Epigenetic loss of the tRNA-modifying enzyme TYW2 induces ROBO1 downregulation and contributes to mesenchymal features in colorectal cancer

Final Bachelor Project

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ABSTRACT

Transfer RNA (tRNA) activity is highly regulated through chemical modifications to control the complex formation between mRNA and ribosome and, in other words, regulate protein translation. In cancer, hypermethylation of some genes can provide growth advantage because of the resulting gene silencing. TYW2 is one of the key enzymes of wybutosine synthesis and induces guanosine hypomodification in phenylalanine-tRNA. We studied TYW2 epigenetic silencing observed in some colorectal cancers, which provokes a downregulation of various transcripts like ROBO1, a known tumour suppressor gene. Analysing various cell models by qRT-PCR, WB and actinomycin D assay, we determined that the mechanism for this downregulation is a decrease in mRNA half-life because of transcript degradation via NMD. The loss of ROBO1 enhances a mesenchymal phenotype in cells, and it was observed in transwell assays, that this promotes an increase in the cell migration ability and enhances metastasis, which is the main cause of death within most cancers. All these findings are a perfect example to understand how epigenetic changes can modify the epitranscriptome and impact the cell phenotype, resulting in various diseases. In this case it promotes the acquisition of malignant features like enhanced migration. Further understanding the mechanisms that cause the cell to become malignant is the first step in finding a treatment.

SUSTAINABLE DEVELOPMENT GOALS

The aim of this project is to study the impact of epigenetics in cancer disease, specifically, the DNA-methylation silencing of TYW2 and how this event regulates other transcripts like ROBO1. The study was performed in human cell lines, so the main impact is towards humans. It is important to mention that according to the World Health Organization, cancer is the second leading cause of death, globally about 1 in 6 deaths is due to cancer, and colorectal cancer is one of the more common and deadly cancers.

The number of people with cancer is increasing year by year, so an increase in the awareness of the importance of prevention is needed in order to reduce cancer prevalence. As the world population is growing and aging, the global number of cancer deaths is rising. An effort in research in how cancer cells functions is needed to further understand the mechanisms that lead a cell to become malignant, so then the transformation can be interrupted with treatments.

The results of the study show that this specific epigenetic event promotes epithelial to mesenchymal transition and an increase in cell motility, in other words, it increases the ability of cancer cells to produce metastasis, which is the main cause of death within most cancers. With this new knowledge, it is tempting to speculate that a treatment with inhibitors of DNA methylation would rescue the expression of the downregulated genes. So long term, this basic research could be applied to new cancer treatments reducing the mortality rate.

For all of the explained above, it is considered that the SDG address in this study fall within the scope of Persons, and is directly framed in SDG 3 “Good Health and Well Being”, specifically the goal 3.4: “By 2030, reduce by one third premature mortality from non-communicable diseases through prevention and treatment and promote mental health and well-being”. This particular goal has its own long-term indicator, the 3.4.1: “Mortality rate attributed to cardiovascular disease, cancer, diabetes or chronic respiratory disease”.

ADAPTATION TO CONFINEMENT

In 14th March 2020 the alarm status was declared because of the sanitary crisis caused by COVID-19. Because of this situation I was forced to stop going to the laboratory and start working from home. As I had been already working there for a month and a half, I started analysing the results I have already obtained.

As the months passed and the situation evolved, it was clear I could not finish the experiments needed for the project in time, as I could not go the laboratory. My supervisor, PhD student Margalida Rosselló guided me and sent me previous results that the group obtained within the same topic, so I can analyse them from home, graph them and elaborate my written memory. The aims of the project, the materials and methodology have not changed. The project is essentially the same, so I followed the standard directions to write the memory and defend it.

Summarising, the main adaptation of my project to confinement was not performing the remaining laboratory work by myself but doing the rest of the work such as bibliographic research, statistical analysis of the results, data-mining, etc.

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ABBREVIATIONS LIST

Abbreviations	Complete name
ATCC	American Type Culture Collection
CDH1	E-cadherin
DMEM	Dulbecco's Modified Eagle's Medium
DNMT	DNA methyltransferases
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
FBS	Fetal bovine serum
LC/MS	Liquid chromatography-mass spectrometry
ncRNA	Non-coding RNA
NCAM	Neural cell adhesion molecule
NMD	Nonsense mediated decay
OHyW	Hydroxylated form of hypermodified wybutosine
O2yW	Peroxidated form of hypermodified wybutosine
RB	Retinoblastoma protein
ROBO1	Rondabout Guidance Receptor 1
TCGA	The Cancer Genome Atlas
TET	Ten-Eleven Translocation enzymes
tRNA	Transfer RNA
$tRNA^{Phe}$	Phenylalanine transfer RNA
TSP-1	Thrombospondin-1
TYW2	tRNA methyltransferase 12 homolog
UPF1	UPF1 RNA helicase and ATPase
VEGF-A	Vascular endothelial growth factor-A
VIM	Vimentin
yW	Wybutosine
-1 PRF	Programmed -1 ribosomal frameshifting

1. INTRODUCTION

Cancer

Cancer is the group of diseases involving abnormal cell growth with the potential to invade and spread to other parts of the body. Over 100 types of cancers can affect humans (NIH, 2015). Cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths in 2018. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervical and thyroid cancer are the most common among women (WHO, 2018).

Hallmarks of cancer

The six hallmarks of cancer refer to the capabilities that enable tumour growth and metastatic dissemination (Hanahan and Weiberg, 2000). Probably the most fundamental trait of cancer cells is the ability to chronically sustain their proliferation. In normal tissue, cell division is strictly regulated through signals such as growth-factors and tyrosine kinase receptors. However, these signals are deregulated in cancer cells, resulting in an escape from cell division control by overproducing growth factors or overactivated growth factor receptors. The overexpression of receptors is also commonly observed in cancer, as it causes the cell to be hyperresponsive to limiting amounts of growth factors. (Perona, 2006). Some cells are growth factor independent; its abnormal growth rates may derive from the constitutive activation of signalling pathways caused by somatic mutations. (Banerjee, 2015).

It is also equally important to inhibit growth supressing and apoptotic genes. Many of the growth-supressing proteins are encoded by tumour suppressor genes. Two of the main tumour suppressor proteins are the retinoblastoma protein (RB) and p53. While RB transduces growth-inhibitory signals that originate outside the cell, p53 receives inputs from cellular stress and genome damage. Both proteins integrate the information and decide the fate of the cell (Sherr and McCormick, 2002). Mutations in tumour suppressor genes can promote the evasion of growth inhibitory signals. Additionally, the disruption of some signalling pathways such as TFG-β enhance to epithelial-to-mesenchymal transition (EMT), that confer cancer cells traits associated with high-grade malignancies (Derynck et al., 2001).

In addition to abnormal cell division, cells must also resist cell death. Programmed cell death by apoptosis is known to be a natural barrier to cancer development (Lowe and Lin, 2000). The apoptotic machinery is composed of two major circuits, one processing extracellular cell-death inducing signals (extrinsic pathway) and one processing signals of intracellular origin (intrinsic pathway). By different mechanisms, they both culminate

in the activation of one caspase protease that ends with the disassembling of the cell and so the cell is consumed by neighbour and phagocytic cells. The fate of the cell is decided through a balance of proapoptotic and antiapoptotic factors such as the family of proteins Bcl-2 (Adams and Cory, 2007). One of the most common disruption of apoptosis in cancer cells is the loss of p53, which sense damage and can lead to activation of apoptosis through diverse mechanisms. The overexpression of antiapoptotic regulators and downregulation of proapoptotic factors are also commonly found in malignant cell populations.

It is accepted since 1997 that cancer cells require replicative immortality to be able to pass through infinite number of cell growth and division cycles. In contrast, normal cell lineages can only go through a limited number of divisions, known as Hayflick limit. (Chiu and Harley, 1997). This limitation is associated to senescence, a state of cell viability but non-proliferation, and to telomere shortening. Telomeres are repetitive regions at the ends of chromosomes, with every division they shorten progressively and eventually lose their ability to protect chromosomal ends. This leads to genomic instability that threatens cell viability. Telomerase is the specialized DNA polymerase that adds repeats to the telomeric ends. This enzyme is almost absent in normal cells, but is commonly functionally expressed in cancer cells, countering the progressive telomeric shortening.

With this abnormally high cell division, tumours grow in size. Therefore, they need a proper vascularization in order to obtain nutrients and evacuate metabolic waste. That's why the ability of inducing angiogenesis is a cancer hallmark. In adult organisms, angiogenesis is active during wound healing and female reproductive cycling, but in tumours, an "angiogenic switch" is almost always activated and remains on (Hanahan and Folkman, 1996). Evidence seems to indicate that this event is regulated by a balance between inducers as vascular endothelial growth factor-A (VEGF-A) and inhibitors as thrombospondin-1 (TSP-1). Tumour microenvironment and inflammatory responses are also contributing to angiogenesis in cancer cells (Baeriswyl and Christofori, 2009).

Activating invasion and metastasis is a widely known characteristic of cancer cells. When epithelial tissues progress to a higher pathological grade of malignancy, they develop alterations in their shape as well as alterations in the attachment to other cells and to extracellular matrix (ECM). The best characterized alteration is the loss of E-cadherin, a key cell-to-cell adhesion molecule (Schmallhofer et al., 2009). Additionally, genes encoding other cell-to-cell and cell-to-ECM adhesion molecules are altered in some cancers, molecules associated with cell migration such as N-cadherin are often upregulated in these cells. Protease genes are upregulated, and protease inhibitors

downregulated, which facilitates cell invasion into the nearby stroma (Coussens and Werb, 1996). Epithelial to mesenchymal transition (EMT) is the transdifferentiation of epithelial cells into motile mesenchymal cells, and is key in the metastatic process in cancer (Lamouille et al., 2014).

The hallmarks mentioned above, are acquired in different tumour types via different mechanisms. This is possible because of the acquisition of two other characteristics: genomic instability and tumour-promoting inflammation (Hanahan and Weinberg, 2011). Cancer progression can be seen as a process of cell clone evolution and selection. Spontaneous mutation is usually very low, so mutation rate is often increased in cancer cells (Negrini et al., 2010). Tumour inflammation can contribute to multiple hallmarks, as it can provide growth factors, proangiogenic factors and inductive signals that lead to EMT (Grievennikov et al., 2010). There are other distinct attributes of cancer cells, yet two attributes are particularly compelling. A major reprogramming of cellular metabolism is needed in order to support continuous cell growth and proliferation (Pavlova and Thompsom, 2016). An active evasion of the immune system seems to play an important role on cancer progression. It was observed that immunodeficient mice often present more aggressive cancers and when transplanted to healthy individuals these tumours often become inefficient at initiating secondary tumours (Teng et al., 2008). Further investigation is needed in order to clarify the role of these emerging hallmarks.

All of these characteristics can be acquired through mutations in DNA sequence or through other alterations such as epigenetic changes.

Epigenetics

Epigenetics is the study of chromatin modifications that do not change the DNA sequence but can affect gene expression. In the past five years several studies have focused their attention on epigenetics, as it became clear that understanding epigenetics was essential in order to understand all aspects of genetics. Gene expression in mammals depends on the epigenetic status of the chromatin, so it determines cell development, cell state and fate, cell cycle regulation, etc.

The most studied modifications are DNA methylation, histone modifications and non-coding RNAs (ncRNAs). DNA is highly compacted in three-dimensional chromosomes, DNA coils around histone octamers to form nucleosomes. This organization not only allows the DNA to compact, but also provides selective accessibility of transcription machinery. Histones are subjected to more than 130 posttranslational modifications such as acetylation, methylation, phosphorylation, sumoylation and ubiquitination. This, in

conjunction with DNA methylation, decides the transcriptional states of all the genomic regions determining the transcriptional programs (Chen et al., 2017).

Approximately, three-quarters of the genome are transcribed into RNA, but only a small portion of genes are translated into proteins. (Djebali et al., 2012). ncRNAs are RNAs that are not translated into proteins. These RNAs comprise another layer of internal signals that control gene expression, they may determine most of our complex characteristics, play a role in disease and in variation between individuals and species (Mattick and Makunin, 2006).

DNA methylation

The most common DNA modification is the cytidine 5'-addition of a methyl group in a CpG dinucleotide. There are CpG-rich regions called CpG islands, which are typically defined as regions of more than 200 nucleotides with a GC content of at least 50%, located in the 5' end of the regulatory region of many genes. They have critical roles in the control of gene activity and the architecture of the nucleus of the cell.

At first, it was thought that this modification was a stable, hereditary trait, but it has been recently discovered that methyl groups can be dynamically added or erased (Wu and Zhang, 2014). This is due to the existence of DNA methyltransferases that introduce the methyl group in the DNA, and DNA demethylases called Ten-Eleven Translocation enzymes (TET) that erase them.

In general, gene promoter CpG-island methylation is associated with gene silencing. It plays a key role in genomic imprinting, where hypermethylation at one of the two parental alleles leads to monoallelic expression. A similar mechanism is observed in gene-dosage reduction in X-chromosome inactivation in females (Li et al., 1993).

DNA methylation is not exclusive to CpG islands. Non-CG methylation has recently been described in humans in stem cells and seems to play a critical role in maintenance of pluripotent state (Laurent et al., 2010)

Cancer epigenetics

Epigenetic processes are natural and essential to many organism functions, but if they occur improperly, there can be major adverse health and behavioural effects. Epigenetic mechanisms account one-third to one-half of known genetic alterations (Weinhold, 2006). Among all epigenetic research, the most studied disease is cancer. In addition to classic mutations, cancer also presents aberrant DNA methylation. It was first discovered in 1983 and has since been observed in many other illnesses and health conditions (Feinberg and Tycko, 2004).

The alteration of DNA methylation patterns leads to disease in the cells, normal patterns and its alterations are shown in the figure below (**Figure 1**). DNA methylation can inhibit or enhance gene expression by recruiting histone-modifying and chromatin remodelling complexes to methylated or unmethylated sites.

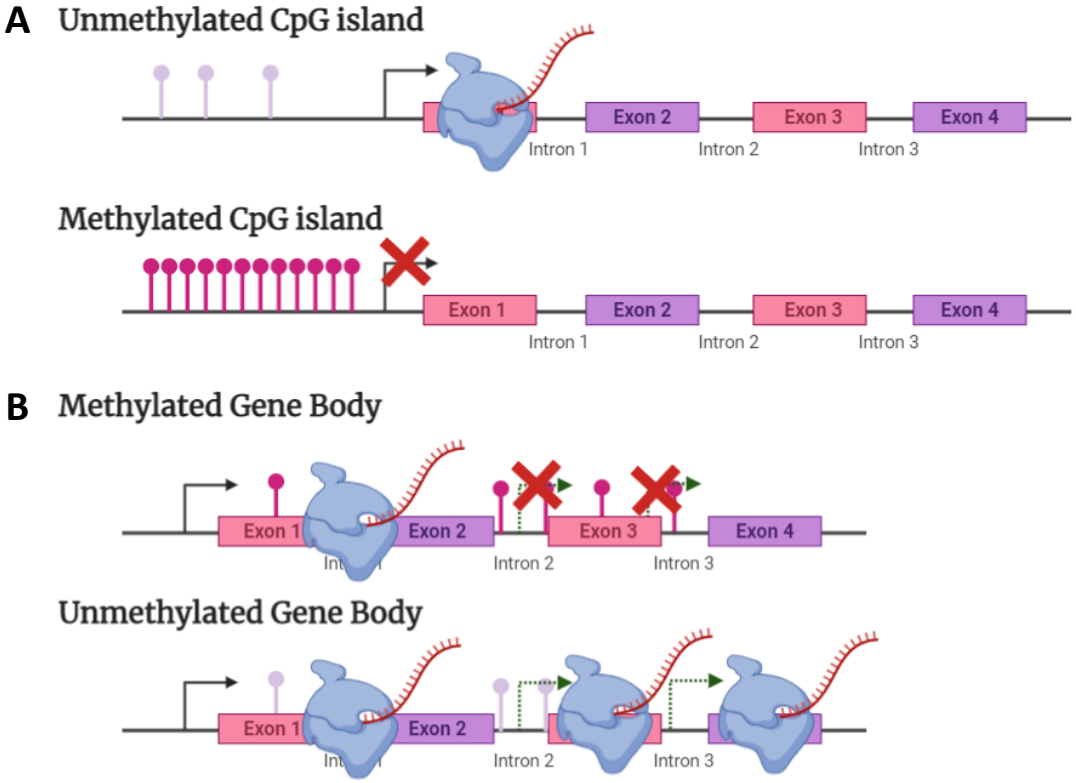

Figure 1: Schematic representation of normal and aberrant methylation patterns. (a) When CpG islands at gene promoters are unmethylated, transcription is allowed. In cancer, aberrant hypermethylation provokes gene silencing and thus lack of transcription. (b) Methylation at the gene body facilitates transcription and prevent aberrant transcription initiation, but in some diseases, gene body is unmethylated allowing transcription to be initiated at incorrect sites. Adapted from Portela and Esteller, 2010.

Cancer cells are characterized by global hypomethylation, with approximately a 20-60% overall loss of methylation. It occurs mainly at repetitive sequences, and promotes chromosomal instability, translocation and gene disruption (Goelz et al., 1985). This loss of methylation can lead to the overexpression of oncogenes (genes with the potential to cause cancer) and facilitate abnormal cell division. In contrast, some gene-specific promoters become hypermethylated, resulting in gene silencing (**Figure 1a**). When a tumour suppressor gene is silenced because of promoter hypermethylation, it stops inhibiting cell proliferation and tumour development (Llinàs-Arias and Esteller, 2017). On the other hand, gene body methylation facilitates a correct transcription, and it prevents aberrant transcription confining transcription initiation to canonical promoters. However, in some diseases gene body methylation is lost leading to abnormal transcription (**Figure 1b**). This genetic and epigenetic reprogramming shifts the cell form a normal phenotype to a malignant phenotype, contributing to the acquisition of all hallmarks of cancer cells.

Recent advances in techniques for methylation detection such as sodium bisulfite conversion and CpG island microarrays, have produced a vast amount of knowledge of methylation patterns in diverse human cancers. Tumour-specific methylation changes in different genes has been identified and documented, this information has potential clinical application in cancer diagnosis, prognosis and therapeutics (Das and Singal, 2004).

Epitranscriptomics and tRNA modifications

Epitranscriptomic is the field that studies the modifications in the RNA. More than 160 RNA chemical modifications have been identified to date. Abundant non-coding RNAs such as tRNAs, rRNAs and spliceosomal RNAs are heavily modified and depend on these modifications for their functions. Dynamic modifications in both coding and non-coding RNAs add another layer of control of genetic information and gene expression (Roundtree et al., 2017).

Transfer RNA (tRNA) is a single RNA strand about 80 nucleotides long that play a central role in translation. It functions as an adapter between the information contained in nucleic acids and the function of proteins. Other possible functions have been recently identified such as adding amino acids to membrane lipids and signalling during stress (Duechler, et al., 2016). tRNA activity is regulated through chemical modifications to control the complex formation between mRNA and ribosome. More than 50 tRNA modifications have been described in eukaryotic cells, and each tRNA molecule contains on average 13 modifications with approximately one-fourth of the nucleosides modified (Hoernes et al., 2017). These modifications play a critical role in translational function of tRNA at multiple levels, such as amino acid loading, wobbling or translation efficiency and fidelity (Duechler et al., 2016).

Previously, it was generally accepted that tRNAs were a housekeeping product with little or no regulatory function. However, nowadays it is known that defects in tRNA modifications are present in some human pathologies, and defects in tRNA modifier enzymes are present in various neurological disorders, mitochondrial diseases or cancer (Torres et al., 2014).

tRNA modification disruption in cancer

The expression of tRNAs is deregulated in cancer cells, but the mechanisms and functional meaning are not well understood. It was thought that tRNA abundance was correlated with tRNA gene copy number, but recent works suggest that tRNA expression regulation is far more complex. Overall, tRNA expression levels vary as much as ten-fold among human tissues, it was found that some oncogenes and tumour suppressor genes

regulate RNA pol III, the polymerase responsible for tRNA expression (Huang et al., 2018). Although the regulatory mechanisms are not fully understood, the same histone modifications upstream of mRNAs and tRNAs can be observed, which may contribute to achieve optimal tRNA supply ratio (Gingold et al., 2014). New data suggests that tRNA deregulation and translational error date have an important role in proteome diversity, cell population heterogeneity, genome instability and drug resistance in tumours, as tRNA expression deregulation influences the main steps of tumorigenesis. tRNAs can impact cancer cells in at least three ways: they can direct translation to the stabilization of transcripts, if the tRNA pool is disrupted they can promote tRNA mischarging, and tRNA imbalance can cause ribosome misreading and premature translation termination (Santos et al., 2019).

Wybutosine-derived nucleosides

Just as DNA itself, tRNAs can also endure nucleotide modifications. One example of tRNA modification is the wybutosine-derived nucleoside in position G37 of phenylalanine tRNA ($tRNA^{Phe}$). Wybutosine (yW), which was first described in 1968, is a tricyclic nucleoside found at the 3' position adjacent to the anticodon of eukaryotic $tRNA^{Phe}$. yW modification of $tRNA^{Phe}$ is known to stabilize codon-anticodon interactions during decoding on the ribosome (Konevega et al., 2004).

The biosynthesis of yW is a multienzymatic process, that originates from the genetically-encoded guanine 37 and spans six different chemical reactions enzymatically catalysed to originate the fully modified yW residue. In yeast only 5 enzymes are needed in order to synthetize wybutosine, but in humans another enzyme called TYW5 is required to produce wybutosine derivatives OHyW and o2yW (**Figure 2**) (Noma et al., 2010).

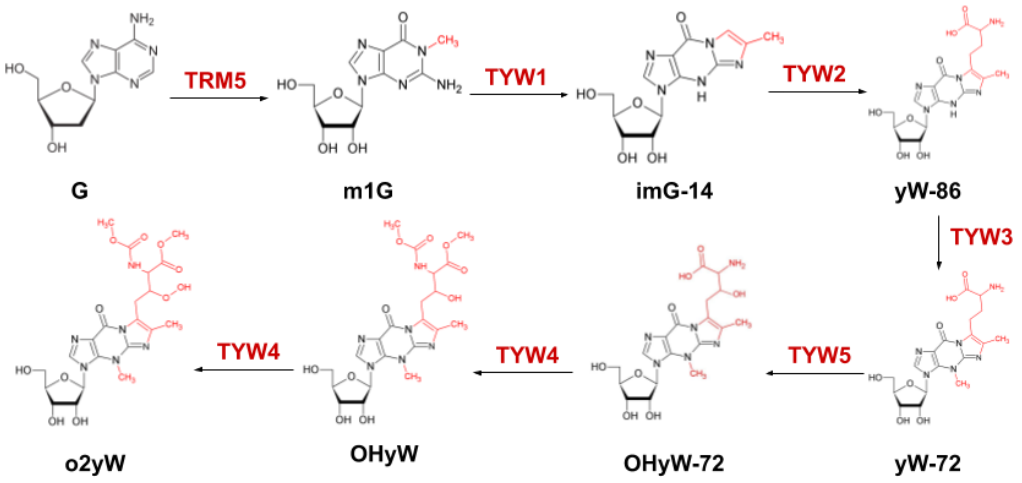

Figure 2: Biosynthetic pathway of wybutosine derivatives OHyW and o2yW in humans. Enzymes that catalyse the reactions and the modifications introduced are shown in red.

It was described that the lack of yW modification in $tRNA^{phe}$, stimulated the level of frameshifting events in retroviral sequences (Carlson et al., 1999). The most well understood frameshift event is a ribosomal slippage by one base in 5' direction, referred as programmed -1 ribosomal frameshifting event (-1 PRF). -1 PRF is commonly used by virus in order to synthesise two or more proteins from a single mRNA. It was found that, unlike in viruses, more than 99% of -1 PRF events that naturally occur in eukaryotic mRNA, direct the protein synthesis to an early STOP codon. Then, this mRNA is degraded through an independent process called the nonsense-mediated mRNA decay pathway (NMD). One of the essential genes of this pathway is the RNA helicase and ATPase UPF1. Thus, in eukaryotes this mechanism appears to be employed as a post-transcriptional regulatory mechanism (Advani and Dinman, 2016).

TYW2 epigenetic silencing in colorectal cancer

In order to further understand possible implications of tRNA modifications in cancer, our research group interrogated the presence of epigenetically inactivated genes encoding the enzymes responsible for yW synthesis.

First, a study of the DNA methylation status of the 5'-end associated CpG islands of all six enzymes that participate in yW synthesis was performed. Through datamining, TYW2 was observed to be methylated in 19.03% of the primary colorectal carcinomas available in The Cancer Genome Atlas (TCGA) and unmethylated in all the normal tissues. Furthermore, datamining of the TCGA RNA-sequencing data available showed that TYW2 hypermethylation was associated with transcript downregulation.

For the purpose to study the link between TYW2 promoter CpG island hypermethylation and transcriptional inactivation of the gene at RNA and protein levels, both methylated and unmethylated colorectal cell lines were used. Bisulfite genomic sequencing was performed on SW48, HT-29, HCT-116 and SW480 cell lines, observing that the 5'-end CpG island of TYW2 in SW48 and HT-29 was hypermethylated, and in HCT-116 and SW480 was unmethylated. Expression of TYW2 was determined by real time-PCR and western blot. Unmethylated cell lines expressed TYW2 mRNA and protein, while the methylated ones did not. Additionally, when DNA methylation inhibitors were used in hypermethylated cell lines, TYW2 expression was recovered, reinforcing the link between TYW2 promoter hypermethylation and transcriptional silencing.

Two stable TYW2 knockout models were generated using CRISPR/Cas9 system to generate a deletion in the gene body. The effectiveness of the knockout was confirmed by western blot, showing no protein expression of TYW2 in these HCT-116 and SW480 TYWO KO cell lines. In addition, in hypermethylated and TYW2-silenced HT-29 silenced

cell line, overexpression of TYW2 was restored by stable transfection. Nucleoside analysis of tRNAs using liquid chromatography-mass spectrometry (LC/MS) demonstrated that fully modified residues OHyW and o2yW were present in TYW2 expressing cells, and the intermediate imG-14, the direct substrate of TYW2, was absent. In contrast, in TYW2 KO cell lines, an accumulation of imG-14 and a loss of OHyW and o2YW was observed.

Hypermodified guanosine at position 37 of tRNA is known to help maintain the reading frame during translation by preventing -1 programmed ribosome frameshifting events at mRNAs containing slippery sequences (Carlson et al., 1999). In order to discover if this phenomenon was also present in human cancer cells, a dual luciferase reporter experiment was performed. In this procedure, when a ribosome frameshift event takes place firefly luciferase activity is abolished while renilla luciferase activity remains active. It was found that TYW2 silenced cell lines present a significant reduction in firefly luciferase activity compared to their TYW2-expressing counterparts. Thus, losing TYW2 and hypermodified o2yW in colon cancer cells confers a phenotype prone to ribosomal frameshifting events.

As explained above, programmed ribosome frameshifting may play a role in mRNA abundance. This is achieved by inducing premature STOP codon, resulting in mRNA degradation via NMD. In order to check if TYW2 epigenetic loss in colorectal cancer cells could induce aberrant mRNA degradation, an RNA-sequencing was performed in HCT-116 expressing TYW2 and in its counterpart the CRISPR knockout cell line. An alteration of 2.370 transcripts was observed, of these transcripts 2.046 were downregulated. In addition, among these downregulated transcripts, 109 contained at least one predicted slippery sequence with UUUU/C, the codon decoded by $tRNA^{Phe}$. Thus, these 109 transcripts were candidates to be directly targeted by TYW2 epigenetic loss in colon cancer cells.

Among this transcripts, roundabout guidance receptor 1 (ROBO1) was selected to further study. ROBO1 gene encodes a receptor that is a member of the neural cell adhesion molecule (NCAM) family of receptors. ROBO1 has been recently classified as a tumour suppressor gene in certain cancers. An association between ROBO1 promoter hypermethylation and the appearance of certain cancer cell characteristics was observed in renal, small lung, gastric and glioblastoma cells (Dallol et al., 2002; Xia et al., 2019; Kouam et al., 2018). ROBO1 was also reported to be lost in some colorectal tumours (Rezniczek et al., 2019). This altogether made ROBO1 a good target for studying the impact of transcript downregulation.

2. OBJECTIVES

The main aim of the project is to further understand the effect of the impairment of yW modification in $tRNA^{Phe}$ and transcript downregulation in colorectal cancer. To address this issue, we focused in studying ROBO1 and developed the following aims:

1. To determine the mechanisms involved in ROBO1 downregulation.
2. To determine the role of ROBO1 as tumour suppressor gene.

3. MATERIALS AND METHODS

Cell culture

The colon cancer cell lines HT-29, HCT-116 and SW480 were purchased from the American Type Culture Collection (ATCC) (**Table 1**). All of them were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37ºC and 5% CO_2. All cell lines were tested for the absence of mycoplasma.

Cell lines	Source	Identifier
HCT-116	ATCC	CCL-247
HT-29	ATCC	HTB-38
SW480	ATCC	CCL-228

Table 1: Cell lines used and their source.

Knockout models

Previously generated HCT-116 and SW480 TYW2 KO model were also used in this study. In these cell lines TYW2 expression was abolished using the CRISP/Cas9 system. Two different sgRNA were cloned into pSpCas9(BB)-2A-GFP vector (Addgene) and transfected using JetPrime® Transfection Reagent. Green positive cell lines were isolated by cell sorting to establish clonal cell lines after 48 hours of transfection and left for clonal expansion for approximately three weeks. Knockout clones were screened by amplification and sequencing of the sgRNA target region within TYW2 gene body. sgRNA constructions and primers used for amplification of the targeted region are listed below (**Table 2**).

Overexpression model

For stable TYW2 restoration in the hypermethylated HT-29 cell line, TYW2 cDNA was obtained from the HCT-116 cell line and cloned into pLVX-IRES-ZsGreen plasmid (Clontech) with a Kozak sequence and a Flag-tag at the C-terminus of the protein. Lentivirus containing this construct were produced by cotransfecting HEK293T cells with recombinant pLVX-IRES-ZsGreen, psPAX2 (Addgene) and pMD2.G (Addgene) using JetPrime® Transfection Reagent. After 72 hours, virus-containing media was collected, filtered and delivered to HT-29 cell lines. Green positive infected cells were purified by cell sorting after five passages. TYW2 cloning primers are listed in (**Table 2**).

Recombinant DNA		Source	Identifier
psPax2		Addgene	12260
pMD2.G		Addgene	12259
pLVX-IRES-ZsGreen1		Clonotech	632187
pSpCas9(BB)-2A-GFP		Addgene	48138
Commercial kits		Source	Identifier
JetPrime Transfection Reagent		Polyplus Transfection	114-75
Primers and sgRNA constructs		Source	Identifier
TYW2_sgRNA1_Fd	CACCGGTAGCGCCACCGAGCCATC	This study	-
TYW2_sgRNA1_Rv	AAACGATGGCTCGGTGGCGCTACC	This study	-
TYW2_sgRNA2_Fd	CACCGAGGCTGATTTGCCCCGATCA	This study	-
TYW2_sgRNA2_Rv	AAACTGATCGGGGCAAATCAGCCTC	This study	-
Cloning_TYW2_Fd	AAAAAAGAATTCGCCGCCACCATGAG AGAGAATGTGGTTGTTAGCAACATGGAGAGAGAAAGTGGGAA GCCCGTGGCTGT	This study	-
Cloning_TYW2_Rv	AAAAAAGCGGCCGCCTACTTATCGT CGTCATCCTTGTAATCGCCGGAGCCGCCAACTGAAGGACAG GGGCAGCATTCCAGATCCAGGACTATGTGATCCACATGGGG	This study	-

Table 2: Primers and kits needed for the cellular models with their commercial reference.

siRNA transfection

For siRNA transfection, 200.000-250.000 cells were seeded in 6-well plates. Transfection was carried out with jetPRIME transfection reagent according to manufacturer's guidelines. Concretely, 100 pmol of siRNA against UPF1 or a negative control (**Table 3**) were diluted in 200 µL of buffer. The mix was vortexed for 10 seconds and 4 µL of jetPRIME reagent were added and pipetted into the wells. Cells were incubated for 48 hours and collected by scrapping, then RNA extraction was conducted as described below.

siRNA	Source	Identifier
UPF1	Thermofisher Scientific	12990
Negative control	Thermofisher Scientific	AM4611

Table 3: List of siRNAs used and their commercial reference.

mRNA decay analyses

For mRNA decay analyses, cells were incubated with Actinomycin D (Sigma, ID: A4262) at 10 µg/mL and collected at 0, 1.5, 3 and 6 hours after treatment release. Then RNA extraction was performed as described below.

Expression analyses

RNA expression was assessed by quantitative reverse transcription PCR (qRT-PCR).

RNA extraction

Total RNA was extracted from cell pellets using SimplyRNA kit (Promega) in the automated Maxwell RSC device (Promega, ID:AS1340) according to manufacturer instructions. The samples were prepared adding 200 µL of homogenization buffer and mixing it. When the cartridges were prepared with plungers, elution tubes and DNAse, 200 µL of lysis buffer were added to the samples, mixed and poured into the first well with the neutralization buffer.

RT-PCR

Total RNA concentration was determined by Nanodrop One, and total RNA was converted to cDNA using the RevertAird First Strand cDNA synthesis kit (Thermofisher Scientific, ID: K1622) according to manufacturer's guidelines.

To prepare the samples, 0,5 µg of RNA were added into each tube and the volume was completed until 11 µL. A mix with buffer, nucleotides, random primers, ribolock (RNase inhibitor) and RevertAid (retrotranscriptase) was prepared, then 9 µL of mix was pipetted into each tube, resulting in a volume of 20 µL. Then SimpliAmpTM Thermal Cycler was programmed with 25º 5 min, 42º 1h, 70º 5 min and 4º infinite.

qPCR

Quantitative PCR was performed using SYBR Green PCR Master Mix (Life Technologies, ID: 4312704) according to manufacturer's specifications.

cDNA samples were diluted first, obtaining 20ng of DNA in 5 µL. Then the mix was prepared with 5 µL of SYBR Green Master Mix and 0,15 µL of primers at a concentration of 10 µM. 5 µL of cDNA and 5 µL of mix were loaded into a 384 well plate. QuantStudioTM 7 RT-PCR System was programmed to perform the quantification. GAPDH expression was used as endogenous control to normalize the results, that were analysed following this formula: $2^{(-\Delta_1\Delta_2 Gt)}$. Δ_1 refers to gene minus GAPDH and Δ_2 refers to sample minus control. The primers were previously tested and showed a good efficiency with r =-3.33.

All primers are listed in **Table 4**.

Oligonucleotides	
qPCR_ROBO1_Fd	GGAGTCAGGGGCACAAGAAA
qPCR_ROBO1_Rv	GGCCTCGTTCATCTTCCTCC
qPCR_VIM_Fd	CTTAAAGGAACCAATGAGTCCCT
qPCR_VIM_Rv	AGTGAATCCAGATTAGTTTCCCTC
qPCR_CDH1_Fd	GGGGTCTGTCATGGAAGGTG
qPCR_CDH1_Rv	GAAACTCTCTCGGTCCAGCC
qPCR_UPF1_Fd	CCATCCCCTTCAACCTGGTC
qPCR_UPF1_Rv	GTTGGGGAGGTTAGTCTGGC
qPCR_GAPDH_Fd	GAAGGTGAAGGTCGGAGTC
qPCR_GAPDH_Rv	TGGACTCCACGACGTACTCA

Table 4: List of primers used and its sequence.

Western Blot

Protein expression was determined by western blotting. Total protein was extracted with RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40), and Lamin B1 (LMNB1), β-actin (ACTB) or Vinculin (VCL) were used as loading controls. For membrane fraction enrichment to perform ROBO1 western blot, fresh cell pellets were fractioned using ProteoExtract® Subcellular Proteome Extraction Kit (Merck) and Calnexin was used as endogenous control. All primary antibodies used are listed in **Table 5**.

Antibodies	Source	Identifier
anti-TYW2	Novus Bio	NBP1-76583
anti-ROBO1	Abcam	Ab7279
anti-Calnexin	Cell Signaling	2679
anti-HA HRP-conjugated	Sigma	H6533
anti-PARP	Cell Signaling	9542
anti-p21	Cell Signaling	2497
anti-B-Actin HRP-conjugated	Sigma	A3854
anti-LaminB1	Abcam	Ab16048
anti-Vinculin HRP-conjugated	CST	18799
anti-rabbit HRP-conjugated secondary antibody	Sigma	A0545

Table 5: List of primary and secondary antibodies used for Western Blotting.

Migration assays

Cell migration capacity was assessed by transwell assay. 100.000 HCT-116 cells and 200.000 SW480 cells were seeded in serum-free media in the upper chamber of an 8 µm pore transwell polycarbonate membrane insert and left for migration to the serum-containing lower chamber (6.5mm Transwell with 8.0 µm pore polycarbonate membrane insert from Corning, ID: 3422). After 48 hours, transwell membranes were fixed with 10% trichloroacetic acid for 1 hour and stained with 0.057% SRB for 30 minutes. ImageJ was used to calculate the percentage of membrane area occupied by cells to assess cell migration.

Cell cycle assays

Cell cycle was analysed on Bromodeoxyuridine (BrdU) labelled cells using an APC antiBrdU/7AAD staining using an APC BrdU Flow Kit (BD Biosciences, 552598). Briefly, cells were incubated with 10 µM BrdU during 1h at 37ºC. Then, cells were washed, fixed and permeabilized following manufacturer's instructions. Samples were incubated with APC-labelled anti-BrdU antibody during 30 minutes at room temperature. Then total DNA was stained using 2uL of 7AAD solution. A minimum of 10,000 cells was analysed per sample. Experiments were performed in triplicate.

ROBO1 restoration

For transient ROBO1 restoration (72h) in the TYW2 silenced cell lines HCT-116 KO and SW480 KO, ROBO1 cDNA was obtained from the SW480 WT cell line and cloned into pcDNA4 T/O plasmid (Thermofisher Scientific) with a Kozak sequence and a HA-tag at the C-terminus of the protein. This construct and the empty pcDNA4 T/O were transfected using JetPrime Transfection Reagent following the manufacturer's indications. Cells were collected after 72 hours and RNA and protein were extracted as previously described. ROBO1 cloning primers are listed in **Table 6**.

Primers
Cloning_ROBO1_Fd AAAAAAAAGCTTCTCGAGGCCGCCACCATGATTGCGGAGCCCGCTCACTTTTACCTGTTTGGATTAATATGTCTCTGTTCAG
Cloning_ROBO1_Rv TTTTTTGCGGCCGCTCAGGCGTAGTGGGCACGTCGTAGGGGTATCCTGATCCGCTTTCAGTTTCCTCTAATTCTTCATTATTATCTTCTCCTCTTTCATATCCTCCAAGTACCTGCATTTCTGCAATATTTCTTCGACCT

Table 6: Primers used in ROBO1 restoration

Statistics

Data was analysed using GraphPad Prism 5. Results are presented as Mean ± SD, for the n independent experiments. Experimental data sets were compared by two-tailed Student's t-test. Values of * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ were considered statistically significant.

4. RESULTS

First, we confirmed by qPCR and WB the ROBO1 downregulation observed in previous RNA-sequencing experiment when TYW2 is silenced. In HCT-116 and SW480 cell lines a significant reduced expression in both mRNA and protein levels is observed (**Figure 3a**). In contrast, in the HT-29 cell line model, when TYW2 is overexpressed, ROBO1 expression increases (**Figure 3b**). The results confirm that TYW2 silencing induces ROBO1 downregulation.

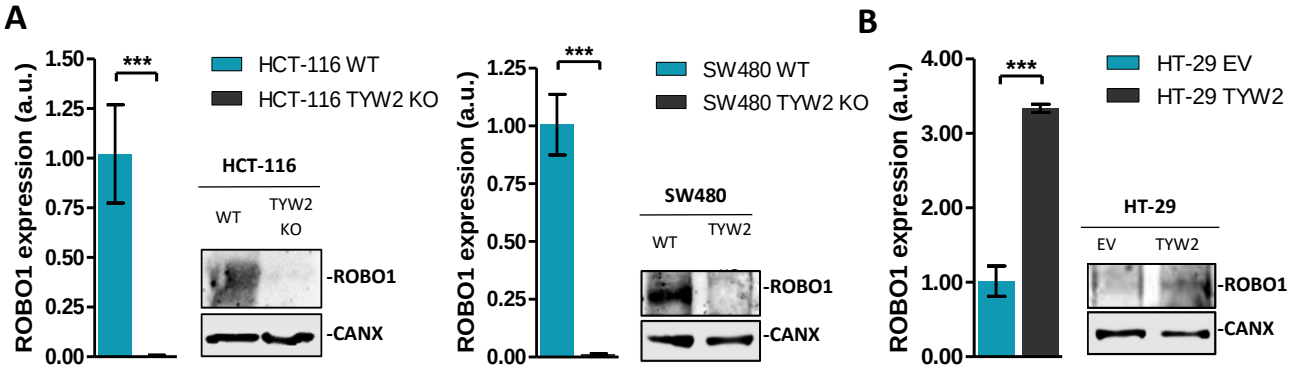

Figure 3: ROBO1 expression levels determined by qPCR and WB. (a) TYW2 unmethylated/expressing cell lines HCT-116 and SW480 and their TYW2-silenced counterparts. (b)TYW2 methylated cell line HT-29 and its TYW2 overexpression counterpart. qPCR data shown represents the mean ±SD of three biological replicates and were analysed using unpaired two-tailed Student's t tests. ***p<0.001.

Once ROBO1 was validated as a potential candidate regulated by TYW2, we wondered what the underlying mechanism was. To address this, we performed an mRNA stability determination by Actinomycin D chase assay (**Figure 4**). When TYW2 is silenced, ROBO1 mRNA half-life is significantly decreased, as it suffers a rapid degradation (**Figure 4a**). In addition, TYW2 overexpression stabilizes ROBO1 transcript (**Figure 4b**).

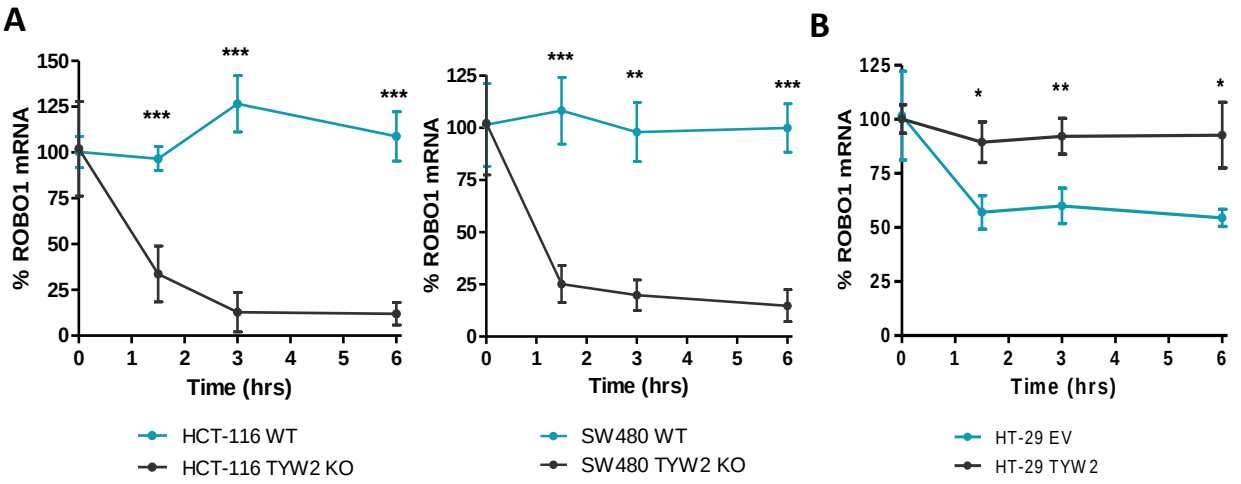

Figure 4: ROBO1 mRNA stability determination by actinomycin D chase assay. (a) TYW2 unmethylated/expressing cell lines HCT-116 and SW480 and their TYW2-silenced counterparts and (b) TYW2 methylated cell line HT-29 and its TYW2 overexpression counterpart. For all cases, data shown represents the mean ±SD of at least four biological replicates analysed by unpaired two-tailed Student's t-test at each time of the point. *p<0.05, ** p<0.01, ***p<0.001.

These results suggest that the reason for this regulation may be a change in mRNA half-life, and coincide with the hypothesis that when G37 is hypomodified a -1 PRF is likely to happen and an early STOP codon might appear. Then, the mRNA is degraded via NMD. In order to test if this degradation is indeed occurring via NMD, a siRNA transfection versus UPF1 was performed to inhibit the pathway. First, we checked by qPCR if the siRNA versus UPF1 worked (**Figure 5a**). Depletion of UPF1 is confirmed in all cell lines transfected with siRNA against UPF1, in comparison to a transfection with a scramble siRNA. Then, we checked ROBO1 mRNA levels, by qPCR (**Figure 5b**). When the NMD pathway is inhibited in TYW2-silenced cell lines (HCT-116 TYW2 KO, SW480 TYW2 KO and HT-29 EV), ROBO1 mRNA is significantly increased compared to when the pathway is not inhibited. These differences are not observed in TYW2-expressing cell lines (HCT-116 WT, SW480 WT and HT-29 TYW2). These results suggest that ROBO1 mRNA depletion in TYW2-silenced cells is due to NMD.

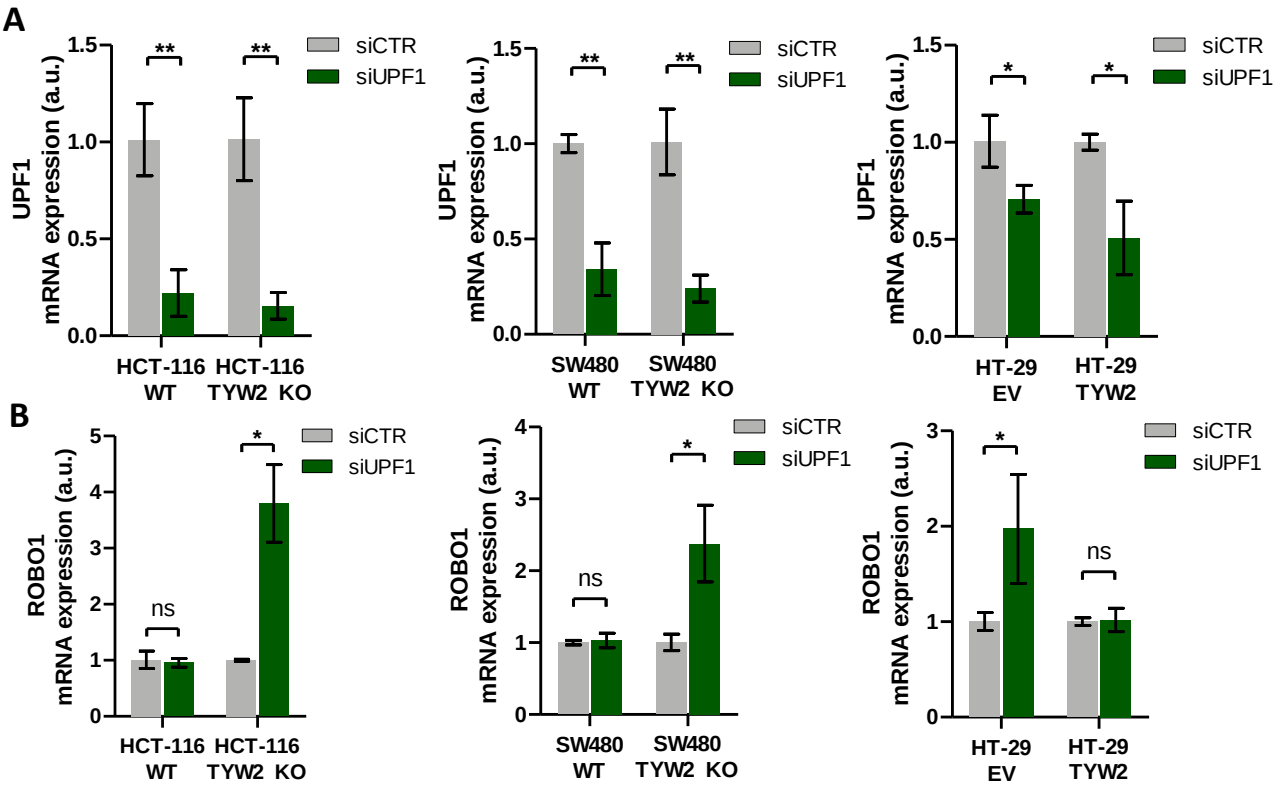

Figure 5: UPF and ROBO1 mRNA expression determination by qPCR upon siRNA transfection. (a) UPF1 expression level determined by qPCR in HCT-116 and SW480 cell lines and their TYW2 silenced counterparts. It is also determined in HT-29 cell line and its TYW2 overexpression counterpart. This experiment was performed under two conditions, one with a siRNA transfection versus UPF1 and one with scramble siRNA. (b) ROBO1 expression level determined by qPCR in the same cell lines and conditions as the UPF1 determination. qPCR data shown represents the mean ±SD of three biological replicates and were analysed using unpaired two-tailed Student's t tests. * $p<0.05$, ** $p<0.01$, ns: non-significant.

Once the connection between TYW2 and ROBO1 was studied, we wondered about the consequences of this ROBO1 downregulation. It has been previously described that

ROBO1 is a tumour suppressor gene and seems to play a role in cell migration. So, we performed a Transwell assay to determine cell migration ability (**Figure 6**). HCT-116 and SW480 TYW2 silenced cell lines with decreased ROBO1 expression showed a significantly higher number of migrated cells compared to WT cells.

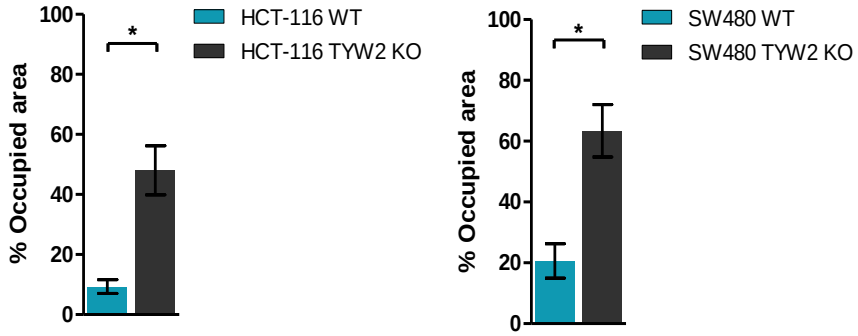

Figure 6: Transwell assay. Performed in HCT-116 and SW480 TYW2 unmethylated/expressing cell lines and their TYW2 silenced counterparts. An increased cell migration ability is observed in TYW2 silenced cell lines compared to the WT ones. p-values are associated with an unpaired two-tailed Student's t-test. *p<0.05.

Looking at the enhanced migration ability, we evaluated by qRT-PCR two classical EMT markers, E-cadherin (CDH1) and Vimentin (VIM). The former is an epithelial marker and the latter is characteristic of mesenchymal cells. We observed an increase in vimentin expression and a downregulation of E-cadherin mRNA, indicating that TYW2-silenced cells display mesenchymal features (**Figure 7**).

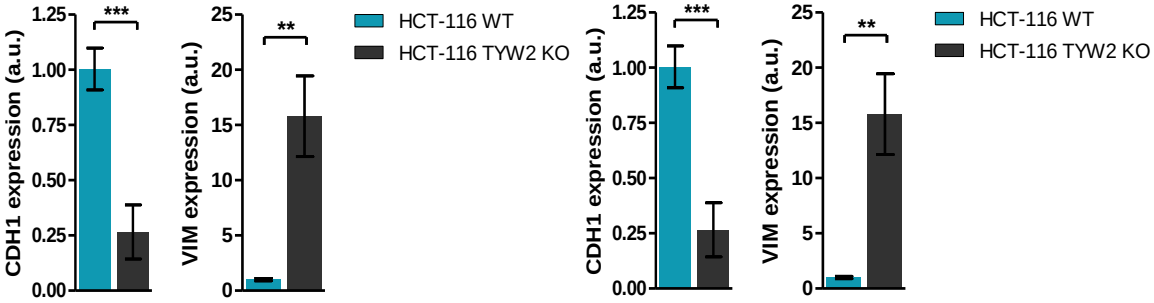

Figure 7: E-cadherin and Vimentin determination by q-PCR. Performed in HCT-116 and SW480 TYW2 unmethylated/expressing cell lines and their CRISPR/Cas9 TYW2 silenced counterparts. An enhanced EMT is observed in TYW2 silenced cell lines as a decrease in E-Cadherin and an increase in Vimentin are observed. qPCR data shown represents the mean ±SD of three biological replicates and were analysed using unpaired two-tailed Student's t tests. ** p<0.01, ***p<0.001.

We wondered if TYW2 silencing has an impact in cell viability, so we analysed cell cycle and apoptosis. No differences in cell cycle were found between unmethylated cell lines and their CRISPR/Cas9-mediated TYW2 KO counterpart (**Figure 8a**). Similar protein levels of p21 were determined by WB, indicating the cell cycle is not arrested because of p21 induction (**Figure 8b**). No differences in PARP cleavage were observed, indicating

that cell death is not increased in TYW2-KO cell lines compared to the TYW2-expressing counterparts (**Figure 8c**).

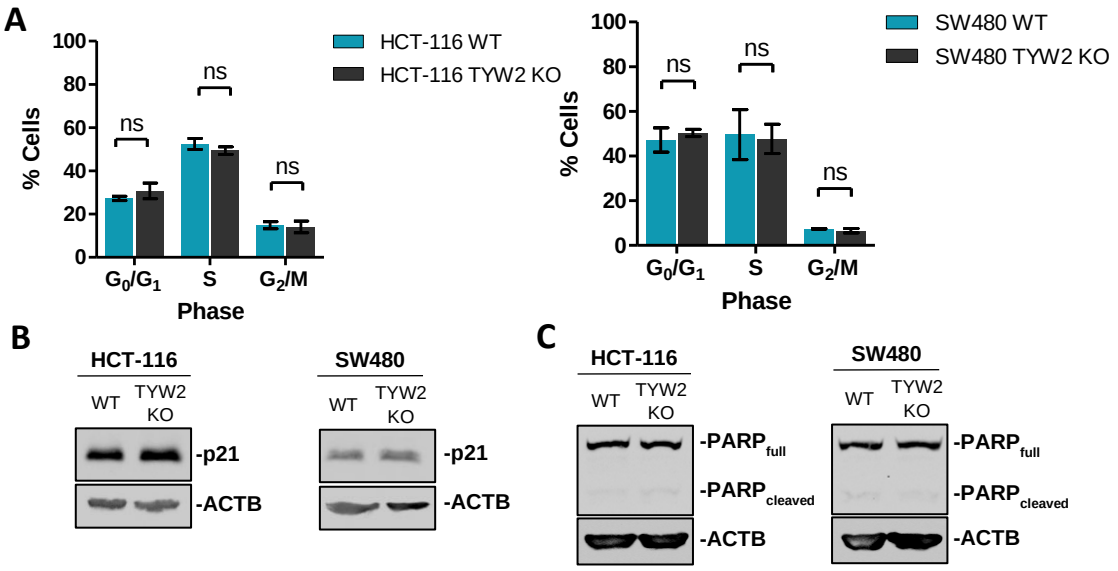

Figure 8: Cell cycle and cell death analysis assessed by BrdU and 7AAD incorporation and WB. Performed in HCT-116 and SW480 cell lines and their CRISPR/Cas9-mediated TYW2 KO counterparts. (a) Cell cycle analysis does not show any differences between cell lines. (b) Similar protein levels of p21 determined by WB indicate no differences in cell cycle arrest. (c) PARP cleavage evaluated by WB indicates no differences in cell death between TYW2-expressing and TYW2 knock-out cell lines. p-values are associated with an unpaired two-tailed Student's t-test. ns: non-significant

As TYW2 silencing affect many transcripts, we performed a ROBO1 expression recovery by transient transfection so we can further understand the role of ROBO1 in this phenotype. This was performed in HCT-116 and SW480 TYW2-silenced cell lines. First, the restoration was confirmed at both transcript and protein level by qPCR and WB respectively (**Figure 9**).

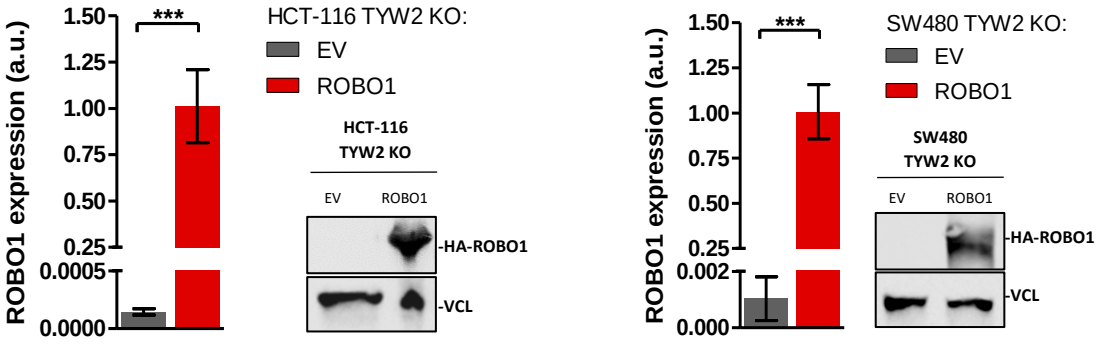

Figure 9: ROBO1 expression levels determined by qPCR and WB. Performed in CRISPR/Cas9 TYW2 silenced cell lines transfected with an empty vector and their ROBO1 transfected counterparts. A significant increase in ROBO1 at both transcript and protein level is observed at the transfected cell lines. qPCR data shown represents the mean ±SD of three biological replicates and were analysed unpaired two-tailed Student's t tests. *p<0.05, ** p<0.01.

Once ROBO1 was confirmed to be expressed, the same E-cadherin and Vimentin determination was performed by qPCR (**Figure 10**). The results obtained matched the previous results. An increase in E-cadherin as well as a decrease in Vimentin was observed in both cell lines. This suggests a reversion of the mesenchymal phenotype.

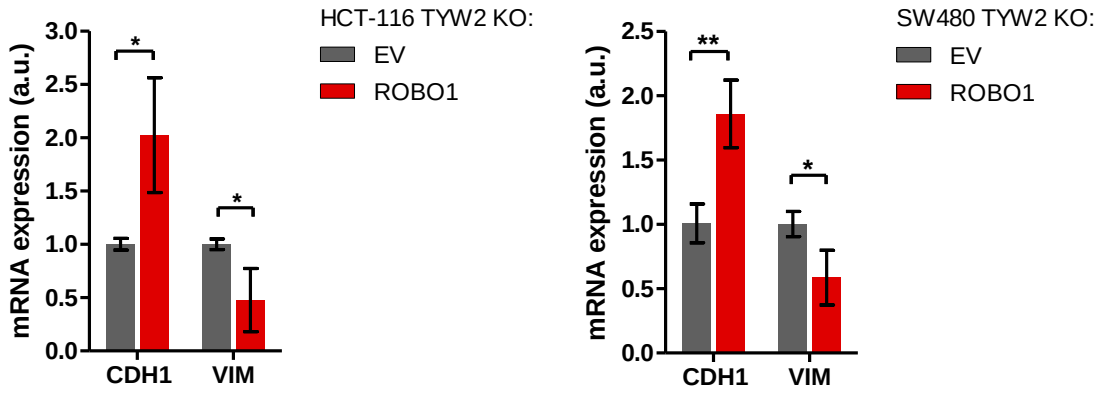

Figure 10: E-cadherin and Vimentin quantification by q-PCR. Performed in HCT-116 and SW480 CRISPR/Cas9 TYW2 silenced cells transfected with an empty vector and their ROBO1 transfected counterpart. A reversion of EMT is observed in ROBO1 restored cell lines as an increase in E-Cadherin and a decrease in Vimentin are observed. qPCR data shown represents the mean ±SD of three biological replicates and were analysed unpaired two-tailed Student's t tests. ***p<0.001, ns: non-significant.

To further evaluate the changes in the migration abilities of the cells after ROBO1 recovery, a Transwell assay was performed (**Figure 11**). The experiment did not show significant differences between ROBO1 expressing or silenced cell lines, so we cannot affirm that restoring ROBO1 expression alone is sufficient to reduce cell migration despite contributing to EMT reversion.

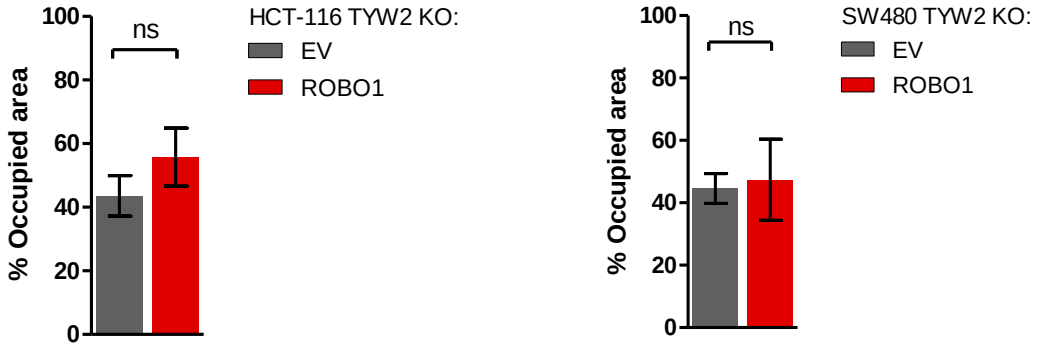

Figure 11: Transwell assay. Performed in HCT-116 and SW480 TYW2 CRISPR/Cas9 silenced cell lines transfected with an empty vector and their ROBO1-transfected counterparts. No differences in cell migration are observed. p-values are associated with an unpaired two-tailed Student's t-test. ns: non-significant.

5. DISCUSSION

Cancer cells acquire some biological capabilities during neoplastic transformation, known as the hallmarks of cancer. Some examples of them are a sustained proliferative signalling, activated invasion and metastasis, genome instability and mutations, and cellular energetics deregulation (Hanahan & Weinberg, 2011). Classical genetics, epigenetics and epitranscriptomics have been studied in the context of cancer, and their alteration contributes to the acquisition of these hallmarks. tRNAs are extremely important for proper cell function, because together with the ribosomes, they allow the translation of genetic code into amino acids and proteins. 47 different types of tRNAs are found in humans, and the amount of each tRNA is tightly controlled to match the codon usage of mRNAs to optimize protein expression according to cellular necessities. For many years, it was generally accepted that tRNAs were a housekeeping product with little regulatory function and were overlooked in cancer. It was thought that tRNA did not play a role in in cellular transformation, and the changes in tRNA levels were just a consequence of the high proliferative status of tumoral cells. However, recent studies shown that dysregulation of tRNAs and tRNA derivatives are involved in proliferation, metastasis, invasiveness of cancer cell, tumour growth and angiogenesis (Huang et al., 2018; Santos et al., 2019). Some data that supports this idea are the findings of mitochondrial tRNA mutations in cancer cells (Florentz et al., 2003) and the detection of tRNA derivatives in transformed cells that exerts oncogenic or tumour suppressor functions (Maute et al., 2012; Balatti et al., 2017). It was also described that tRNAs both cytosolic and mitochondrial can bind to the apoptotic mediator cytochrome c, having an anti-apoptotic effect (Mei et al., 2010).

The epitranscriptomics field is quickly growing as new data is available and RNAs regulatory function is starting to be understood. A lot of chemical modifications have been described, such as 5-methylcitosine, N1-methyladenosine, pseudouridine… The proteins which function is adding, removing or binding to these modifications are starting to be found altered in many pathologies like cancer (Esteve-Puig et al., 2020). Among all RNAs, tRNAs are one of the most modified ones and are tightly regulated through chemical modifications. More than 50 tRNA modifications have been described in eukaryotic cells (Hoernes et al., 2017). These modifications play a critical role in translational function of tRNA at multiple levels, and aberrant modification patterns are observed in cancer. Disruptions in tRNA modifiers can be involved in carcinogenesis and tumour progression, for example, ELP3 acts in the Wnt pathway, is upregulated in some tumours and has been linked to metastasis (Delaunay et al., 2016). The tRNA methylatransferase 9B seems to exert a role in tumour suppression, and is

downregulated in breast, bladder, colorectal, cervix and testicular carcinomas (Begley et al., 2013). These are only few examples of the increasing amount of new data about RNA modifications, which leads us to conclude that RNA modifications play critical roles in cell function such as control of gene expression, cellular differentiation and tumorigenesis.

Modifications at wobble position 34 and purine 37 of tRNA restrain the dynamics of the anticodon loop, in other words, they change the conformation of the tRNA towards the canonical structure (Agris, 2008). Decades ago, hypermodification of G37 in $tRNA^{Phe}$ was reported deficient in some cancer models (Grunberger et al., 1975; Mushinski and Marini, 1979; Kuchino et al., 1982). Our group studied this phenomenon and demonstrated that the hypomodification is due to the cancer specific inactivation of the tRNA modifier enzyme TYW2. Among a wide spectrum of human cancer types, a promoter CpG island hypermethylation-associated silencing of TYW2 was observed, including colorectal cancer, the most common type of cancer where this epigenetic defect was described. The loss of TYW2 was associated with the lack of hypermodified forms of G37 such as OHyW and o2yW. More importantly, it was linked with increased ribosome frameshift leading to transcript degradation. The transcriptomic assay showed that there are many RNAs targeted by this tumour-specific event induced upon TYW2 silencing. In this project we determined that the tumour suppressor ROBO1 is a robust example of key genes that can be hampered by this process. We also determined the mechanisms acting in the transcript degradation.

For years, it has been known that the pathway Slit-ROBO directs axon guidance and neuronal precursor cell migration. Slit proteins are secreted into the extracellular space and mediate chemorepulsive signals in cells expressing ROBO receptors. In 2006 a new potential role for Slit/Robo signalling pathway was proposed, as it was reported that ROBO and Slit families regulate cell migration in endothelial cells. Some studies found strong correlation between the expression of ROBO4 and an increased angiogenesis in several types of solid tumours. An upregulation of ROBO1 in colorectal cancer was also determined (Gröne et al., 2006). This suggested Slit/ROBO as a potential selective target for cancer treatment with fewer side effects than chemotherapy. However, the role of Slit/ROBO in cell migration was not fully elucidated, and further studies regarding functional analyses were needed (Fujiwara et al., 2006).

Two years later, Slit2 and ROBO1 expression was studied through qRT-PCR and immunohistochemistry in order to better understand their role in tumour biology. It was found that astrocytic tumour cells and glioblastoma cells overexpressed ROBO1 at

mRNA and protein levels, suggesting that the chemorepulsive effect mediated by Slit2 and ROBO1 participates in glioma cell guidance in the brain. In the study, they suggested that expressing Slit 2 may stimulate invasion of tumour cells from the main tumour mass (Mertsch et al., 2008). This was not the first time ROBO1 was studied in the context of cancer, a study performed in mice proposed ROBO1 as a tumour suppressor gene. When a ROBO1 homozygote deletion was introduced into the mice germ line, the descendant died at birth, and their lungs showed hyperplasia and increased mesenchyme (Xian et al., 2001). The study continued with the focus in the heterozygous mice and their tumour susceptibility. It was found that mice with the deletion present a 3-fold increase chance to develop spontaneous cancer in their second year of life. It was also determined that the structurally normal allele presented substantial methylation in the gene's promoter (Xian et al., 2004).

After these findings, many groups studied the role of ROBO1 in the context of various cancers. It was found that Slit/ROBO proteins are expressed in both normal and malignant ovarian tissues, and it seems that they do not play an important role in regulating human ovarian cancer cell proliferation and migration (Dai et al., 2011). In breast cancer, it was found that Slit/ROBO1 signalling limits cell proliferation in the basal layer inhibiting canonical WNT signalling and influencing branching morphogenesis (Macias et al., 2011), it was later found that this was due to the blocking of PI3K/Akt/β-catenin pathway (Chang et al., 2012). Additionally, another independent study in breast cancer patients correlated low expression of Slit2 and ROBO1 with poor prognosis and increased brain metastasis (Qin et al., 2015). In cervical cancer, a study of Slit/ROBO1 promoter hypermethylation showed that the pathway inactivation significantly contributes to the cancer pathogenesis (Nayaran et al., 2006).

These findings pinpoint ROBO1 as a tumour suppressor gene, and are compatible with our own study, as we found that in colorectal cancer the loss of ROBO1 contributes to the acquisition of mesenchymal features and an increase in migration ability. However, not all studies suggest the same. The first time that ROBO1 was observed to be overexpressed was in hepatocellular carcinoma, and the study suggested this gene as a potential serologic marker and therapeutic target (Ito et al., 2006). Later, when MiR-218 was studied in the context of gastric cancer and nasopharyngeal carcinoma, the overexpression of ROBO1 triggered tumour metastasis and was related to poor survival. The suppression of ROBO1 inhibited tumour cell invasion and metastasis in vitro and in vivo (Alajez et al., 2011; Tie et al., 2010). This phenomenon was also observed in breast cancer (Yang et al., 2012), pancreatic cancer (He et al., 2014), and glioma (Gu et al., 2015). In osteosarcoma, slit2/ROBO1 axis seems to contribute to Warburg effect, which

promotes cancer progression (Zhao et al., 2018). Slit2/Robo1 signalling is also oncogenic in intestinal tumorigenesis (Zhang et al., 2015).

These studies do not necessarily contradict our work, as ROBO1 was studied under different conditions, and it is known to be differentially expressed in human cancers. We studied ROBO1 in the context of colorectal cancer, in lines with an aberrant expression of TYW2. TYW2 malfunction potentially affects hundreds of transcripts, as it provokes an hypomodification of $tRNA^{Phe}$, and ROBO1 is one of them. Through TYW2 modulation we determined that in our colorectal cancer cell line models, ROBO1 acts as a tumour suppressor gene. Further studies are needed in the future to better understand the controversial role of ROBO1 in cancer.

From the clinical side, it was observed that the DNA methylation-linked loss of TYW2 is associated with poor clinical survival in colorectal cancer patients at early stages of the disease. We determined that a possible cause for this poor outcome might be an increase in metastasis, as TYW2 silencing and the consequent reduction in ROBO1 contribute to EMT and enhances the migration ability of the cells. However, our group determined that 109 transcripts are potential targets for the epigenetic loss of TYW2, and that is a possible explanation for the not fully recover of the cells when ROBO1 is restored. Thus, TYW2 methylation status could be proposed as a biomarker of early dissemination for tumours.

In the last decades many drugs with DNA methyltransferases inhibitory effect have been discovered. One example of hypomethylating agent is 5-Azacytidine, which is approved by the FDA for treatment of myelodysplastic syndrome (Christman, 2002). Given the emergence of many epigenetic drugs that restore the activity of genes inactivated by DNA hypermethylation, it is tempting to speculate that a treatment with inhibitors of DNA methylation might rescue TYW2 deficiency, restoring ribosome and RNA base pairing, and avoid migration prone features of these cells, thus, reducing the mortality of the identified high-risk patients that present otherwise an apparent localized site. Although these hypomethylation therapy has less side effect than conventional cytotoxic therapy, they still have some side effects that limits doses and duration of the treatment and have the potential to form mutagenic lesions, so better understanding of how mechanism based inhibitors interact with DNA methyltransferases is needed in order to develop better drugs (Issa, 2007).

6. CONCLUSIONS

- ROBO1 expression is TYW2 dependent: when TYW2 is silenced, ROBO1 is downregulated and when TYW2 is overexpressed, ROBO1 is upregulated.
- TYW2 regulates ROBO1 expression through mRNA surveillance via NMD.
- TYW2 silencing does not affect cell cycle and cell death.
- Cells with low ROBO1 expression tend to express mesenchymal features and so are more prone to migrate.
- ROBO1 rescue in TYW2 silenced cells lightly reverts EMT but is not enough to reduce cell migration.

7. BIBLIOGRAPHY

Adams, J. M., & Cory, S. (2007). The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene, 26(9), 1324-1337.

Advani, V. M., & Dinman, J. D. (2016). Reprogramming the genetic code: The emerging role of ribosomal frameshifting in regulating cellular gene expression. Bioessays, 38(1), 21-26.

Agris, P. F. (2008). Bringing order to translation: the contributions of transfer RNA anticodon-domain modifications. EMBO reports, 9(7), 629-635.

Alajez, N. M., Lenarduzzi, M., Ito, E., Hui, A. B., Shi, W., Bruce, J., ... & O'Sullivan, B. (2011). MiR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2-ROBO1 pathway. Cancer research, 71(6), 2381-2391.

Baeriswyl, V., & Christofori, G. (2009, October). The angiogenic switch in carcinogenesis. In Seminars in cancer biology (Vol. 19, No. 5, pp. 329-337). Academic Press.

Balatti, V., Nigita, G., Veneziano, D., Drusco, A., Stein, G. S., Messier, T. L., ... & Palamarchuk, A. (2017). tsRNA signatures in cancer. Proceedings of the National Academy of Sciences, 114(30), 8071-8076.

Ballard, M. S., & Hinck, L. (2012). A roundabout way to cancer. In Advances in cancer research (Vol. 114, pp. 187-235). Academic Press.

Banerjee, K., & Resat, H. (2016). Constitutive activation of STAT 3 in breast cancer cells: A review. International journal of cancer, 138(11), 2570-2578.

Begley, U., Sosa, M. S., Avivar-Valderas, A., Patil, A., Endres, L., Estrada, Y., ... & Begley, T. (2013). A human tRNA methyltransferase 9-like protein prevents tumour growth by regulating LIN9 and HIF1-α. EMBO molecular medicine, 5(3), 366-383.

Cancer. (2018). Retrieved from: https://www.who.int/health-topics/cancer#tab=tab_3. Retrieved date: June, 2020.

Carlson, B. A., Kwon, S. Y., Chamorro, M., Oroszlan, S., Hatfield, D. L., & Lee, B. J. (1999). Transfer RNA modification status influences retroviral ribosomal frameshifting. Virology, 255(1), 2-8.

Chang, P. H., Hwang-Verslues, W. W., Chang, Y. C., Chen, C. C., Hsiao, M., Jeng, Y. M., & Lee, W. H. (2012). Activation of Robo1 signaling of breast cancer cells by Slit2 from stromal fibroblast restrains tumorigenesis via blocking PI3K/Akt/β-catenin pathway. Cancer research, 72(18), 4652-4661.

Chen, Z., Li, S., Subramaniam, S., Shyy, J. Y. J., & Chien, S. (2017). Epigenetic regulation: a new frontier for biomedical engineers. Annual review of biomedical engineering, 19, 195-219.

Chiu, C. P., & Harley, C. B. (1997). Replicative senescence and cell immortality: the role of telomeres and telomerase. Proceedings of the Society for Experimental Biology and Medicine, 214(2), 99-106.

Christman, J. K. (2002). 5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. Oncogene, 21(35), 5483-5495.

Coussens, L. M., & Werb, Z. (1996). Matrix metalloproteinases and the development of cancer. Chemistry & biology, 3(11), 895-904.

Dai, C. F., Jiang, Y. Z., Li, Y., Wang, K., Liu, P. S., Patankar, M. S., & Zheng, J. (2011). Expression and roles of Slit/Robo in human ovarian cancer. Histochemistry and cell biology, 135(5), 475-485.

Dallol, A., Forgacs, E., Martinez, A., Sekido, Y., Walker, R., Kishida, T., ... & Latif, F. (2002). Tumour specific promoter region methylation of the human homologue of the Drosophila Roundabout gene DUTT1 (ROBO1) in human cancers. Oncogene, 21(19), 3020-3028.

Das, P. M., & Singal, R. (2004). DNA methylation and cancer. Journal of clinical oncology, 22(22), 4632-4642.

Delaunay, S., Rapino, F., Tharun, L., Zhou, Z., Heukamp, L., Termathe, M., ... & Desmet, C. J. (2016). Elp3 links tRNA modification to IRES-dependent translation of LEF1 to sustain metastasis in breast cancer. Journal of Experimental Medicine, 213(11), 2503-2523.

Derynck, Rik, Rosemary J. Akhurst, and Allan Balmain. "TGF-β signaling in tumor suppression and cancer progression." Nature genetics 29.2 (2001): 117-129.

Djebali, S., Davis, C. A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., ... & Xue, C. (2012). Landscape of transcription in human cells. Nature, 489(7414), 101-108.

Duechler, M., Leszczyńska, G., Sochacka, E., & Nawrot, B. (2016). Nucleoside modifications in the regulation of gene expression: focus on tRNA. Cellular and Molecular Life Sciences, 73(16), 3075-3095.

Esteve-Puig, R., Bueno-Costa, A., & Esteller, M. (2020). Writers, readers and erasers of RNA modifications in cancer. Cancer Letters, 474, 127-137.

Feinberg, A. P., & Tycko, B. (2004). The history of cancer epigenetics. Nature Reviews Cancer, 4(2), 143-153.

Feng, Y., Feng, L., Yu, D., Zou, J., & Huang, Z. (2016). srGAP1 mediates the migration inhibition effect of Slit2-Robo1 in colorectal cancer. Journal of Experimental & Clinical Cancer Research, 35(1), 191.

Ferguson-Smith AC (2011). "Genomic imprinting: the emergence of an epigenetic paradigm". Nature Reviews Genetics. 12 (8): 565–75. doi:10.1038/nrg3032. PMID 21765458.

Florentz, C., Sohm, B., Tryoen-Toth, P., Pütz, J., & Sissler, M. (2003). Human mitochondrial tRNAs in health and disease. Cellular and Molecular Life Sciences CMLS, 60(7), 1356-1375.

Fujiwara, M., Ghazizadeh, M., & Kawanami, O. (2006). Potential role of the Slit/Robo signal pathway in angiogenesis. Vascular Medicine, 11(2), 69-74.

Gingold, H., Tehler, D., Christoffersen, N. R., Nielsen, M. M., Asmar, F., Kooistra, S. M., ... & Andersen, L. D. (2014). A dual program for translation regulation in cellular proliferation and differentiation. Cell, 158(6), 1281-1292.

Goelz, S. E., Vogelstein, B., & Feinberg, A. P. (1985). Hypomethylation of DNA from benign and malignant human colon neoplasms. Science, 228(4696), 187-190.

Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, inflammation, and cancer. Cell, 140(6), 883-899.

Gröne, J., Doebler, O., Loddenkemper, C., Hotz, B., Buhr, H. J., & Bhargava, S. (2006). Robo1/Robo4: differential expression of angiogenic markers in colorectal cancer. Oncology reports, 15(6), 1437-1443.

Grunberger, D., Weinstein, I. B., & MUSHINSKI, J. F. (1975). Deficiency of the Y base in a hepatoma phenylalanine tRNA. Nature, 253(5486), 66-67.

Gu, J. J., Gao, G. Z., & Zhang, S. M. (2015). miR-218 inhibits the migration and invasion of glioma U87 cells through the Slit2-Robo1 pathway. Oncology letters, 9(4), 1561-1566.

Hanahan, D., & Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. cell, 86(3), 353-364.

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

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. cell, 144(5), 646-674.

He, H., Hao, S. J., Yao, L., Yang, F., Di, Y., Li, J., ... & Fu, D. L. (2014). MicroRNA-218 inhibits cell invasion and migration of pancreatic cancer via regulating ROBO1. Cancer biology & therapy, 15(10), 1333-1339.

Hoernes, T. P., & Erlacher, M. D. (2017). Translating the epitranscriptome. Wiley Interdisciplinary Reviews: RNA, 8(1), e1375.

Huang, S. Q., Sun, B., Xiong, Z. P., Shu, Y., Zhou, H. H., Zhang, W., ... & Li, Q. (2018). The dysregulation of tRNAs and tRNA derivatives in cancer. Journal of Experimental & Clinical Cancer Research, 37(1), 101.

Issa, J. P. J. (2007). DNA methylation as a therapeutic target in cancer. Clinical Cancer Research, 13(6), 1634-1637.

Ito, H., Funahashi, S. I., Yamauchi, N., Shibahara, J., Midorikawa, Y., Kawai, S., & Iwanari, H. (2006). Identification of ROBO1 as a novel hepatocellular carcinoma antigen and a potential therapeutic and diagnostic target. Clinical Cancer Research, 12(11), 3257-3264.

Iyer, L. M., Abhiman, S., de Souza, R. F., & Aravind, L. (2010). Origin and evolution of peptide-modifying dioxygenases and identification of the wybutosine hydroxylase/hydroperoxidase. Nucleic acids research, 38(16), 5261-5279.

Kato, M., Araiso, Y., Noma, A., Nagao, A., Suzuki, T., Ishitani, R., & Nureki, O. (2011). Crystal structure of a novel JmjC-domain-containing protein, TYW5, involved in tRNA modification. Nucleic acids research, 39(4), 1576-1585.

Konevega, A. L., Soboleva, N. G., Makhno, V. I., Semenkov, Y. P., Wintermeyer, W., Rodnina, M. V., & Katunin, V. I. (2004). Purine bases at position 37 of tRNA stabilize codon–anticodon interaction in the ribosomal A site by stacking and Mg2+-dependent interactions. Rna, 10(1), 90-101.

Kong, R., Yi, F., Wen, P., Liu, J., Chen, X., Ren, J., ... & Fan, D. (2015). Myo9b is a key player in SLIT/ROBO-mediated lung tumor suppression. The Journal of clinical investigation, 125(12), 4407-4420.

Kouam, P. N., Rezniczek, G. A., Kochanneck, A., Priesch-Grzeszkowiak, B., Hero, T., Adamietz, I. A., & Bühler, H. (2018). Robo1 and vimentin regulate radiation-induced motility of human glioblastoma cells. PloS one, 13(6).

Kuchino, Y., Borek, E., Grunberger, D., Mushinski, J. F., & Nishimura, S. (1982). Changes of post-transcriptional modification of wye base in tumor-specific tRNAPhe. Nucleic acids research, 10(20), 6421-6432.

Lamouille, S., Xu, J., & Derynck, R. (2014). Molecular mechanisms of epithelial–mesenchymal transition. Nature reviews Molecular cell biology, 15(3), 178.

Laurent, L., Wong, E., Li, G., Huynh, T., Tsirigos, A., Ong, C. T., ... & Wei, C. L. (2010). Dynamic changes in the human methylome during differentiation. Genome research, 20(3), 320-331.

Li, E., Beard, C., & Jaenisch, R. (1993). Role for DNA methylation in genomic imprinting. Nature, 366(6453), 362-365.

Li, X., Xiong, X., & Yi, C. (2017). Epitranscriptome sequencing technologies: decoding RNA modifications. Nature methods, 14(1), 23.

Llinas-Arias, P., & Esteller, M. (2017). Epigenetic inactivation of tumour suppressor coding and non-coding genes in human cancer: an update. Open biology, 7(9), 170152.

Lowe, S. W., & Lin, A. W. (2000). Apoptosis in cancer. Carcinogenesis, 21(3), 485-495.

M. Duechler, G. Leszczynska, E Sochaka…. Nucleoside modifications in th eregulation of gene expresión: focus on tRNA.

Macias, H., Moran, A., Samara, Y., Moreno, M., Compton, J. E., Harburg, G., & Hinck, L. (2011). SLIT/ROBO1 signaling suppresses mammary branching morphogenesis by limiting basal cell number. Developmental cell, 20(6), 827-840.

Mattick, J. S., & Makunin, I. V. (2006). Non-coding RNA. Human molecular genetics, 15(suppl_1), R17-R29.

Maute, R. L., Schneider, C., Sumazin, P., Holmes, A., Califano, A., Basso, K., & Dalla-Favera, R. (2013). tRNA-derived microRNA modulates proliferation and the DNA damage response and is down-regulated in B cell lymphoma. Proceedings of the National Academy of Sciences, 110(4), 1404-1409.

Mei, Y., Yong, J., Liu, H., Shi, Y., Meinkoth, J., Dreyfuss, G., & Yang, X. (2010). tRNA binds to cytochrome c and inhibits caspase activation. Molecular cell, 37(5), 668-678.

Mertsch, S., Schmitz, N., Jeibmann, A., Geng, J. G., Paulus, W., & Senner, V. (2008). Slit2 involvement in glioma cell migration is mediated by Robo1 receptor. Journal of neuro-oncology, 87(1), 1-7.

Mushinski, J. F., & Marini, M. (1979). Tumor-associated phenylalanyl transfer RNA found in a wide spectrum of rat and mouse tumors but absent in normal adult, fetal, and regenerating tissues. Cancer research, 39(4), 1253-1258.

Narayan, G., Goparaju, C., Arias-Pulido, H., Kaufmann, A. M., Schneider, A., Dürst, M., & Murty, V. V. (2006). Promoter hypermethylation-mediated inactivation of multiple Slit-Robo pathway genes in cervical cancer progression. Molecular cancer, 5(1), 16.

Negrini, S., Gorgoulis, V. G., & Halazonetis, T. D. (2010). Genomic instability—an evolving hallmark of cancer. Nature reviews Molecular cell biology, 11(3), 220-228.

Noma, A., Ishitani, R., Kato, M., Nagao, A., Nureki, O., & Suzuki, T. (2010). Expanding role of the jumonji C domain as an RNA hydroxylase. Journal of Biological Chemistry, 285(45), 34503-34507.

Noma, A., Kirino, Y., Ikeuchi, Y., & Suzuki, T. (2006). Biosynthesis of wybutosine, a hyper-modified nucleoside in eukaryotic phenylalanine tRNA. The EMBO journal, 25(10), 2142-2154.

Pavlova, N. N., & Thompson, C. B. (2016). The emerging hallmarks of cancer metabolism. Cell metabolism, 23(1), 27-47.

Perona, R. (2006). Cell signalling: growth factors and tyrosine kinase receptors. Clinical and Translational Oncology, 8(2), 77-82.

Peters J (August 2014). "The role of genomic imprinting in biology and disease: an expanding view". Nature Reviews Genetics. 15 (8): 517–30. doi:10.1038/nrg3766. PMID 24958438.

Portela, A., & Esteller, M. (2010). Epigenetic modifications and human disease. Nature biotechnology, 28(10), 1057.

Qin, F., Zhang, H., Ma, L., Liu, X., Dai, K., Li, W., & Ma, Y. (2015). Low expression of Slit2 and Robo1 is associated with poor prognosis and brain-specific metastasis of breast cancer patients. Scientific reports, 5(1), 1-11.

Rezniczek, G. A., Grunwald, C., Hilal, Z., Scheich, J., Reifenberger, G., Tannapfel, A., & Tempfer, C. B. (2019). ROBO1 Expression in Metastasizing Breast and Ovarian Cancer: SLIT2-induced Chemotaxis Requires Heparan Sulfates (Heparin). Anticancer research, 39(3), 1267-1273.

Robertson, K. D. (2005). DNA methylation and human disease. Nature Reviews Genetics, 6(8), 597-610.

Roundtree, I. A., Evans, M. E., Pan, T., & He, C. (2017). Dynamic RNA modifications in gene expression regulation. Cell, 169(7), 1187-1200.

Santos, M., Fidalgo, A., Varanda, A. S., Oliveira, C., & Santos, M. A. (2019). tRNA deregulation and its consequences in cancer. Trends in molecular medicine.

Schmalhofer, O., Brabletz, S., & Brabletz, T. (2009). E-cadherin, β-catenin, and ZEB1 in malignant progression of cancer. Cancer and Metastasis Reviews, 28(1-2), 151-166.

Sherr, C. J., & McCormick, F. (2002). The RB and p53 pathways in cancer. Cancer cell, 2(2), 103-112.

Teng, M. W., Swann, J. B., Koebel, C. M., Schreiber, R. D., & Smyth, M. J. (2008). Immune-mediated dormancy: an equilibrium with cancer. Journal of leukocyte biology, 84(4), 988-993.

Tie, J., Pan, Y., Zhao, L., Wu, K., Liu, J., Sun, S., ... & Li, Q. (2010). MiR-218 inhibits invasion and metastasis of gastric cancer by targeting the Robo1 receptor. PLoS Genet, 6(3), e1000879.

Torres, A. G., Batlle, E., & de Pouplana, L. R. (2014). Role of tRNA modifications in human diseases. Trends in molecular medicine, 20(6), 306-314.

Weinhold, B. (2006). Epigenetics: the science of change.

What is cancer?. (2015). Retrieved from: https://www.cancer.gov/about-cancer/understanding/what-is-cancer. Retrieval date: June, 2020.

Wu H, Zhang Y. 2014. Reversing DNA methylation: mechanisms, genomics, and biological functions.Cell 156:45–68

Xia, Y., Wang, L., Xu, Z., Kong, R., Wang, F., Yin, K., ... & Xu, H. (2019). Reduced USP33 expression in gastric cancer decreases inhibitory effects of Slit2-Robo1 signalling on cell migration and EMT. Cell proliferation, 52(3), e12606.

Xian, J., Aitchison, A., Bobrow, L., Corbett, G., Pannell, R., Rabbitts, T., & Rabbitts, P. (2004). Targeted disruption of the 3p12 gene, Dutt1/Robo1, predisposes mice to lung adenocarcinomas and lymphomas with methylation of the gene promoter. Cancer research, 64(18), 6432-6437.

Xian, J., Clark, K. J., Fordham, R., Pannell, R., Rabbitts, T. H., & Rabbitts, P. H. (2001). Inadequate lung development and bronchial hyperplasia in mice with a targeted deletion in the Dutt1/Robo1 gene. Proceedings of the National Academy of Sciences, 98(26), 15062-15066.

Yang, L., Li, Q., Wang, Q., Jiang, Z., & Zhang, L. (2012). Silencing of miRNA-218 promotes migration and invasion of breast cancer via Slit2-Robo1 pathway. Biomedicine & Pharmacotherapy, 66(7), 535-540.

Zhang, Q. Q., Zhou, D. L., Lei, Y., Zheng, L., Chen, S. X., Gou, H. J., ... & Li, J. C. (2015). Slit2/Robo1 signaling promotes intestinal tumorigenesis through Src-mediated activation of the Wnt/β-catenin pathway. Oncotarget, 6(5), 3123.

Zhao, S. J., Shen, Y. F., Li, Q., He, Y. J., Zhang, Y. K., Hu, L. P., ... & Wang, Y. H. (2018). SLIT2/ROBO1 axis contributes to the Warburg effect in osteosarcoma through activation of SRC/ERK/c-MYC/PFKFB2 pathway. Cell death & disease, 9(3), 1-13.