the HLH-LZ domain. NTS -nuclear localization site, TRD-transactivation domain [56].

c-Myc plays different roles within the cell and is involved in many processes including apoptosis, proliferation, cell cycle progression and DNA replication (Figure 6). In the small intestine c-Myc is important for the survival and growth of enterocytes [67]; this has been demonstrated by the use of conditional protein mutants [68] [69]: intestinal cells deficient in c-Myc proliferate less than wild-type [69].

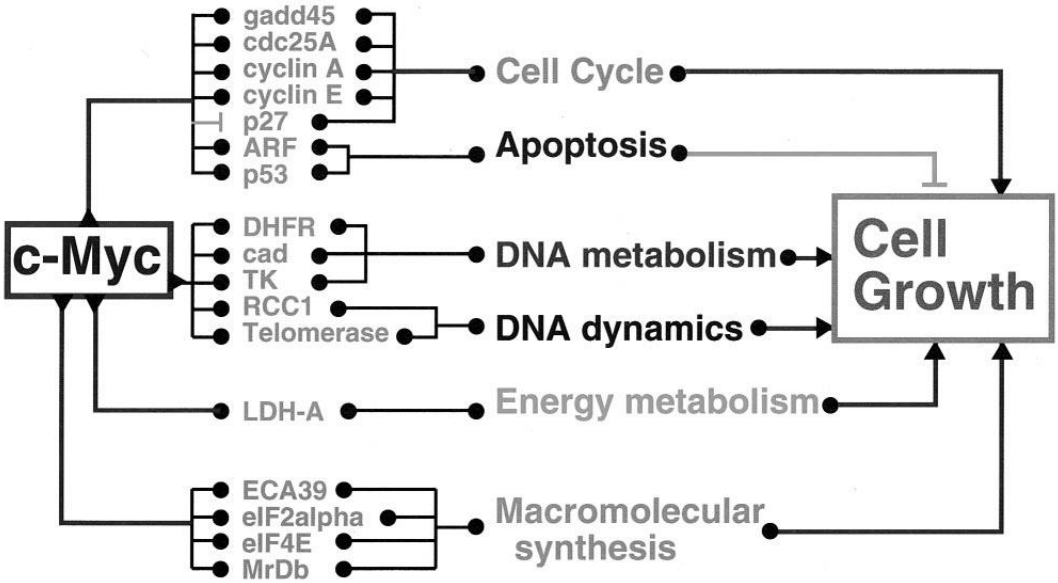



Figure 6: Interconnection between c-Myc, its putative target genes and cell cycle [56]

## c-Myc in colorectal cancer

The overexpression of c-Myc and the activation/suppression of its targets is commonly found in colorectal cancer [70]. Evidence of the importance of the protein in this pathology has been shown that deletion of the c-Myc gene causes inhibition of tumour progression in mouse models that perfectly mimic human disease [70]. In addition, the c-Myc protein is a target gene of the Wnt pathway (Figure 7), one of the major deregulated signaling in colorectal cancer. Indeed, it has been shown that the c-Myc promoter contains an element of response to Wnt pathway (WRE) and that its expression contributes to determining the tumour phenotype due to APC mutations [71]; in fact, the deletion of both APC and c-Myc allows cells to proliferate and differentiate just like wild-type [67]. The expression profiles of knockout cells for these two proteins indicate that about 1/3 of the genes upregulated after APC loss require the intervention of c-Myc: this might suggest that c-Myc is the “driving force” in colorectal tumor after the Wnt pathway hyperactivation [67]. To support this thesis

there is evidence that the 50% reduction in the expression of c-Myc attenuates the phenotype due to the loss of APC, reducing colorectal tumorigenesis [72].

In CRC, as in other cancers, the cell replication process is deregulated [73]. Also in this context, c-Myc plays a key role since cyclin D2 and its molecular partners cdk4/6, important mediators of cell cycle, are located downstream of its signaling [74] [67] (figure 3). It has been shown that in CRC the overexpression of cycline D2 and cdk4/6, following the loss of APC, is dependent on the expression of c-Myc [67] and also the hyperproliferation that accompanies the loss of APC is partly due to the presence of cycline D2 and cdk4/6. C-myc also drives tumor growth by inhibiting the suppressor of proliferation p21 [75] by binding directly to its promoter and blocking its transcription.

Given the key role of c-Myc in regulating many critical functions of the cell, it is not surprising that its intracellular levels are subject to fine regulation; in fact its presence and its activity are regulated at the transcriptional, translational and post translational level by enzymes, miRNA and other proteins [67].

In conclusion c-Myc appears as a key factor in colorectal tumorigenesis and cooperation with the Wnt pathway is critical to the onset of the disease. During tumour progression, mutations of additional factors, such as P53, may contribute to increase its activity levels; under physiological conditions P53 causes apoptosis in cells with high levels of c-Myc. Global deregulation of c-Myc functions results in the activation of a large number of target genes involved in the processes of invasion, angiogenesis and tumour metastasis [67].

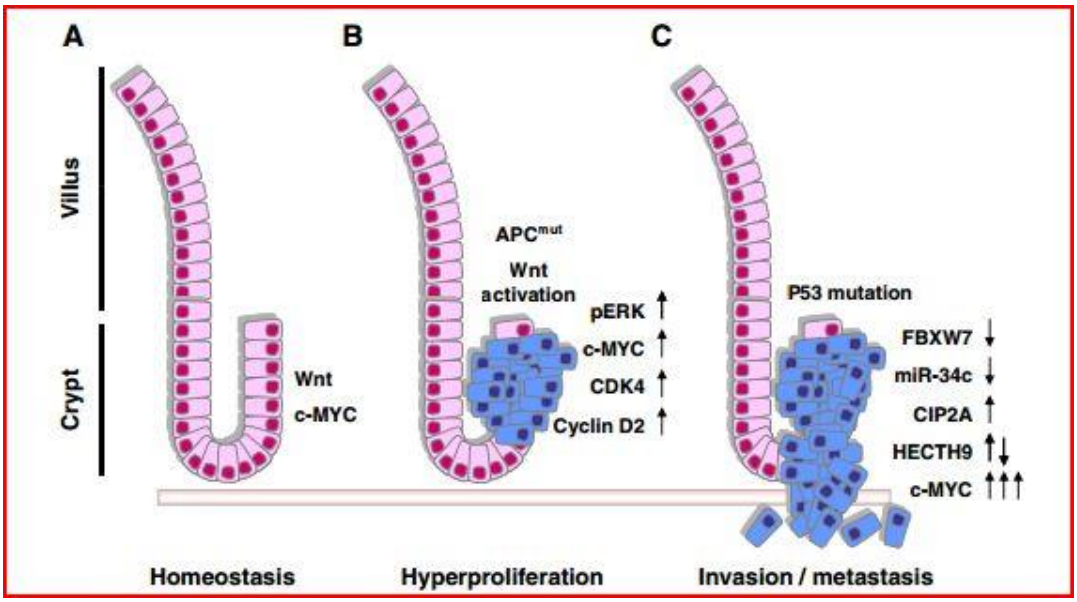



Figure 7: Role of Wnt/c-Myc signaling during CRC progression [67]

# POLYAMINE

## Metabolism, Mechanism of Action and Role in Tumorigenesis

Polyamines are ubiquitous, small, positively charged polycations required for both eukaryotic and prokaryotic cellular growth and differentiation. They are low molecular weight, water soluble, aliphatic amines, with pK values between 8.3 and 10.9. Polyamines bind negatively charged molecules under physiological conditions, including DNA, RNA, ATP, proteins or phospholipids [76] [77] and are important regulators of various physiological processes. The naturally occurring polyamines spermine and spermidine are the biosynthetic products of putrescine, which is derived from ornithine by the action of ornithine decarboxylase (ODC), one rate-limiting enzyme, together with S-adenosyl-methionine decarboxylase(SAMDC) [78] [79] [80] (Figure 8).

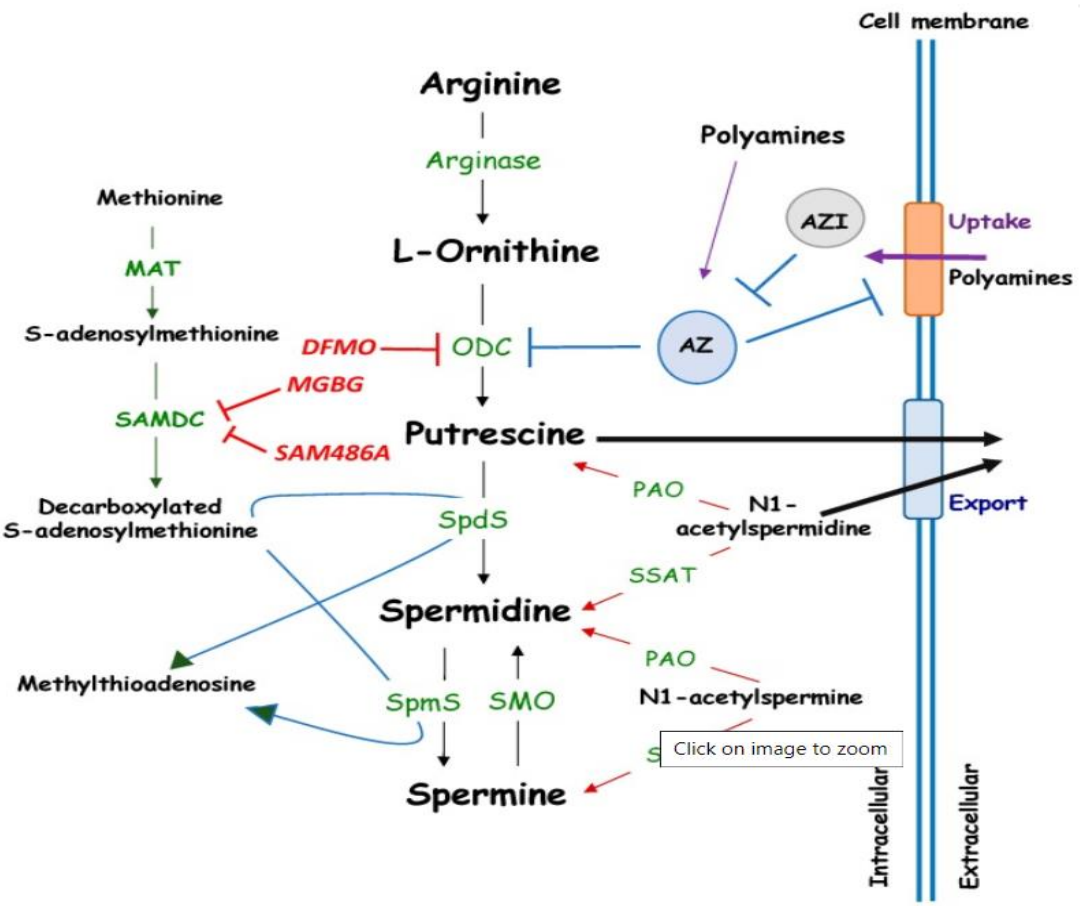



Figure 8: Metabolism of polyamines. MAT: Methionine adenosyl-transferase; ODC: Ornithine decarboxylase; AZ: Antizyme; AZI: Antizyme Inhibitor; PAO: Polyamine oxidase; SAMDC: S-Adenosyl-l-Methionine Decarboxylase; SMO: Spermine oxidase; SSAT: Spermidine/Spermine-N(1)-acetyltransferase; SpdS: Spermidine Synthase; SpmS: Spermine Synthase. Pharmacological inhibitors of ODC (DFMO) and SAMDC (MGBG, SAM486A) are indicated in red [80].

During cancer cell growth, the polyamine biosynthetic pathway is very active and polyamine content is increased in tumor cells and tissues, such as breast, colon, skin and prostate cancers [81]. The elevated polyamine content is often linked to increased putrescine synthesis by ornithine decarboxylase, as well as to increased polyamine uptake [82]. Several studies indicate that depletion of polyamines leads to inhibition of tumor growth [83] [84] [85] [80].

A key role in regulating intracellular polyamine homeostasis is played by the ornithine decarboxylase enzyme (ODC), which is tightly regulated at multiple levels in normal and

cancer cells to promptly adjust the levels of polyamines in response to the specific cellular needs [86] [80]. A first level of control of ODC content is through its stability. Indeed, ODC is a very short-lived protein and its rapid turnover is mediated by the proteasome in a ubiquitin-independent manner [87]. A protein called antizyme (AZ) associates with ODC and increases its targeting to the proteasome and consequent degradation [88]. Interestingly, AZ synthesis is induced by polyamines, leading to enhanced association to ODC monomers to form heterodimers and preventing the formation of functional ODC homodimers [87] [80]. A second level of control of ODC content is through its transcriptional regulation. ODC mRNA levels are regulated by various cues and transcription factors, being the oncogene c-Myc the best-characterized and more relevant regulator [89] [90]. Indeed, the promoter region of the *Odc* gene contains two canonical E boxes (CACGTG) that bind c-Myc/Max transcription factors. Consistently, increased ODC expression is observed when c-Myc is upregulated, such as in cancer [91] [92] [80]. A third level of control of ODC expression is via its CAP dependent or IRES dependent translation [93] [94] [80].

Another key regulator of polyamine metabolism with relevance in tumor disease is the SAMDC enzyme, which catalyses the decarboxylation of S-Adenosylmethionine (SAM) into decarboxylated SAM (dc-SAM). Dc-SAM is the aminopropyl donor for the synthesis of spermidine and sperimine, catalysed by SpdS and SpmS respectively. SAMDC has been recently found upregulated by mTORC1 in prostate cancer via phosphorylation-mediated stabilization, thus providing an important link between the oncogenic nutrient-sensing machinery and polyamine metabolism and suggesting the potential therapeutic benefit of its targeting [80].

## Polyamines and translation

Within cells, polyamines contribute to both the efficiency and fidelity of protein synthesis. In addition to directly acting on the translation apparatus to stimulate protein synthesis, the polyamine spermidine serves as a precursor for the essential post-translational modification of the eukaryotic translation factor 5A (EIF5A), which is required for synthesis of proteins containing problematic amino acid sequence motifs, including polyproline tracts, and for termination of translation [95].

In humans, the two isoforms of eukaryotic translation initiation factor 5A (EIF5A), EIF5A1 and EIF5A2, are the only two eukaryotic proteins that undergo a post-translational modification coined hypusination. This unique modification involves the unusual amino acid hypusine, and is mediated by two highly conserved and essential enzymes, deoxyhypusine synthase (DHPS) and deoxyhypusine hydroxylase (DOHH) [96] [97] [98] (Figure 9).

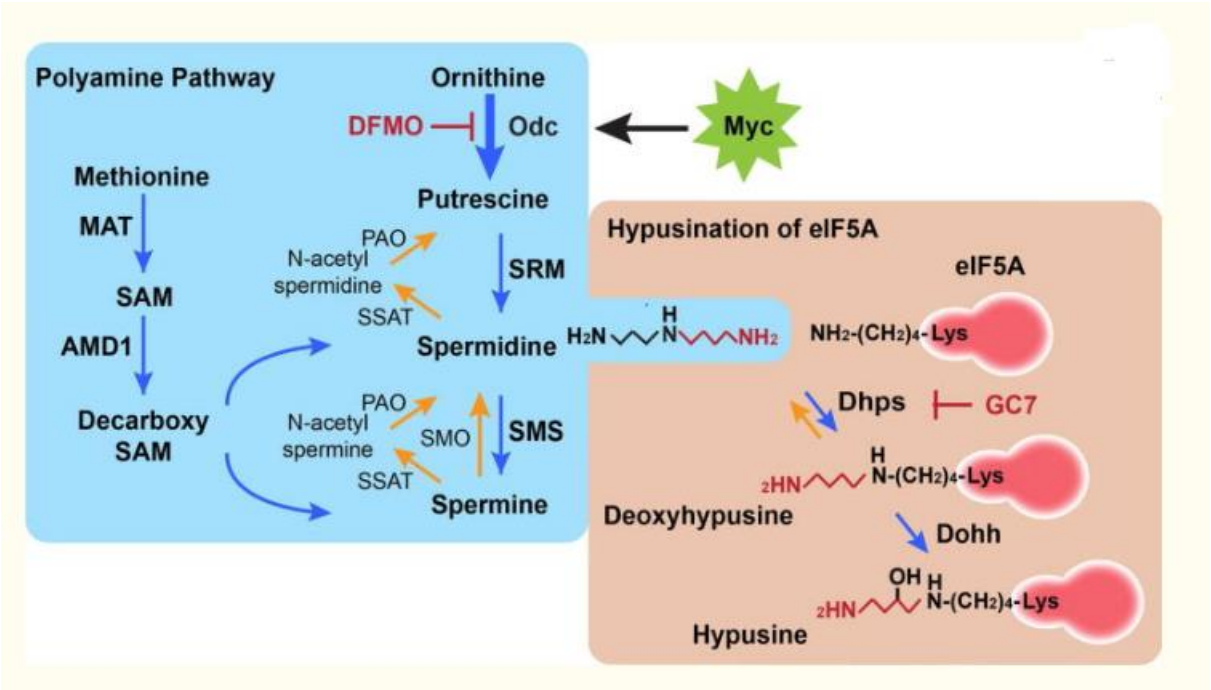



Figure 9: The polyamine-hypusine pathway. MAT-methionine adenosyl transferase, SAM-S-adenosyl-l-methionine, AMD1-AdoMet decarboxylase, SRM-spermidine synthase, SMS-spermine synthase, SMO-spermine oxidase, SSAT-spermidine/ spermine N1-acetyltransferase, PAO-polyamine oxidase [99].

First, the 4-aminobutyl moiety of the polyamine spermidine is transferred to the epsilon amino group of a specific EIF5A lysine residue (lysine-50 in human EIF5A) by DHPS to form

deoxyhypusinated EIF5A. Second, deoxyhypusine is hydroxylated by DOHH to generate hypusinated EIF5A. Like DHPS and DOHH, EIF5A is also conserved in all eukaryotes, underscoring its importance for development, growth and survival [99].

EIF5A1 and EIF5A2 proteins have 84 % homology, yet these isoforms have distinct patterns of expression, where EIF5A2 is expressed in select tissues, such as the brain and testis, while EIF5A1 is ubiquitously expressed. Furthermore, the *EIF5A2* gene is located in chromosome 3q26.2, a region that is frequently amplified in lung squamous, ovarian, esophageal, gastric, bladder, colorectal, breast, pancreatic and liver cancers [99].

EIF5A1 is increasingly recognized as a critical regulator of tumor cell growth. Furthermore, some EIF5A1 translational targets appear to directly link EIF5A to tumor development and identifying such targets will advance our knowledge of the roles of hypusination in cancer [99].

## Clinical correlations

The expression of EIF5A1 and EIF5A2 is highly correlated with patient prognosis in many tumor types. For example, in a study comparing the expression of different genes from sporadic colorectal cancer (CRC) patients with first diagnosis less than 50 years of age vs. normal colon mucosa tissues [100], EIF5A1 was one among the top four up-regulated genes in colon tumors. In addition, elevated levels of EIF5A1 were associated with poor prognosis in early onset CRC. Immunohistochemistry (IHC) analysis of human gastric cancer (GC) and its adjacent normal tissues revealed that elevated levels of EIF5A2 and its potential target metastasis-associated protein (MTA1) correlate with more advanced stages of disease, and with lymphovascular invasion [101]. Furthermore, elevated levels of EIF5A2 are highly correlated with poor prognosis of GC patients and with advanced clinical stage in non-small cell lung carcinoma [102] and ovarian cancer [103]. Therefore, EIF5A2 may serve

as a prognostic marker of some tumor types. Finally, survival analysis of neuroblastoma has shown that high levels of DHPS expression correlates with poor prognosis [104] [99].

## The polyamine-hypusine circuit as a therapeutic target in cancer

Agents that block the function of the key enzymes of the circuit, DHPS, and DOHH, as well as their substrates spermidine and EIF5A, markedly impair tumor cell growth, progression and maintenance of the malignant state. Hypusination of EIF5A is the sole role of DHPS and DOHH, and no other enzymatic targets have been found. Thus, the polyamine-hypusine pathway is attractive as a prognostic, prevention and therapeutic target [99]. SNS01-T is a polyethylenimine (PEI)-based nanoparticle containing both siRNA targeting EIF5A1 and an overexpression plasmid expressing the non-modifiable EIF5A-K50R mutant under the regulation of B-cell specific promoter [105]. This nanoparticle was developed based on the finding that overexpression of unhypusinated EIF5A induces apoptosis [106]. SNS01-T has been shown to impair tumor growth in xenograft models of B-cell cancers (multiple myeloma, mantle cell lymphoma, and diffuse large B-cell lymphoma), and is currently being tested in a Phase1b/2a clinical trial for these B-cell cancers. In addition, combinatorial treatment of SNS01-T with the current standards of adjuvant therapy, lenalidomide or bortezomib, have shown synergistic effects and enhanced survival relative to the treatment with either drug alone [99].

Several studies have shown that the DHPS inhibitor GC7 inhibits the growth of many cancer types. Furthermore, a few combinatorial treatments with GC7 have been tested. For example, patients with chronic myeloid leukemia (CML) are commonly treated with imatinib, which inhibits BCR-ABL tyrosine kinase. Interestingly, proteomics analyses of imatinib-treated BCR-ABL-positive leukemia cells (K562 cells) revealed the down-regulation of

EIF5A1 [107]. Furthermore, co-treatment of imatinib and GC7 or with siRNA targeting EIF5A1 showed synergistic effects in inhibiting leukemia cell proliferation.

Although GC7 is commonly used to inhibit DHPS enzymatic activity, its clinical utility is unclear given concerns regarding specificity of this agent. Specifically, GC7 is spermidine analogue, and thus, it can potentially affect other key targets of spermidine, including SAT1 (spermidine/spermine N(1)-acetyltransferase), a key regulator of polyamine catabolism [108], inward rectifying Kir2.1 potassium channels [109], and flux through the autophagy pathway [110]. Indeed, GC7 has documented effects on autophagy when used at higher concentrations (200 μM) [111] [99].

CPX and DEF inhibit DOHH, which, in turn, inhibits hypusination of EIF5A, and both drugs have anti-proliferative, anti-tumor [112] and anti-angiogenic [113] effects. Specifically, CPX impairs lymphangiogenesis by inhibiting tube formation of lymphatic endothelial cells, possibly through suppression of a VEGFR3-mediated ERK signaling pathway [114]. Based on these promising results, the iron chelators CPX and DEF are being considered as potential therapeutic agents for treating cancers. Indeed, CPX has been tested in the phase 1 clinical trial for individuals with relapsed or refractory hematologic malignancies, and these patients displayed some haematological improvements [115]. However, as an iron chelator, CPX has many cellular targets, for example, iron-dependent enzymes, such as ribonucleotide reductase [116], and the Wnt signaling pathway [117]. Thus, the anti-tumor activity of CPX most likely reflects pleiotropic effects on diverse cellular pathways [99].

Among the inhibitors of polyamine synthesis there is DFMO which is a specific inhibitor of ornithine decarboxylase and currently it has been clinically tested in gliomas, neuroblastoma, colon, prostate and non melanoma skin cancer [118]; methylglyoxal-bis-guanidylhydrazone (MGBG), an inhibitor of S-adenosyl-methionine decarboxylase [78], which reduces spermidine and spermine levels but elevates putrescine levels [119]. Although MGBG is an effective SAMDC inhibitor, its use in chemotherapy is restricted

because of its mitochondrial toxicity [79]. SAM486A (4-amidinoindan-1-one-2′-amidinhydrazone) is a derivative of MGBG and it was tested in various cancer cells and animal systems, as well as in phase I and II clinical trials for activity against adult cancers. Unfortunately it resulted ineffective [119] probably because of the induction of compensatory mechanisms, which preserve the intracellular concentrations of polyamines [83] [80].

# MATERIALS AND METHODS

## Cell cultures and drug treatments

HT29, SW480, LOVO and HCT116 colorectal cancer cells are cultured in Dulbecco's Modified Medium (DMEM) with addition of 10% fetal bovine serum (FBS), 1% antibiotics (100U/ml penicillin and 100U/ml streptomycin) and 1% glutamine.

Drug treatments were performed using 100µM, 200µM, 500µM, 1mM and 5mM DFMO (Sigma-Aldrich) and 0,1µM, 1µM, 10µM, 50µM and 100µM GC7 (LGC bioserch technology) for 24, 48 and 72 hours.100 µg/ml Cycloesimide (CHX) (Sigma-Aldrich) was used in HCT116 cells for 5,10,15,40,60 minutes. 10 µM spermidine and putrescin (Sigma-Aldrich) were used for 4 hours with prior addition of 1mM DFMO for 72h. 10µM Cisplatinum was used for MTT assay. All drugs were added in DMEM medium.

## Plasmids and antibodies

Lentiviral plasmids for RNA interference were produced in our laboratory using pLKO.1 backbone (Addgene) as starting vector.

The plasmid Flag-c-Myc mouse was provided by an external laboratory and was used as a template to generate mutant plasmids for the 5 polyproline sites (Flag c-Myc 5M and the single point mutants). Reporter vectors M50 Super 8x TOPFlash (12456 Addgene), M51 Super 8x FOPFlash (12457 Addgene) and TK renilla were used for luciferase assay.

The following antibodies were used: anti-vinculin (sc-73614 Santa Cruz), anti-c-Myc (sc-9402 Cell signalling), anti-hypusine (Lab Mirmira, Indianapolis, USA), anti-DHPS (ab-

202133), anti-EIF5A1 (ab32443 Abcam), anti-p53 (Santa Cruz) anti-beta catenin (sc-7963 Santa Cruz ), anti-pGSK3 (9331 Cell signalling), anti-GSK3 total (5676 Cell signalling), anti-pERK(9101 Cell signalling ), anti-total ERK (9102 Cell signalling), anti-FlagM2 (A8592; Sigma- Aldrich) e anti-GFP (sc-9996 Santa Cruz).

## Cell transfection and proliferation assays

HCT116 cell were transfected with mFlag-c-Myc WT and its single point mutant using 4µl/µg DNA DreamFect transfection reagent (OZ biosciences) according to the manufacturer's protocol, for 24-48h.

The proliferation assays were performed for 24h,48h,72h plating 20,000 cells/cm$^2$. Every time point was plated in triplicate. Drug treatment with GC7 or DFMO was performed 24h after plating cells. For counting cells were diluted in TrypanBlue (thermo Fisher Scientific) and counted using a Burker chamber.

## Lentivirus production and cell infection

50% confluent HEK293 cells were co-transfected by calcium phosphate precipitation with 20µg of different pLKO.1 vectors (encoding for different shRNAs) together with 15µg PCMV-R 8.74 (encoding for factors involved in virion assembly) and 10µg PMDG (encoding for virus envelope proteins), to produce lentivirus. After 24h medium (DMEM) was discarded ad replaced. The supernatant, containing lentivirus, was collected after 48h and 72h after transfection. MOI was determined using the HIV-1 kit p24 ELISA (NEK050B001KT, Perkin-Elmer), according to the manufacturer's protocol. The following vectors pLKO.1 (Sigma-Aldrich) were used for lentivirus production:

- ShDHPS Fw: CCGGAGTGCACTGGGATGATCATTCTCTAGAGAATGATC
- ShDHPS Rv: AATTCAAAAAAGTGCACTGGGATGATCATTCTCTAGAGA
- ShEIF5A1 Fw: CCGGGCATTACGTAAGAATGGCTTTTCTAGAAAAGCCAT
- ShEIF5A1 Rv: AATTCAAAAAGCATTACGTAAGAATGGCTTTTCTAGAAA

HCT116 cells were infected with supernatant containing lentivirus for 72h (supernatant was discarded and replaced with DMEM after 24h). After 72h cells were treated with 5µg/ml Ampicillin for 72h. MOI ≥1 was used.

## Western blot

Protein extracts for western blot (WB) were obtained by lysing the different cells in denaturing buffer SDS-urea (50 mm TrisHCl pH 7.8, 2% SDS, 10% glycerol, 10 mm Na4P2O7, 100 mm naf, 6 M urea, 10 mm EDTA); the protein extract was then sonicated, to make the lysate non-viscous, and quantized by nanodrop. The samples were mixed with loading buffer 5x (1M Tris hcl pH 6.8, 10% glycerol, 6% SDS, 10% βmercaptoethanol, 0.2% Bromophenol blue in EtOH), maintained at 100°C for 5 minutes, loaded on polyacrylamide gel in denaturing conditions and transferred to a nitrocellulose filter. The filters were then incubated with a solution of milk 5% in TBS-T for 30 minutes, to saturate the non-specific sites, and then with the different primary antibodies diluted in milk 5% over night. After 24h the filters were washed, incubated with specific secondary antibody for 30 minutes and the chemioluminescence was detected by chemidoc with the use of ECL (ECL – enhanced chemiluminescent substrate for horseradish peroxidase HRP) (Thermofisher).

## MTT assay

HCT116 cells were plated in 96 well tissue culture dishes at a density of 360.000 cells/cm$^2$. Drug treatment with GC7, DFMO and Cisplatinum were performed 24h after plating cells. 0.5mg/ml MTT solution (Sigma Aldrich) was added to the culture medium for 3h at 37°C. After 3h the medium was removed and 100µl/well of DMSO were added. The absorbance was detected using the spectrophotometer at 600nm.

## Luciferase assay

HCT116 cells were plated in 24 well tissue culture dishes at a density of 20000 cells/cm$^2$, each experimental point in triplicate. The following day the cells were transfected using DreamFect transfection reagent (OZ biosciences) according to the manufacturer's protocol with reporter vector M50 Super 8x TOPFlash (50ng) (12456 Addgene), control reporter vector M51 Super 8x FOPFlash (50ng) (12457 Addgene) and reporter plasmid encoding TK renilla (20). After 24 hours the cells were incubated with starvation medium (Optimem, FBS 0.5%, penicillin 1%, streptomycin 1%, sodium pyruvate 1%, nonessential amino acids 1%) for 8 hours. Then cells were treated with Lithium chloride (60mm) for 24 hours in order to activate the WNT/beta-chain pathway. The following day the cells were treated with GC7 at a concentration of 100 µM for 24h. Cell extracts were prepared with lysis Buffer (passive lysis buffer 5x, Biotium) according to the manufacturer's protocol. Luciferase assay was performed with Firefly & Renilla Luciferase Single Tube Assay Kit (Biotium #30081-T) according to the manufacturer's protocol.

## RNA immunoprecipitation (RIP)

HCT116 cells were plated in 56 tissue culture dishes with a confluence of 100%, cross-linked with 1% formaldehyde solution for 10 minutes at room temperature and then incubated with glycine (125mm) for 5 minutes at room temperature. Pellets were lysed with FA Buffer (50mm HEPES-KOH pH 7.5, 140 mm nacl, 1 mm EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitors, 50U/ml rnase inhibitor SupeRNase, ambion) and sonicate. The extracts obtained were treated with DNase (invitrogen) and 25mM MgCl2, 5mM CaCl2 and SupeRNAse for 10 minutes at 37°C. The reaction was interrupted with the addition of 20 mM EDTA. 10% of the final volume was kept as input. Immunoprecipitation was performed with the specified antibodies overnight. The following day prot A-agarose resin was added to the lysate for one hour at 4°C in agitation; subsequently samples were washed extensively with three different solutions (**Low salt**: 0.1% SDS, 1% TRITON X-100 2mm, EDTA 20mm Tris-HCl pH 8.1, 150mm nacl; **High salt**: 0.1% SDS, 1% TRITON X-100, 2mm EDTA, 20mm Tris HCl pH 8.1, 500mm nacl; **Licl buffer**: 0.1 mm EDTA, 1% EDTA, 1. and elutes (1% SDS, 0.1M, NaHCO3, SuperRNAsi 50U/ml). Elution was performed with **Elution buffer** (1% SDS, 0,1M $NaHCO_3$ , SuperRNasi 50U/ml) for 15 minutes in agitation at room temperature. The extraction of RNA was performed with the TriZol reagent, the cDNA was obtained using the BIOLINE kit (BIO-65053 bioline) and then analyzed by real-time PCR.

## RNA extraction, retro-transcription and qPCR

RNA was extracted with the TRIzol reagent (Invitrogen). 1 µg of total RNA was reverse-transcribed with SuperScript II reverse transcriptase and random examers (Invitrogen).

qPCR was performed using Sybr Green enzyme (Sensifast SYBR Lo-ROX kit, Bioline) according to the manufacturer's protocol and complementary primers to the indicated target genes:

- Human c-Myc Fw: CACCACCAGCAGGCACTC
- Human c-Myc Rv: TTCCACAGAAACAACATC
- Human β-ACTIN Fw: CACCCTGAAGTACCCCATCGAG.
- Human β-ACTIN Rv: TGATCTGGGTCATCTTCTCGCG
- Human L32 Fw: CCCTTGTGAAGCCCAAGATC
- Human L32 Rv: TCTGGGTTTCCGCCAGTTAC

## Nanostring

HCT116 cells were transfected with PLKO.1 vector against DHPS enzyme or a PLKO.1 control vector. The samples were analysed using nCounter PanCancer pathway panel according to the manufacturer's protocol. Quality control and normalization were performed as recommended by manufacturer (using nSolverAnalysis Software version 3.0), and data points with the extreme low counts (<83) were removed. Genes of interest were selected using the following parameters:

A) Upregulated genes in DHPS depleted cells compared to control cells (fold change: ≥ 1,2)
B) Downregulated genes in DHPS depleted cells compared to control cells (fold change: ≤ 0,85)
C) Genes not regulated between DHPS depleted cells compared to control cells (fold change between 1,2 and 0,85)

Hierarchical clustering was used to identify the top 10 upregulated and downregulated genes (top 10 with the higher or lower fold change).

Generation of the heatmaps was performed using Glucore software.

For transcription factor analysis was used Genomatix software.

## Colorectal cancer xenograft models

$2x10^6$ HCT116 cells were suspended in equal volumes of PBS and Matrigel (BD Pharmingen) and implanted subcutaneously in both flanks in adult athymic nude mice (Charles River Laboratories). Animals were divided in two subgroups (control: n=6; GC7 treated: n=6). Treatment initiated when tumor volume reached 100 $mm^3$. Mice were treated with GC7 4mg/kg (IP injection) every 2 days and tumors size were measured every 2 days using a caliper.

For xenograft with DHPS depleted cells, HCT116 cells were transfected with PLKO.1 vector against DHPS enzyme or a PLKO.1 control vector. Animals were divided in two subgroups (control: n=6; DHPS: n=6). After 72h from transfection followed by 72h of selection with puromycin 5µg/ml, $2x10^6$ HCT116 cell DHPS depleted or control cells were suspended in equal volumes of PBS and Matrigel (BD Pharmingen) and implanted subcutaneously in both flanks in adult athymic nude mice (Charles River Laboratories). Measurements began when tumor volume reached 100 $mm^3$ and tumors size were measured every 2 days using a caliper.

Volume was calculated as $V = (L \times W2)/2$. Growth patterns were summarized graphically by plotting the mean and SD for each treatment group over time.

## Statistic analysis

Statistical analysis was carried out using Graphpad Prism (version 7) software. Results are expressed as mean ± s.d. of at least 3 separate experiments, each performed in triplicate. Statistical differences were analyzed with the Mann–Whitney U test for non-parametric values and a $P < 0.05$ was considered significant.

# RESULTS

## Inhibition of hypusination limits CRC cell growth in vitro and in vivo

We first studied the effect of the irreversible ODC inhibitor DFMO on the growth of various human colorectal cancer cell lines (HT29, HCT116, SW480 and LoVo). All tumor cells were significantly inhibited by DFMO in a dose-dependent fashion, indicating their dependence on polyamine metabolism (figure 10A-B-C-D). Next, we tested the effect of the DHPS inhibitor GC7 on the growth of the same CRC cells. As shown in figure 11(A-B-C-D), the drug inhibited all cell lines tested.

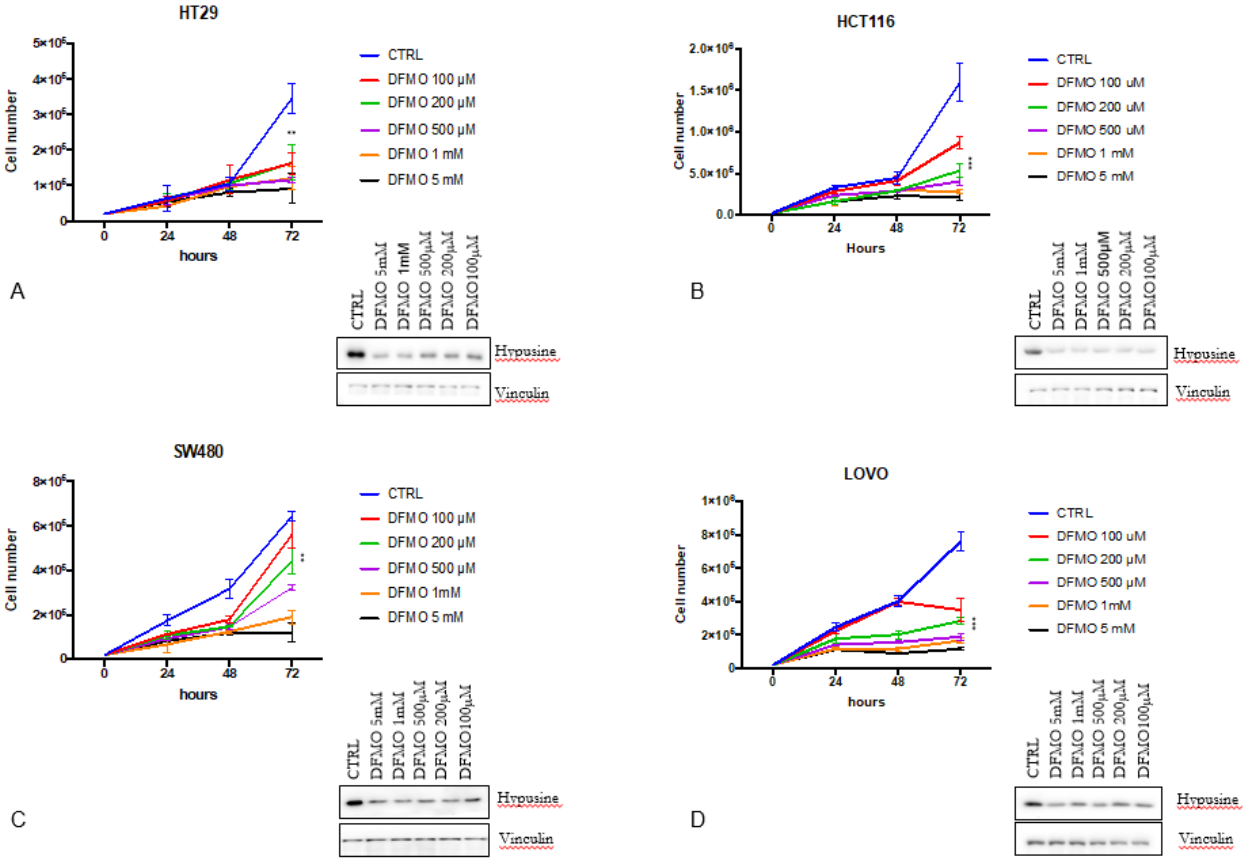


Figure 10: HT29 (A), HCT116 (B), SW480 (C), LOVO (D) growth was significantly inhibited by DFMO in a dose-dependent fashion. DFMO effect on polyamine metabolism was evaluated by Eif5A1 hypusination protein levels.

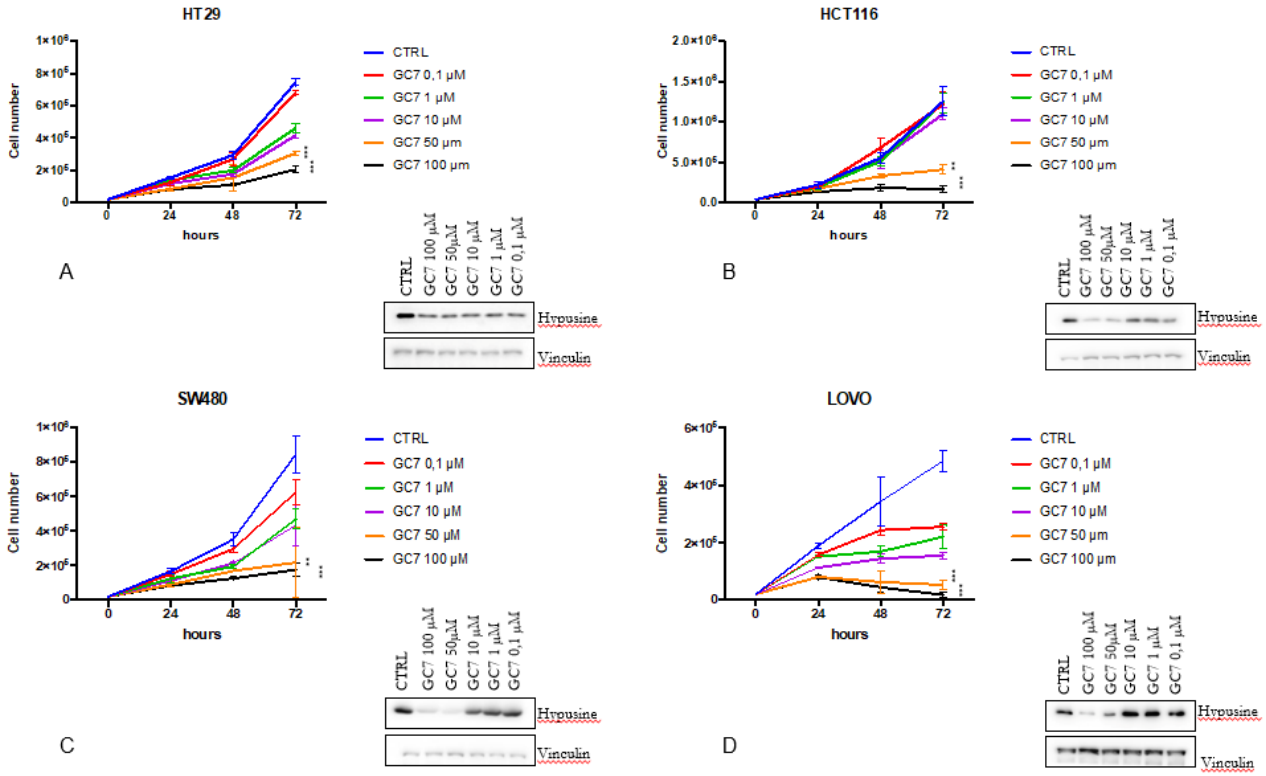



Figure 11: DHPS inhibitor GC7 inhibited HT29 (A), HCT116 (B), SW480 (C), LOVO (D) growth. GC7 effect was evaluated by Eif5A1 hypusination protein levels.

The growth inhibitory effect of GC7 and DFMO could not be attributed to promotion of apoptosis, as shown by the lack of PARP cleavages, as well as by the unmodified percentage of apoptotic cells observed by flow cytometry analysis of annexin V and propidium iodide double stained cells (figure 12A-B-C). Instead the two drugs caused inhibition of cell cycle, as documented by an increase of the percentage of cells in G0/G1 phase at the expense S and G2/M phases (figure 12D). Therefore, these data indicate that pharmacological blockade of the hypusination axis mimics general polyamine inhibition.

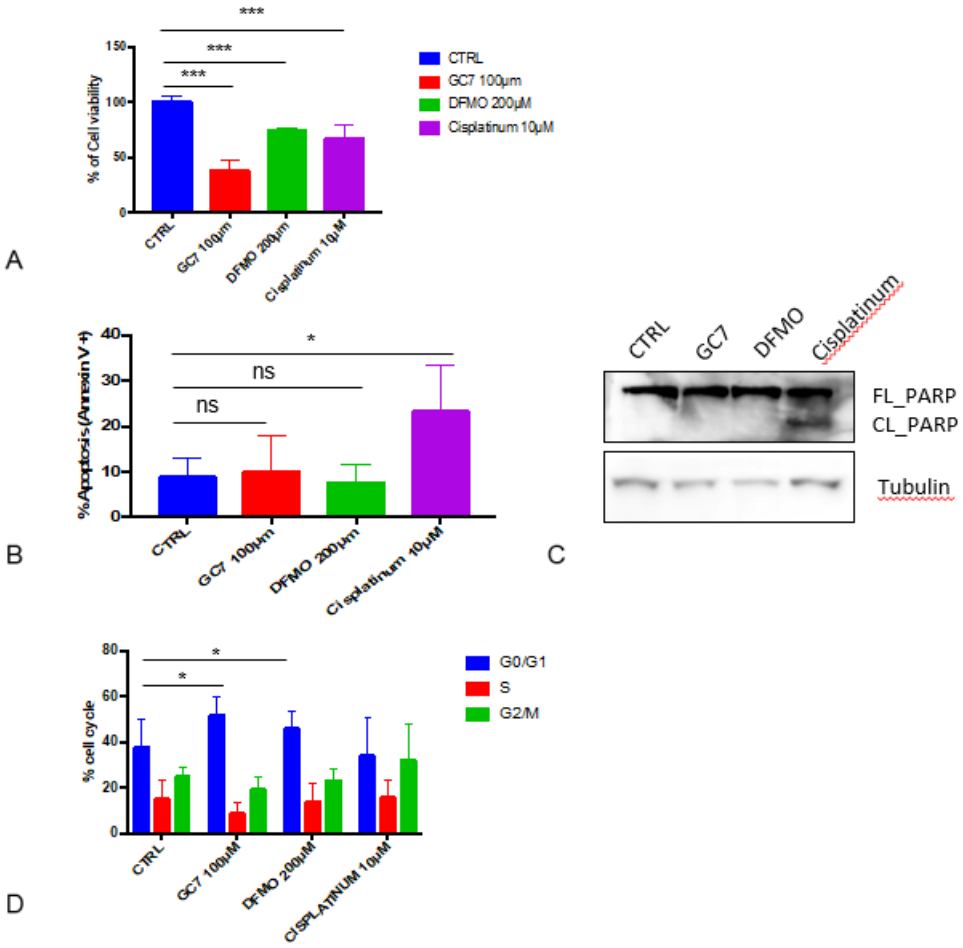



Figure 12: The growth inhibitory effect of GC7 and DFMO could not be attributed to promotion of apoptosis (A-B-C). The two drugs caused inhibition of cell cycle as documented by an increase of the percentage of cells in G0/G1 phase (D).

To determine whether GC7 treatment had antitumor effects also in preclinical models, we grafted HCT116 cells into the flanks of athymic nude mice. When the tumor volume reached 100 $mm^3$, mice were IP-injected daily with 4mg/kg GC7 or DMSO for 15 days and the growth of tumor volumes monitored every 3 days. Treatment with GC7 significantly lowered tumor growth and caused reduction of average tumor weight and size, compared to control (figure 13A-B-C).

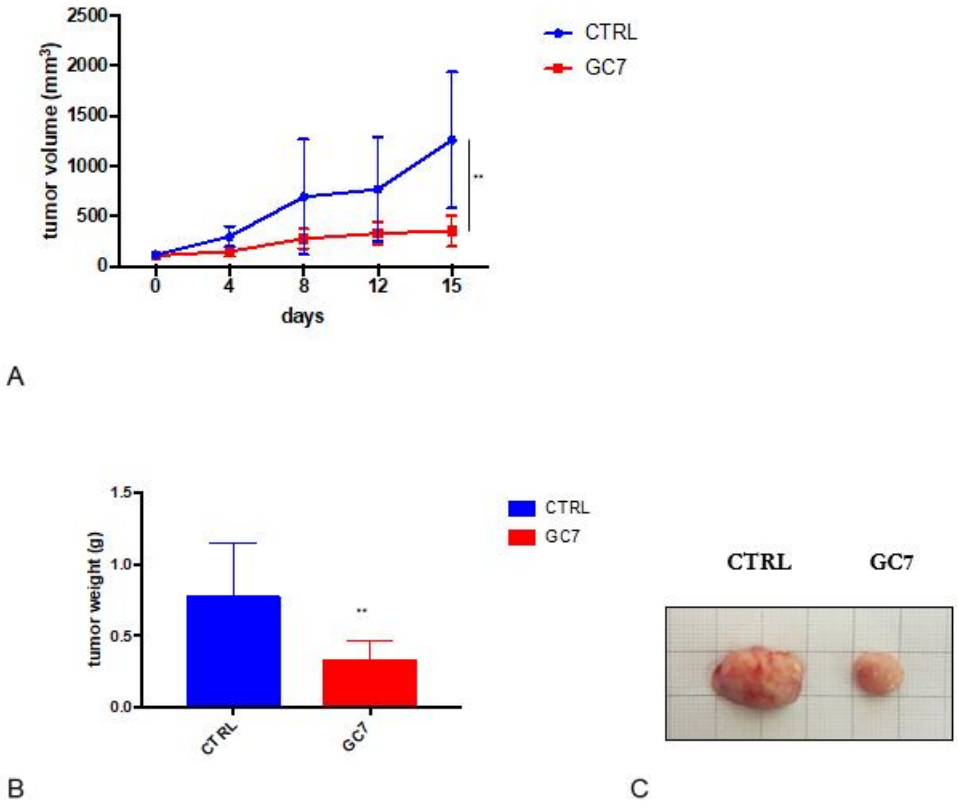



Figure 13: *In vivo* treatment with GC7 significantly lowered tumor growth (A) and caused reduction of average tumor weight and size, compared to control (C-D).

To ascertain that the observations made with GC7 were not due to off target effects of the drug, we performed lentiviral-mediated knockdown of DHPS in HCT116 cells. After transduction of lentiviral particles, the levels of DHPS protein were reduced more than 95% and this was associated to a strong decrease of the hypusine levels, confirming the efficacy and specificity of the knockdown (figure 14B-D).

Proliferation rate and viability of cells lacking DHPS were strongly reduced compared to control cells and the inhibitory effect was comparable to that obtained with GC7 treatment (figure 14A-B-C-D)

Of note, when implanted into the flanks of nude mice, DHPS deficient CRC cells grew significantly slower and at the end of the experiment tumor sizes and volumes were greatly reduced compared to controls (figure14E-F-G)

Collectively, these results demonstrate that inhibition of DHPS elicits significant antitumor effects in CRC cells.

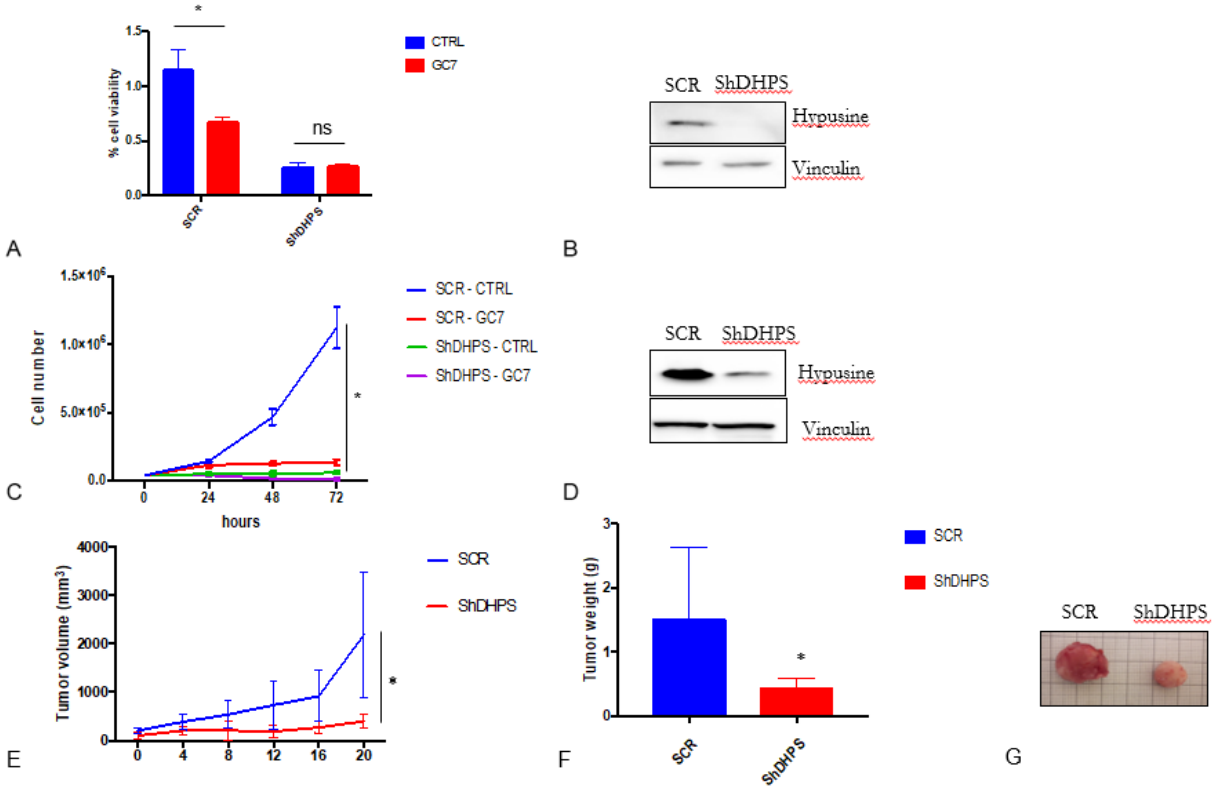



Figure 14: Genetic inhibition of polyamine/hypusine pathway through DHPS RNAi blocks CRC cell growth in vitro (A-B-C-D) and in vivo (E-F-G)

## DHPS inhibition down-regulates c-Myc, but not other key CRC oncogenic pathways

A number of driver mutations are found in CRC, most of them causing an aberrant function of a few key signaling pathways such as Wnt/beta catenin, MAPKs, TP53, Pi3K-AKT, among the others and consequent dependence on these alterations for growth and survival [8] [12] [13] [3] [29] [30] [31] [32] [33] [34] [35] [8].

To understand the mechanisms underlying the antitumor properties of DHPS inhibition, we performed multiplex gene expression analysis of 730 genes from 13 cancer-associated canonical pathways including those that are typically altered in CRC. We analyzed mRNA

extracted form DHPS-deficient or control CRC cells: 188 genes (26%) were upergulated, 104 (14%) were downregulated, while 126 (17%) were not modulated by the lack of DHPS. The remaining 312 (42%) genes were not significantly expressed (figure 15).

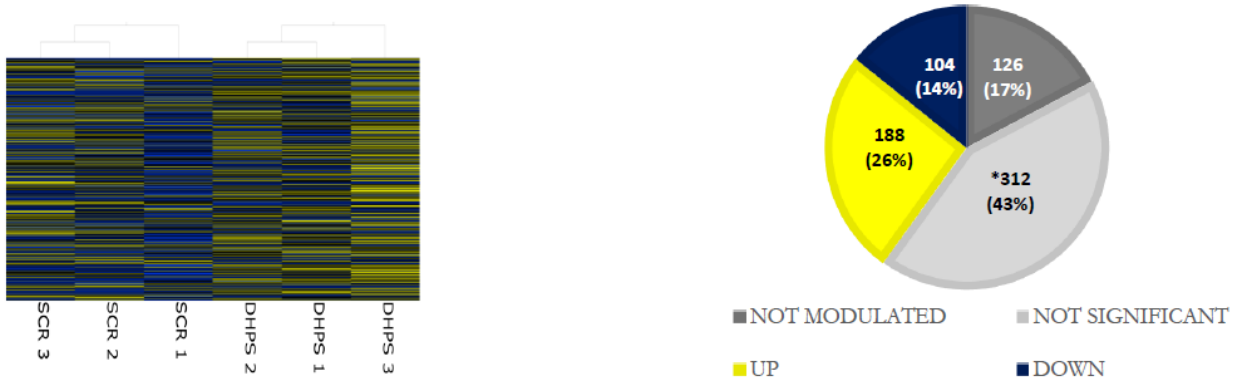
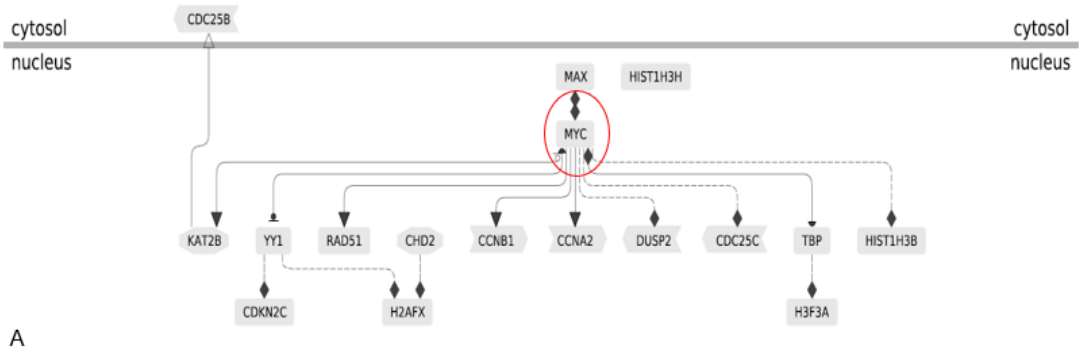
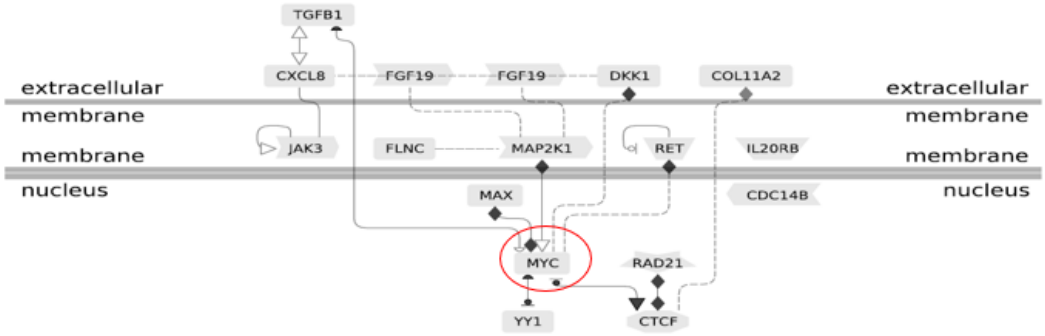



Figure 15: Nanostring analysis. Modulation of 730 genes mRNA from 13 cancer-associated canonical pathways in DHPS deficient cells compared to control cells.

To search common regulators, we selected the 10 most upregulated and dowregulated genes and performed a transcription factor analysis. Notably, a key transcription factor directly or indirectly involved in the transcriptional regulation of most of the genes from both groups was the oncogene c-Myc (figure 16A-B).




A




B

Figure16: Transcription factor analysis. The lack of DHPS enzyme affects expression of genes under the control of c-Myc transcription factor. The most 10 upregulated genes (A) and the most 10 downregulated genes (B).

We further studied the involvement of the most common altered pathways in CRC by analyzing the activation status of Wnt/beta catenin, EGFR/MAPK, P53, PI3K/AKT pathways in CRC cells infected with shDHPS or sh control (shSCR) expressing lentiviruses or treated with GC7.

Wnt/beta catenin signaling, a pathway aberrantly activated in the majority of CRCs, was not affected by impaired hypusination as shown by the unchanged activity of TFC/LEF-Luc reporter and beta catenin protein levels in treated cells (figure 17A-B). The total levels of TP53 were also not altered by DHPS inhibition as well as phosphorylated ERK and GSK3beta, two markers of activation of the RAS-BRAF-MAPK and PI3K/AKT pathways, respectively. Conversely, the levels of c-Myc were strongly downregulated in CRC cells upon blockade of the hypusine signaling (figure 17B), further supporting the mRNA expression profiles.

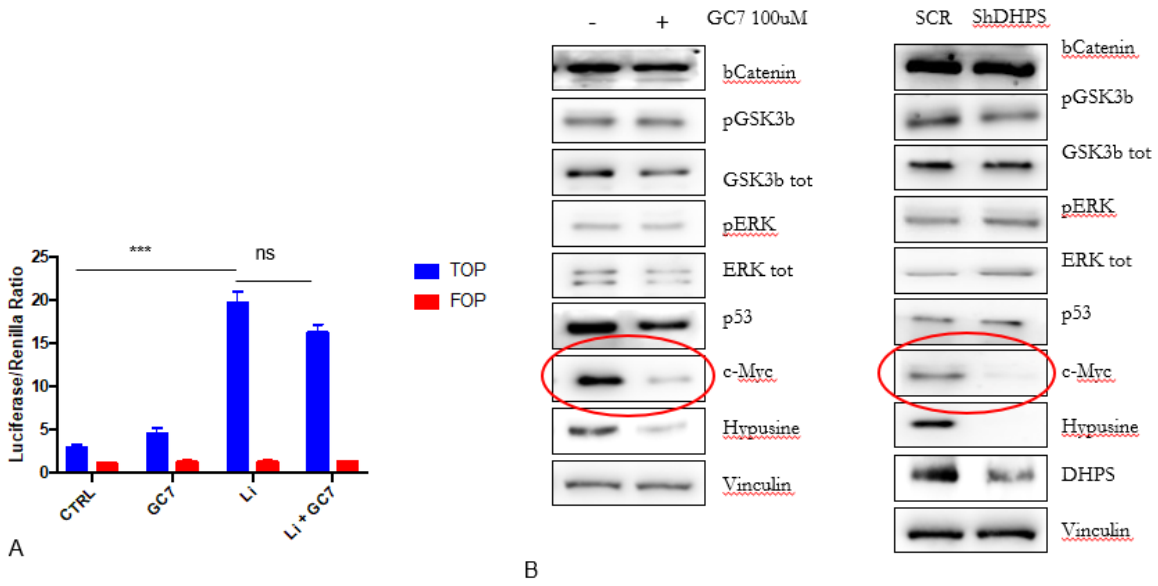



Figure 17: The pharmacological inhibition of Eif5A-Hyp through GC7 does not affect activity of TFC/LEF-Luc reporter (A).The lack of DHPS enzyme or GC7 does not affect Wnt, RAS-BRAF-MAPK and PI3K/AKT pathways and the total levels of TP53, but affect c-Myc protein (B).

The effect was not related to transcriptional or mRNA stability mechanisms, as documented by the unchanged mRNA levels upon GC7 treatment (figure 18A). Also, the drug did not affect c-Myc protein stability, as indicated by its unchanged half-life in cells treated with the protein synthesis inhibitor cycloheximide (CHX) (figure 18B).

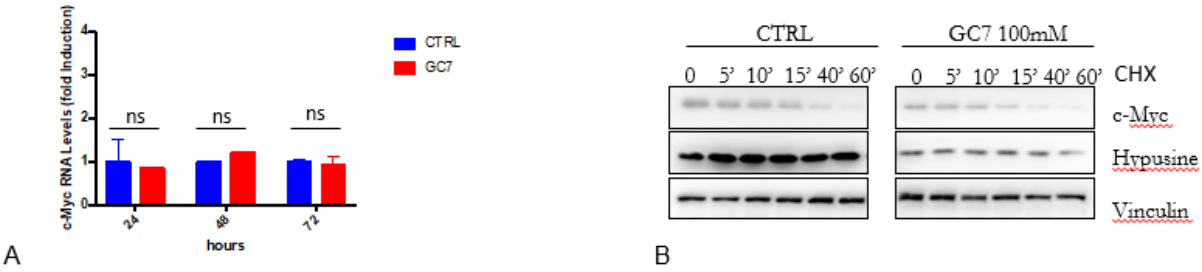



Figure 18: GC7 did not affect c-Myc mRNA transcription (A) and c-Myc protein stability (B)

## Hypusinated EIF5A promotes c-My translation

Having found that hypusination controls c-Myc at post-transcriptional levels and independently of mRNA or protein stabilization mechanisms, we wondered whether the regulation occurs through a direct EiF5A-mediated translational process.

In analogy with other EiF5A-regulated targets (e.g. ODC or AZ1 and AZIN1) [95], addition of putrescine (PUT) or spermidine (SPD) to polyamine-depleted media caused a marked increase of EIF5A1 hypusination and c-Myc protein, but not of c-Myc mRNA levels. The upregulation was abrogated by the ablation of EIF5A1, indicating the involvement of the translation factor in this process (figure 19A-B). Also, in this case, the effect was not related to a change in c-Myc stability as demonstrated by the unmodified half-life of c-Myc after incubation of cells with SPD in CHX-treated cells (fig 19C). The addition of the protein synthesis inhibitor cycloheximide to CRC cells prevented c-Myc upregulation, supporting the hypothesis that the SPD increases c-Myc protein levels by promoting its translation but not its stability.

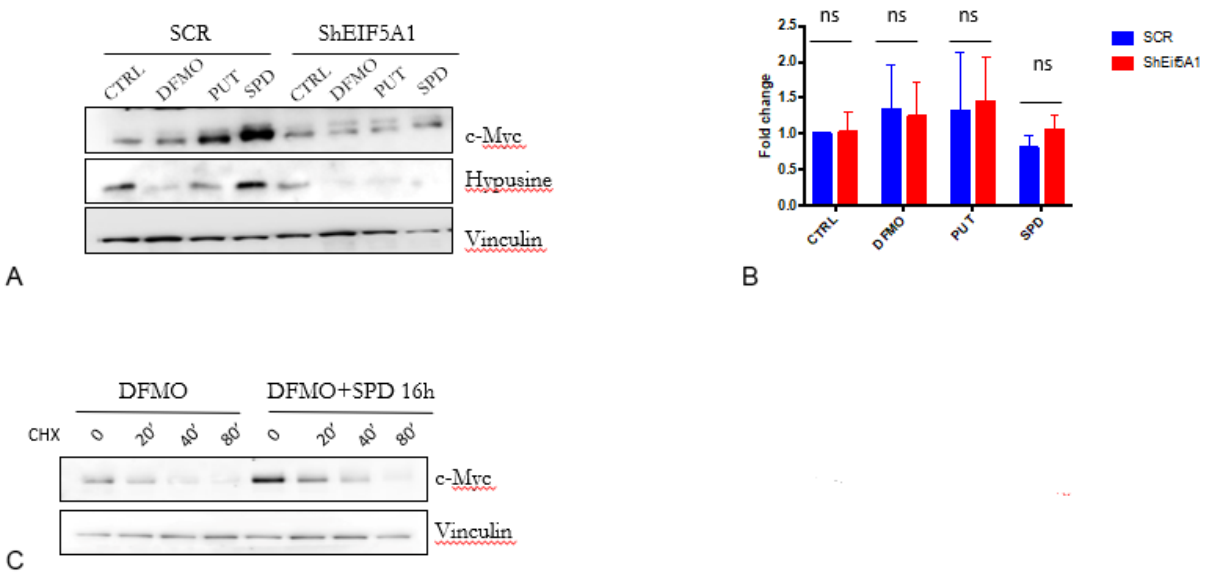



Figure 19: Addition of putrescine (PUT) or spermidine (SPD) to polyamine-depleted media caused a marked increase of EIF5A1 hypusination that was abrogated by the ablation of EIF5A1 (A). c-Myc mRNA levels was

no affected by the addition of SPD or PUT (B). The effect of SPD or PUT is no related to a change in c-Myc protein stability (C).

Since the hypusinated EIF5A1 has been shown to regulate translational elongation by relieving ribosome stalling, we analyzed polyribosome fractions in DHPS-deficient vs WT CRC cells.

As shown in figure 20, cells lacking DHPS showed an increase of the polysome fraction compared to control cells, in keeping with increased ribosome mRNA occupancy, an effect typically observed upon ribosome stalling [95]. By contrast the content of c-Myc mRNA in the same fractions was not significantly different, while c-Myc protein levels were reduced (figure 20A-B-C). These results were consistent with the hypothesis that inhibition of hypusination causes an increased ribosome stalling, thereby reducing c-Myc biosynthesis.

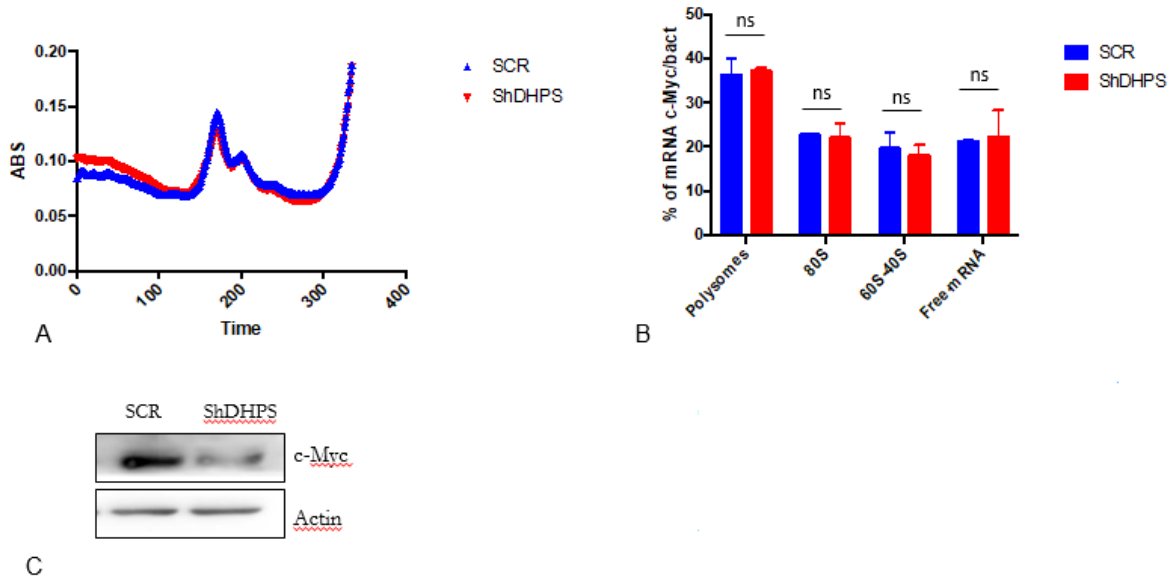



Figure 20: Cells lacking DHPS showed an increase of the polysome fraction compared to control cells (A). The content of c-Myc mRNA (B) and c-Myc protein levels (C) in the same polysome fractions.

Since EiF5A has been described to regulate translation through association with mRNA untranslated regions [95] o with coding regions [95], we next sought to identify the mRNA

region required for c-Myc translational regulation. To this end we tested the effect of DHPS inhibition in cells where the c-Myc 5' or 3'UTRs were deleted through the CRISPR/Cas9 approach.

In both cell lines, inhibition of hypusination continued to cause a decrease of c-Myc protein levels compared to control cells, indicating that the regulation does not require c-Myc UTRs but rather its coding region (Figure 21A-B).


Figure 21: Eif5A1 regulation on c-Myc translation does not require 5'UTR (A) or 3'UTR regions (B)

Consistent with this hypothesis, GC7 and DHPS knockdown caused a significant decrease of exogenous c-Myc protein levels encoded by a plasmid containing c-Myc coding region but not its UTRs (Figure 22A-B). Consistently, SPD supplementation of polyamine-depleted CRC cells, induced a marked increase of exogenous c-Myc protein. (figure 22C).

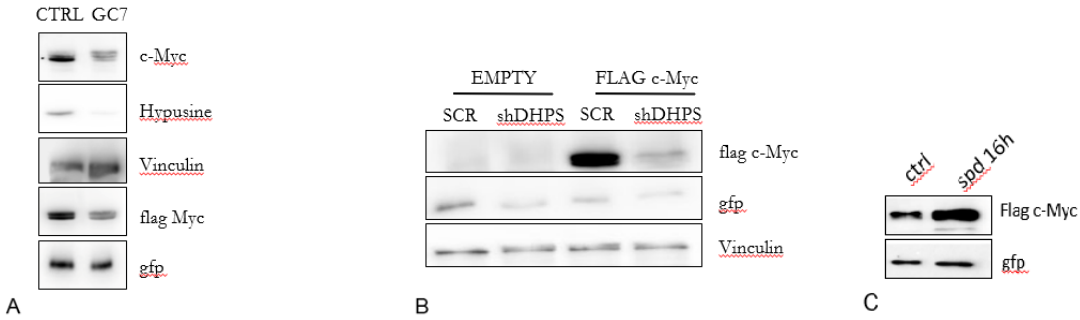


Figure 22: GC7 (A) and DHPS knockdown (B) caused a significant decrease of exogenous c-Myc protein levels. SPD supplementation of polyamine-depleted CRC cells induced a marked increase of exogenous c-Myc protein (C).

## Tripeptide pausing motifs in c-Myc CDS are required for EIF5A-mediated regulation

Previous work demonstrated that EIF5A alleviates translational pausing of the ribosomes at specific stalling motifs [95] in yeast. In particular, through ribosome-profiling screening, it was demonstrated that the absence of EIF5A causes a robust stalling of ribosomes at the level of 29 tripeptide motifs [95]. Based on this, we analyzed the amino acid sequence of c-Myc and found the presence of 5 potential stalling sites, with the first AA in position 170, 220, 780, 910 and 980.

Mutation of these five pausing sites (c-Myc 5MUT) prevented the decrease of c-Myc protein levels induced by DHPS inhibition (figure23A), confirming the involvement of these motifs in the observed effect. By contrast, individual mutation of each sequence did not modify the inhibition of c-Myc protein levels (figure 23B).

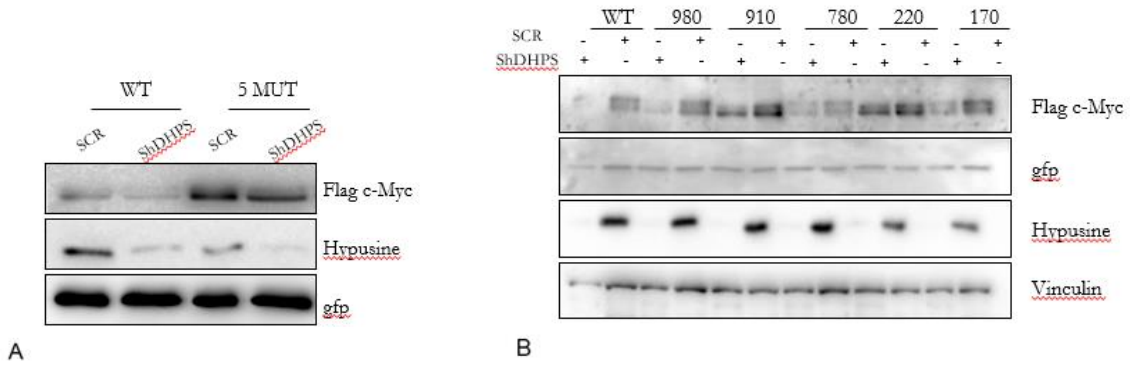



Figure 23: Mutation of all five pausing sites (c-Myc 5MUT) prevented the decrease of c-Myc protein levels induced by DHPS inhibition (A). Individual mutation of each sequence did not modify the inhibition of c-Myc protein levels (B).

# DISCUSSION

In this paper we have demonstrated a new regulatory mechanism of c-Myc translation that could have important applications in colorectal cancer therapy.

In particular we have demonstrated an anti-cancer property of the drug GC7 that is due to the blocking of EIF5A hypusination, followed by its functional inactivation and the subsequent inhibition of c-Myc translation (Figure 24).

Colorectal cancer has a very high incidence in the world and the disease-related mortality rate is still one of the highest [2]. The current therapeutic approach of tumors at non-metastatic stages involves the use of surgery and, where appropriate, the combination of chemotherapeutic agents [120].

Like most neoplastic diseases, however, the prognosis is quite severe in the case of metastatic disease, where the estimated survival at the time of diagnosis is 25-30 months.

Innovative therapeutic approaches are being sought to ensure an increase in survival rates and, in particular, new possible pharmacological targets and new molecular markers are emerging. Among these, polyamines have been considered as potential therapeutic targets of new anti-tumour drugs. The increase in the concentration of polyamines in tumour tissues is associated with a de-regulation of their metabolism [121]. Some inhibitors of the metabolism of polyamines or of their intracellular transport have been identified, and they all have the ability to counteract tumour cell growth. This reduction correlates with a decrease in both the development and progression of neoplastic disease [122]. One of these drugs, extensively characterized pharmacologically, is the D,L-alpha-difluoromethlornitin (DFMO) which acts by blocking the ornithine de-carboxylase enzyme (ODC).

The use of DFMO as an anticancer agent in mono-therapy has not been satisfactory, probably due to the ability of cancer cells to restore intracellular polyamine levels by

increasing their uptake from the extracellular space [80]. For this reason, DFMO is mainly used as a chemo-preventive agent in various clinical trials for diseases such as familial adenomatous polyposis (FAP) or prostate tumours [123] or to avoid relapses in patients with neuroblastoma [124]. However, the identification of the specific mechanisms regulated by polyamines and necessary for tumour growth could allow the generation of new anticancer drugs that could stably counteract tumour growth, without generating resistance, such in the case of DFMO.

Polyamines are de-regulated in different types of cancer, including colorectal cancer, where the increase in their concentration is also associated with an increase in the concentration of EIF5A [125]. This factor is involved in both the initiation and elongation process of the translation of many proteins, including several oncogenes [126]. The EIF5A factor needs a post-translation modification to be functionally active. This modification involves the introduction of an unusual modification, the hypusination, within its amino acid sequence. The substrate of the hypusination reaction, which occurs at the lysine level, is the polyamine spermidine and the reaction is mediated by two enzymes: DHPS and DOOH [127].

In clinics, DOOH enzyme inhibitors are mainly used against other types of diseases, such as malaria [128].

The inhibitor of the DHPS enzyme, GC7 (N1-guanyl-1,7-diamineoheptane), exerts its function by competitive inhibition. In the literature, the efficacy of GC7 as co-adjuvant for other chemotherapeutics has been demonstrated by *in vitro* studies. Fangs and collaborators, for example, describe how GC7 succeeds in increasing cis-platin efficiency in squamous cell oral carcinoma (OSCC) [129]; a similar effect is also shown by Xu G. and co-workers in lung cancer cells [130].

GC7 has been tested in combination with some antibodies in order to block tumour cells proliferation. For example, in lung cancer it has been tested together with a chimeric monoclonal antibody, cetuximab [131]. Starting from these data in the literature, we focused

on assessing whether GC7 could play an anti-proliferative action also in colorectal cancer cells, a hypothesis that has been confirmed by our data.

One of the main problems encountered with GC7 are its non-specific effects [122], especially when used at high concentrations. For this reason, we have also assessed if there was an anti-cancer effect following the silencing of both DHPS and the translation factor EIF5A. In both cases, we observed a reduced cell proliferation that was similar to that obtained after treatment with the drug, thus supporting the specificity of the biological effects observed against the DHPS-EIF5A axis.

Our observations suggest that the inhibitory effect of GC7 is related to its ability to inhibit the translation of the oncogene c-Myc. In particular, treatment with GC7 causes a decrease in the levels of this protein, without affecting the amount of its mRNA. A previous study suggested that polyamines regulate c-Myc neosynthesis favoring the link between HUR, chk1 kinase and the 3'UTR sequence of the oncogene. However, our data have led us to rule out the involvement of this mechanism, at least in our model of study. In fact, the deletion of the 3'UTR sequence by CRISPR/Cas9 method did not involve changes in the response to the drug, thus allowing us to conclude that the regulation took place in another region of the transcript. With a similar approach we have also excluded the involvement of the 5'UTR sequence.

Using a plasmid construct expressing only the coding sequence of c-Myc, we were able to verify if the involved sequence was the coding region. We found five pausing motifs whose translation has been shown to be regulated by EIF5A [95]. We have demonstrated that mutation of these five pausing sites, (c-Myc 5MUT), prevented the decrease of c-Myc protein levels induced by DHPS inhibition (figure23A), confirming the involvement of these motifs in the observed effect. By contrast, individual mutation of each sequence did not modify the inhibition of c-Myc protein levels (figure 23B).

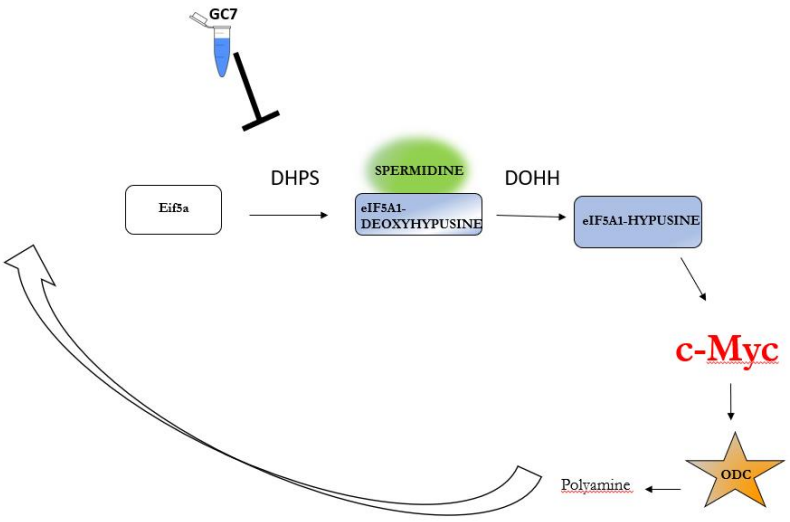



Figure 24: Diagram of the model that we assumed based on the obtained data and the literature.

A relevant aspect of this work is the identification of a novel pharmacological strategy to inhibit the c-Myc oncogene, a protein difficult to target with direct inhibitors because of its flat structure. Hence, the identification of molecules that induce a decline of its content, by downregulating its neosynthesis may represent a valuable starting point to generate novel therapeutic approaches against colorectal carcinoma, and, potentially, for all those tumors characterized by an increase in the levels of c-Myc. Also, it will important to investigate the potential synergy between GC7 and others chemotherapy drugs, currently used in clinical practice, to propose GC7 as co-adjuvant for the treatment of colorectal cancer and other diseases related to the overespression of this oncogene.

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