Universidade de Aveiro Departamento de Biologia
Ano 2013/2014

Ana Cláudia Faria Curinha de Sousa

Regulation of *MCL1* alternative polyadenylation-derived mRNA isoforms by microRNAs in human T cells

Regulação por microRNAs das isoformas de mRNA do *MCL1* produzidas por poliadenilação alternativa em linfócitos T humanos

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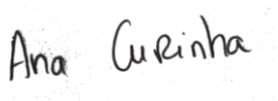

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Isabel Pereira de Castro, Investigadora Pós-Doc no Instituto de Biologia Molecular e Celular da Universidade do Porto, da Doutora Maria Alexandra Marques Moreira Mourão do Carmo, Investigadora Principal no Instituto de Biologia Molecular e Celular da Universidade do Porto, e do Doutor Manuel António da Silva Santos, Professor Associado do Departamento de Biologia da Universidade de Aveiro

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“Somewhere, something incredible is waiting to be known.”
Carl Sagan

o júri
presidente

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I. Pereira-Castro, **A. Curinh**a, A. Neves-Costa, L. F. Moita, J. R. Neilson, A. Moreira. *A lentivirus-based shRNA screen identifies integrator complex subunit 9 (INTS9) has a regulator of MCL1 alternative polyadenylation in human T cells.* I^{3}S 4th Annual Meeting, Póvoa do Varzim, Portugal, Outubro 2014

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palavras-chave

MCL1, poliadenilação alternativa, região 3’ não traduzida, regulação pós-transcricional, microRNAs

resumo

A poliadenilação é um passo de processamento fundamental da maturação do mRNA, essencial para o seu transporte, estabilidade e tradução. Análises bioinformáticas têm demonstrado que cerca de 70% dos genes humanos têm vários sinais de poliadenilação na região 3’ não traduzida (3’UTR). Estes sinais são usados para produzir várias isoformas de mRNA por poliadenilação alternativa (APA), um processo com um papel fundamental na expressão génica em programas celulares bem como em condições patológicas e não patológicas. Quando ocorre na região 3’ não traduzida, a APA dá origem a transcritos com diferentes tamanhos da 3’UTR. Os microRNAs (miRNAs) e as proteínas que se ligam ao RNA (RBPs) ligam-se frequentemente a sequências presentes nesta região e portanto isoformas de mRNA com 3’UTRs mais longas contêm mais locais onde estes reguladores se podem ligar e, por isso, estão mais sujeitos a regulação. Os miRNAs são reguladores pós-transcricionais da expressão génica com cerca de 23 nucleótidos, que têm sido envolvidos numa variedade de condições celulares. No sistema imune, tem sido demonstrado que após activação dos linfócitos T passa a haver uma maior seleção dos sinais de poliadenilação proximais em vez dos distais, originando mRNAs com 3’UTRs mais curtas e consequentemente com menos locais onde se possam ligar miRNAs e RBPs. O gene *MCL1* (Myeloid Cell Leukemia Sequence 1) codifica uma proteína (Mcl-1) com função anti-apoptótica essencial para o desenvolvimento e manutenção dos linfócitos T em animais, que faz parte da família proteica da Bcl-2, uma família de reguladores da apoptose. Tem sido demonstrado que o *MCL1* é altamente regulado tanto ao nível transcricional como pós-transcricional. Os objetivos do nosso estudo são determinar o padrão de APA que ocorre no *MCL1* em linfócitos T humanos e caracterizar o papel dos miRNAs na regulação das isoformas de mRNA do *MCL1* produzidas por APA. Verificamos que o *MCL1* produz quatro isoformas de mRNA pelo uso de quatro sinais de poliadenilação canónicos localizados na 3’UTR e que esses sinais são altamente conservados nos mamíferos. Observamos que a isoforma de mRNA mais longa é regulada ao nível pós-transcricional nos PBMCs ativados. Nos nossos resultados demonstramos também que as 3’UTR mais curtas dão origem a uma maior actividade de luciferase do que o mRNA mais longo. Isto sugere que durante a ativação dos PBMCs o aumento na proteína Mcl-1 é devida à tradução das isoformas mais curtas. Na segunda parte do estudo identificamos o miRNA-17 como um possível regulador da isoforma longa após ativação dos linfócitos T. A expressão deste miRNA está aumentada após ativação dos linfócitos T e quando é mutado o seu local de ligação ao mRNA putativo na 3’UTR do *MCL1* observou-se um aumento na atividade da Luciferase na linha celular HeLa. Também realizamos a sobreexpressão do miRNA-17, do miRNA-29b e do miRNA-92a o que levou a uma diminuição na expressão endógena do Mcl-1. A partir deste estudo concluímos que a isoforma do *MCL1* gerada por APA mais longa é regulada negativamente ao nível pós-transcricional após ativação celular de modo a aumentar a expressão do Mcl-1 e que o miRNA-17 poderá ser o regulador chave deste mecanismo.

keywords

MCL1, alternative polyadenylation, 3' untranslated region, post-transcriptional regulation, microRNAs

abstract

Polyadenylation is a fundamental processing step of mRNA maturation, essential for its export, stability and translation. Bioinformatic analyses have shown that 70% of human genes have several polyadenylation signals (pA signals) in the 3'untranslated region (3'UTR) that are used to produce multiple mRNA isoforms by alternative polyadenylation (APA). This process has a fundamental role in gene expression in a variety of cellular programs as well as in non-pathological conditions and diseases. When it occurs in the 3'UTR, APA gives rise to transcripts with different 3'UTR lengths. MicroRNAs (miRNAs) and RNA-binding proteins (RBPs) often bind to sequences present in that region and thus mRNA isoforms with longer 3'UTRs have more sites where these regulators can bind, being more prone to regulation. miRNAs are 23 nucleotides in length post-transcriptional regulators of gene expression that have been implicated in a variety of cell conditions. In the immune system, it has been shown that upon T cell activation there is a global switch in pA signal selection from a distal to a proximal pA signal, originating mRNAs with shorter 3'UTRs and, consequently, with less miRNAs and RBPs target sites. The *MCL1* (Myeloid Cell Leukemia Sequence 1) gene encodes an anti-apoptotic protein (Mcl-1), which is a member of the Bcl-2 (B cell lymphoma-2) family of apoptosis regulators. *MCL1* is essential for development and maintenance of both B and T lymphocytes in animals. It has been demonstrated that *MCL1* is highly regulated at both transcriptional and post-transcriptional levels. The aims of our study were to characterize the pattern of *MCL1* APA and to unravel the role of miRNAs in the regulation of *MCL1* APA-derived isoforms. We discovered that *MCL1* produces four mRNA isoforms by the usage of four canonical pA signals located in the 3'UTR and that these pA signals are highly conserved in mammals. We verified that the longest mRNA isoform is regulated at a post-transcriptional level in activated PBMCs. We also demonstrated that the shortest 3'UTRs give rise to higher amounts of luciferase activity than the longest mRNA. Additionally, we showed that Mcl-1 protein levels increase upon PBMCs activation. This suggests that during PBMCs activation the increase in Mcl-1 protein is due to the translation of the shortest isoforms. In the second part of this study we identified miRNA-17 as a putative regulator of the longest isoform upon T cell activation. The expression of this miRNA increased upon activation of T cells and when we mutated its putative-binding site on *MCL1* 3'UTR we observed an increase in luciferase activity in HeLa cells. We also overexpressed miRNA-17, miRNA-29b and miRNA-92a and showed that this causes a decrease in endogenous Mcl-1 expression. From this study we conclude that the *MCL1* longest APA-derived isoform is down-regulated at a post-transcriptional level upon T cell activation in order to increase Mcl-1 expression and that miRNA-17 may be the key regulator in this mechanism.

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Abbreviations

AGO	Argonaute
ALL	Acute lymphoblastic leukemia
APA	Alternative polyadenylation
Bcl-2	B-cell lymphoma 2
CFIIm	Cleavage factor II
CFIm	Cleavage factor I
CLL	Chronic lmphocytic leukemia
CPSF	Cleavage and polyadenylation specificity factor
CstF	Cleavage stimulating factor
DN	Double negative
DP	Double positive
DSE	Downstream sequence element
EGF	Epithelial growth factor
EST	Expressed sequence tags
FBS	Fetal bovine serum
HIF-1α	Hypoxia-inducible factor 1-alpha
IL	Interleukin
MCL1	Myeloid cell leukemia 1
miRISC	microRNA-mediated silencing complex
miRNA	microRNA
mRNA	messenger RNA
mTORC1	mammalian target of rapamycin complex 1
nt	nucleotide
pA	polyA
pA signal	Polyadenylation signal
pA site	Polyadenylation site
PAB	PolyA binding protein
PABPC1	Poly(A) binding protein, cytoplasmic 1
PAP	polyA polymerase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PHA	Phytohemagglutinin
Pre-mRNA	Precursor mRNA
RBP	RNA-binding protein
SP	Single positive

STAT	Signal transducer and activator of transcription
TLI	Tandem UTR length index
TRBP	Transactivating response RNA-binding protein
USE	Upstream sequence element
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
XRN1	Exoribonuclease 1

INTRODUCTION

Gene Expression

Gene expression is a biological process highly regulated at multiple steps. These steps include modulation of the chromatin structure by the interaction of regulatory proteins with specific DNA motifs of the target genes, and also messenger RNA (mRNA) synthesis and processing in the nucleus, mRNA transport, and ultimately translation and decay of the mRNA in the cytoplasm.[1, 2] These steps, in which information flows from DNA to RNA (transcription and precursor mRNA (pre-mRNA) processing) and finally to protein (translation and mRNA decay), are remarkably elaborate and highly interconnected in eukaryotic cells (**figure 1**).[3, 4]

Pre-mRNA processing is a complex mechanism crucial to mRNA metabolism. The RNA molecule resulting from transcription (pre-mRNA) contains both coding and non-coding sequences. Before it can be translated into protein, introns have to be removed (splicing) and both ends of the pre-mRNA are modified by capping on the 5'-end and polyadenylation on the 3'-end. These processes are known as post-transcriptional events and occur co-transcriptionally being also highly regulated and interconnected. Thus, it is now well-stablished that pre-mRNA processing regulates downstream events affecting the fate of the transcript and thus modulating gene expression.[5-8]

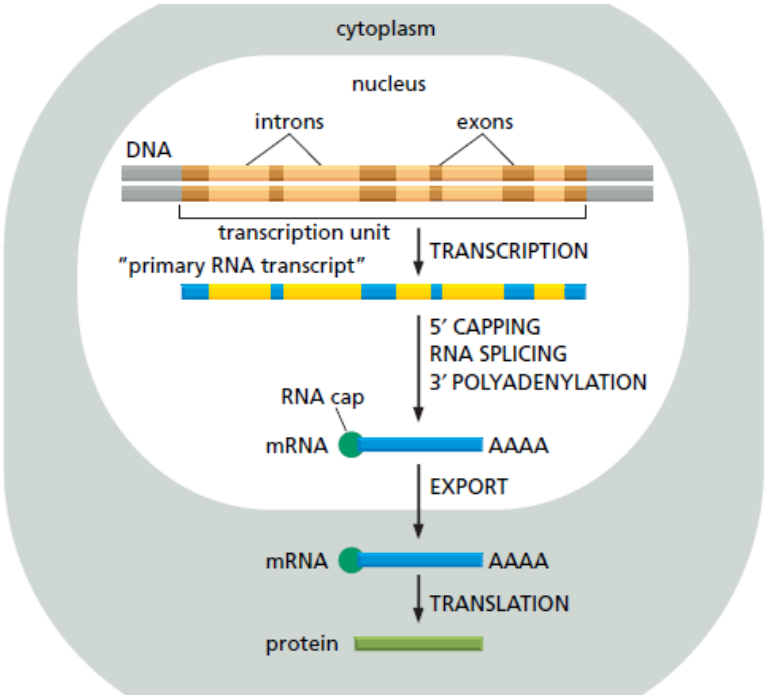


Figure 1 - Schematic illustration of the steps from DNA transcription to protein translation in eukaryotes. The mechanisms by which a protein is produced starts with transcription of DNA by RNA polymerase II to produce a pre-mRNA. Before it can be translated into a protein, the pre-mRNA has to be processed, forming the mature RNA that is transported to the cytoplasm where translation takes place. The mechanisms of RNA processing include 5' capping, splicing and 3' polyadenylation. Adapted from Alberts *et al*.[6]

Polyadenylation

Polyadenylation is a pre-mRNA 3'end processing step that consists in the addition of a polyA (pA) tail[9] by polyA polymerase (PAP) upon an endonucleolytic cleavage of the transcript.[10-12] The length of the pA tail (about 250-300 adenines in humans) is crucial for the transport of mature mRNAs to the cytoplasm, their translation efficiency and for the quality control and degradation of mRNAs.[1, 9, 11] As reviewed in Curinha *et al.*[1], recent studies have now challenged what is known about the length of the pA tail. The pA tail is a dynamic region of the mRNA[13] that is controlled differently depending of the developmental stage. Also, it has been shown that an increase in PAP activity is associated with poor prognosis in certain cancers[14] and also that usage of PAP inhibitors affect some genes involved in inflammatory conditions[15]. Thus, a tight control of the pA tail length may be a determinant factor in the development of some diseases.

Several *cis*- and *trans*-acting RNA elements (sequences in the 3' untranslated region (3'UTR) of the pre-mRNA and molecules that bind to these sequences, respectively) are core components of the polyadenylation process. The most important *cis*-acting element is a hexanucleotide sequence, which is known as the polyadenylation signal (pA signal) located 15-30 nucleotides (nt) upstream of the cleavage site (preferably a CA dinucleotide). The AAUAAA sequence is the canonical pA signal and the strongest in defining the place where polyadenylation takes place, but this signal can also adopt more than ten weaker variants. In order to enhance the cleavage efficiency, two more U or GU-rich sequences located upstream and downstream of the pA signal, the upstream sequence element (USE) and downstream sequence element (DSE), are needed.[9-12] The most important *trans*-acting elements in the pA signal recognition are the cleavage and polyadenylation specificity factor (CPSF), which binds to the pA signal, and the cleavage stimulating factor (CstF), that binds to the DSE sequence. Additionally, the cleavage factors I and II (CFIm and CFIIm), that bind to the USE sequence and are essential for the cleavage step, the PAP, responsible for the pA tail addition, and the pA-binding protein (PAB), are also core elements of the polyadenylation machinery (**figure 2**).[9, 12]

Alternative Polyadenylation

Some protein-coding genes can harbor two or more pA signals in their sequences and thus polyadenylation can occur in different points of the gene, a process known as alternative polyadenylation (APA).[9-12] Different studies have demonstrated a role for APA in regulating 70% of the mammalian genes, increasing immensely the transcriptome diversity.[16, 17] The recognition of one pA signal over

another is due to the strength of the *cis* and *trans*-acting elements that surround each pA signal, the strength of the pA signal itself, and the regulation at the DNA level, such as chromatin remodeling and epigenetic marks (**figure 3**).[9, 10, 12]

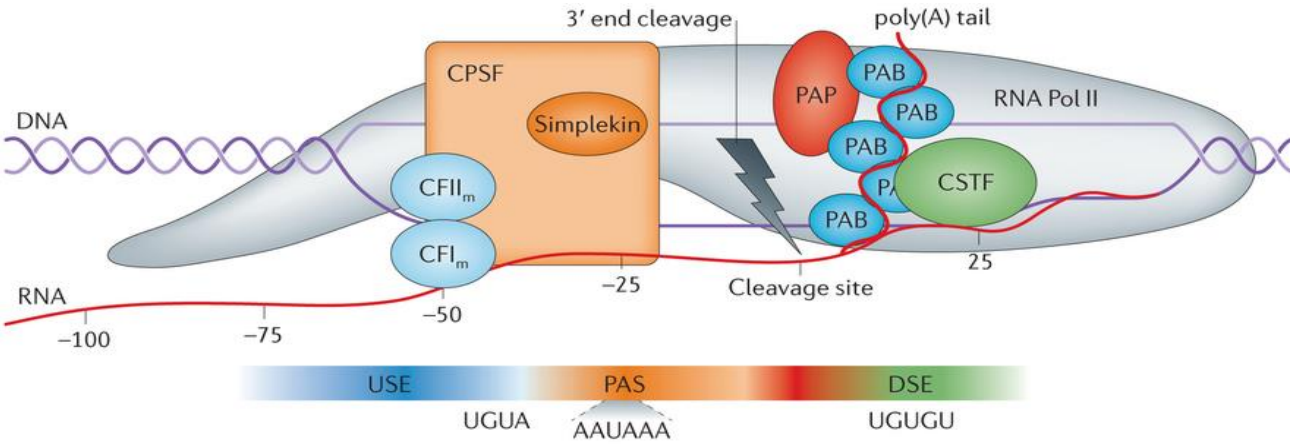


Figure 2 - Schematic illustration of the polyadenylation core elements. The *cis*- and *trans*-acting elements involved in the polyadenylation process are represented. The color of the t*rans*-acting factors and the *cis*-acting elements to which they bind are the same. Adapted from Elkon *et al*.[9]

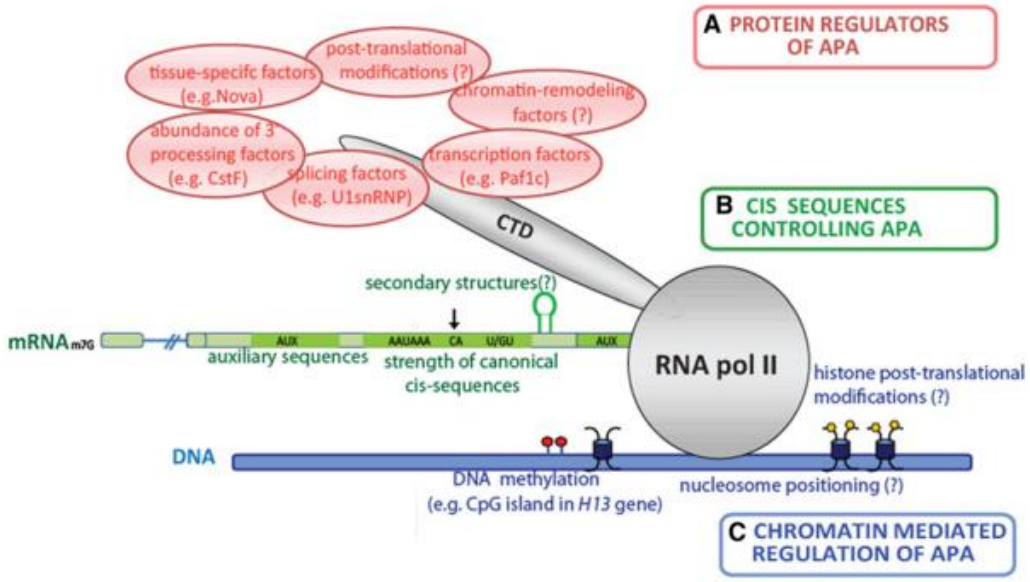


Figure 3 – Regulatory mechanisms of alternative polyadenylation. The production of different APA-derived mRNA isoforms from the same transcriptional unit is tightly regulated by *trans*-acting factors **(A),** such as polyadenylation and splicing factors; *cis*-acting sequences **(B)**, such as RBPs and miRNAs-binding sites; and by chromatin remodeling **(C)**, such as the nucleosome positioning in the vicinity of the polyadenylation sites (pA sites). Adapted from Giammartino *et al*.[12]

The pA signals can be present in the coding or intronic region, giving rise to transcripts with different coding sequences and thus affecting the function of the gene, or be present in the 3'UTR, giving rise to transcripts with the same coding region but different 3'UTR lengths.[9-12] In this last case, since 3'UTR harbor putative-binding sites for several microRNAs (miRNAs) and RNA-binding proteins (RBPs), longer 3'UTRs are more prone to suffer regulation affecting their stability, cellular localization and/or translation efficiency (**figure 4**). Thus, the length of the 3' UTR affect the fate of the transcript produced and ultimately modulates the expression of the gene.[10-12]

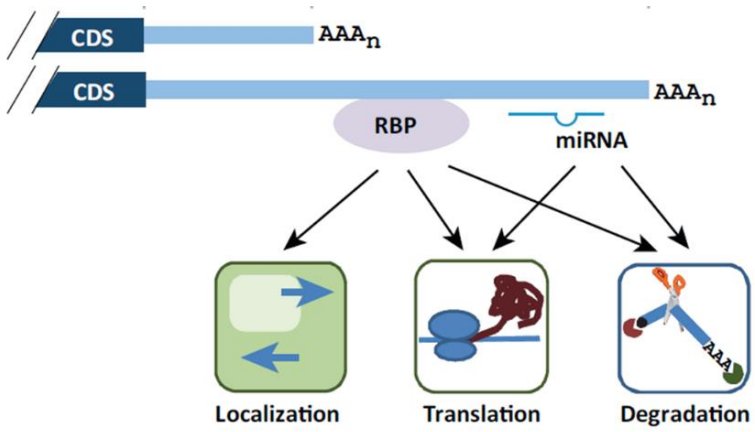


Figure 4 - Regulation of alternative polyadenylated mRNAs by cis and trans-acting factors. The presence of RBPs and miRNAs-binding sites in the 3'UTR of different mRNA isoforms promotes the binding of these regulators to their specific *cis*-elements and thus modulate the fate of a specific transcript. mRNA isoforms with longer 3'UTRs are more prone to undergo this type of regulation since they have more of these *cis*-elements present in their sequence. Adapted from Tian *et al*.[10]

Several studies have been made in the APA field and it is now known that the APA pattern tends to be gene-, tissue-, or disease-specific. The usage of one pA site over another depends on how the regulatory features mentioned above behave in the type of cell where the process is occurring, in the health or disease condition that the organism is suffering, and ultimately, in the gene being regulated (**figure 5**). Some examples of the implications of APA in health and disease will be further described ahead.

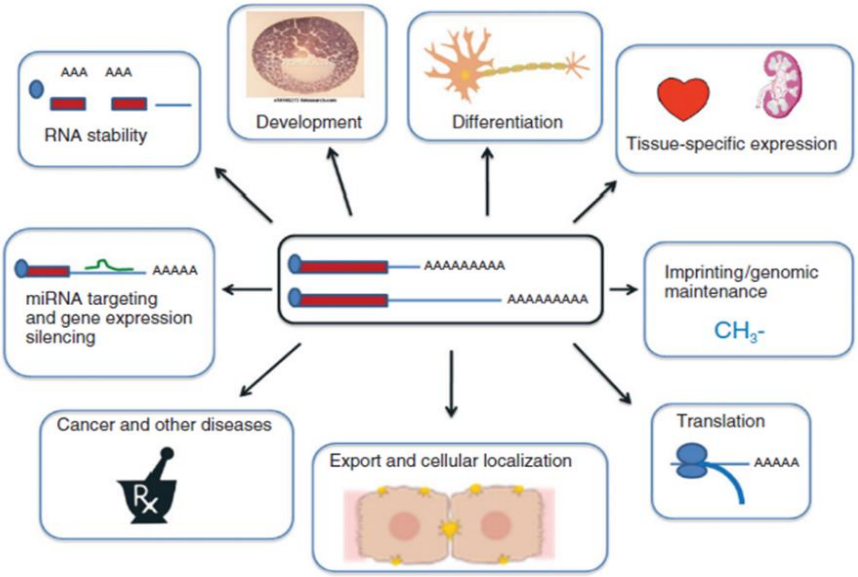


Figure 5 - APA in a variety of cellular and organismal events. It is well-described that the usage of one pA site over another is actively regulated in a cellular, health and/or disease-specific manner. Adapted from Lutz *et al*.[11]

Polyadenylation and Alternative Polyadenylation in Health and Disease

Besides exciting results in the past few years have highlighted the crucial roles of APA in the control of gene expression, major efforts are needed to characterize the APA regulatory mechanisms in a variety of genes and its importance in health and disease. Several studies have reported a role for polyadenylation and APA in oncological, immunological, neurological and haematological diseases, as well as in cellular and molecular conditions important for cell homeostasis. It has been described a preferential usage of proximal pA signals resulting in 3'UTR shortening in proliferative cells, such as under activation of T lymphocytes, and in tumour cells. In contrast, in development, differentiation processes and neurological tissues, there is a preferential usage of distal pA signals which gives rise to a lengthening of the 3'UTR (**figure 6**).[1] Some examples reviewed in Curinha *et al*.[1] are described below.

An important study in proliferation field is the one made by Sandberg *et al.*[18] in T lymphocytes. In this study they developed a tandem UTR length index (TLI) that assessed the expression of extended 3'UTR relative to total gene expression levels. They have found that the TLI is decreased after 48h upon activation of T lymphocytes, representing a decrease in the relative expression of isoforms with longer 3'UTRs. Additionally, it was established in the same study a negative correlation between proliferative index and TLI.[18] As B and T lymphocyte activation is a key immune

response to several stimuli, this may represent an efficient mechanism to escape regulation and respond quicker when lymphocyte activation is necessary.

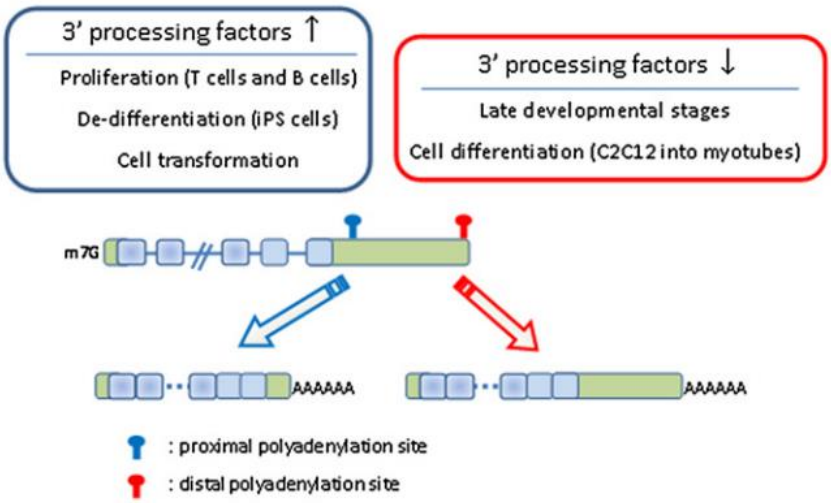


Figure 6 - APA in proliferative and developmental conditions. According to a variety of cellular states, as well as diverse diseases, the choice of one pA site over another is differentially regulated. The choice of proximal pA signals occurs preferentially in proliferative conditions and cell transformation while the choice of distal pA sites occurs mostly in cell differentiation and late developmental stages. Adapted from Di Giammartino *et al*.[12]

In what concerns the oncological field, Mayr *et al*.[19] demonstrated that besides nontransformed cells and cancer cells have similar proliferation rates, cancer cells tend to produce more levels of transcripts with shorter 3'UTRs, and thus the correlation between shorter 3'UTRs and cell transformation is higher than shorter 3'UTRs and proliferation. Also in this study it was demonstrated that isoforms with shorter 3'UTRs produce more protein due to its higher stability, which may be a relevant activation mechanism employed by some oncogenes in cancer cells. This was demonstrated using the proto-oncogene insulin-like growth factor 2 mRNA binding protein 1. The shortest mRNA isoform was shown to promote higher oncogenic transformation levels than its longest mRNA isoform. This could be explained by the fact that shorter 3'UTRs have less binding sites to negative regulators such as miRNAs and thus are less prone to regulation.[19] Also in this field, Fu *et al.*[20], described an opposite pattern of 3'UTR length in two different breast cancer cell lines. In MCF7 cell line it was seen an elevated production of mRNA isoforms with shorter 3'UTRs whereas in MB231 cell line it was seen high production of longer 3'UTR mRNA isoforms.[20] What it can be concluded from this observation is that in cancer, APA is regulated at a cell type-specific manner.

In contrast to cell proliferation and tumour cells, it has been shown that during development and cell differentiation there is an increase in the usage of distal PAS. Hoque *et al.*[16], analyzed C2C12 and 3T3-L1 cells, which were induced to differentiate and represent a model for myogenesis and adipogenesis respectively, and also mouse embryos as a model for embryogenesis. In this study it was demonstrated an increase in the levels of mRNAs produced by the usage of distal PAS both in mouse embryos and during cell differentiation.[16]

In what concerns neurological system, it has been shown that an *aSyn* mRNA isoform with a longer 3' UTR is more highly expressed in brain tissues of Parkinson disease patients compared with unaffected brains.[21] Another study in this field demonstrated that *COX-2,* which is expressed in the brain, produces two different mRNAs with 2.8 kb and 4.6 kb by APA. It has been demonstrated that the neocortex, which is affected in Alzheimer disease, expresses high levels of the 4.6 kb *COX-2* mRNA, and therefore a possible association between COX-2 APA pattern, its expression levels and Alzheimer disease phenotype may be foreseen.[22]

Myeloid Cell Leukemia-1 gene

Myeloid cell leukemia-1 (*MCL1*) gene was first described to be up-regulated in ML-1 cells, a human myeloid leukemia cell line, in an early differentiation stage. It encodes Mcl-1, a member of the B-cell lymphoma 2 (Bcl-2) protein family. The Bcl-2 protein family is known to be involved in the regulation of apoptosis, a programmed form of cell death crucial for tissue homeostasis, development, inflammation and safe removal of unwanted or damaged cells. An aberration in this physiological event could lead to human disease, such as cancer, once it promotes cell immortalization. The regulation of apoptosis by Bcl-2 protein family members is achieved throughout the balance between the pro- (e.g., Bax and Bad) and anti-apoptotic proteins (e.g., Bcl-2, Bcl-X_L and Mcl-1) of this family, which determines the flow of cells through proliferation and differentiation.[23-26]

Mcl-1 is an anti-apoptotic protein known to provide short-term enhancement of cell viability influencing cell fate transitions and also life and dead-decisions. Mcl-1 exerts its anti-apoptotic function by sequestering Bax and Bak proteins, pro-apoptotic members of the Bcl-2 protein family. Mcl-1 is a ubiquitous protein, however, its expression varies in a tissue- and differentiation-specific manner according to its physiological role.[23-25, 27] A variety of cell types depend on Mcl-1 expression for their survival and development, such as B and T-lymphocytes and neutrophils. Also, it is described that Mcl-1 is very important for macrophage effector function.[23, 25, 28]

Contribution of Mcl-1 to T cells biology and pathophysiology

T lymphocytes are core elements of immunity that confer specificity to the immune response. These cells are produced in bone marrow and mature in the thymus where differentiate from double negative (four stages: DN1, DN2, DN3, DN4), $CD4^-CD8^-$, to double positive (DP), $CD4^+CD8^+$, and then to single positive (SP), $CD4^+CD8^-$ or $CD8^+CD4^-$ cells. From the thymus, SP thymocytes transit to peripheral blood where after activation and subsequent proliferation, differentiate from naïve T cells to effector T cells.[29, 30] Mcl-1 is expressed by T cells at all developmental stages and is necessary for their survival and, consequently, their maturation.[29, 31] In order to determine the role of Mcl-1 in T cell development and function, a variety of studies have been made and demonstrated the role of *Mcl-1* in regulating T cells biology. For example, Opferman *et al*.[31] and Dzhagalov *et al*.[29] verified a dependence of T lymphocytes on Mcl-1 for their development, survival and maintenance. In the Opferman *et al*.[31] study they used a knockout mouse for Mcl-1 and found a decrease in the overall expression of thymocytes: double positive cells and single positive cells. Also, it was demonstrated that the loss of Mcl-1 increased apoptosis at DN2 thymocytes and arrested the development of DN3 thymocytes. Moreover, in this study it was shown a role for Mcl-1 in maintaining the existing mature T lymphocytes since the deletion of Mcl-1 lead to the depletion of T cells from the spleen.[31] Dzhagalov *et al*.[29] went further and demonstrated also a role for Mcl-1 in the survival of activated T cells and thus this study together with the Opferman *et al.*[31] study demonstrates that Mcl-1 promotes the survival of DN, DP, SP, naïve and activated T cells. In Dzhagalov *et al*.[29] study they confirmed that *MCL1* deletion resulted in the blockage of DN2/DN3 to DN4 transition and also in the decreased of SP cells. It was also demonstrated *in vitro* that DP cells depend on Mcl-1 for their survival, but *in vivo* the loss of Mcl-1 did not have an effect in the DP survival cells since other anti-apoptotic molecules of this family also have important roles in the survival of T cells at this developmental stage. The authors also searched for the role of Mcl-1 in activated T cells and in contrast with DP cells *in vitro*, activated T cells from a *MCL1* knockout mouse undergone apoptosis demonstrating the importance of Mcl-1 in the maintenance of activated T cells. Also, they have stimulated T cells with an anti-CD3 antibody, which binds to CD3 on the surface of T cells, and a strongly up-regulation of Mcl-1 was seen, demonstrating a role for Mcl-1 in the survival and function of activated T cells.[29]

It was also demonstrated that *MCL1* has a relevant role under pathological conditions. Particularly, *MCL1* is highly expressed in diverse human cancers with poor prognosis, a variety of which hematopoietic cancers, such as acute lymphoblastic

leukemia (ALL) and chronic lymphocytic leukemia (CLL), contributing to malignant cell growth and evasion of apoptosis.[25] Therefore, compounds that inhibit *MCL1* could be an efficient therapeutic agent in cancers with *MCL1* overexpression.

MCL1 gene characterization and post-transcriptional regulation

The human *MCL1* gene comprises 3 exons, all of which encode the information that gives rise to the anti-apoptotic protein Mcl-1. This gene is actively regulated at both transcriptional and post-transcriptional levels.[23, 24] A variety of interleukins (IL-3[32], IL-5[33], IL-6[34]) as well as growth factors, such as epidermal growth factor (EGF)[35] and vascular endothelial growth factor (VEGF)[36], were described to induce transcriptional up-regulation of *MCL1*. At transcriptional level, a variety of transcription factors are implicated in *MCL1* regulation, being the signal transducers and activators of transcription (STAT) an important family of these regulators. In response to IL-6[37], IL-3[32], and VEGF[38], STAT3 was described to up-regulate the transcription of this gene. Other transcription factors involved in *MCL1* transcription regulation are the PU.1[39] and hypoxia-inducible factor 1-alpha (HIF-1α)[40], described to up-regulate transcription of *MCL1*, and E2F-1[41], that down-regulate *MCL1* through its binding to the *MCL1* promoter.[23, 24, 27]

At post-transcriptional level, *MCL1* is initially regulated by alternative splicing, which gives rise to three mRNA isoforms. By the skipping of exon 2 or by the skipping of a portion of exon 1, it is generated two shortened mRNA isoforms, Mcl-1S and Mcl-1ES, respectively. Contrary to the anti-apoptotic isoform, these two alternative splicing-derived isoforms have a pro-apoptotic role by sequestering the Mcl-1 anti-apoptotic isoform and because they are unable to interact and sequester the pro-apoptotic Bcl-2 family members (**figure 7**). *MCL1* is also down-regulated by miRNAs, being the most known miRNA-29[23, 42], as will be further explained below. At translation level, one example of *MCL1* regulation is by the PI3K-AKT-mammalian target of rampamycin complex 1 (mTORC1) pathway, which induces *MCL1* translation (**figure 8**).[23, 24, 27, 42]

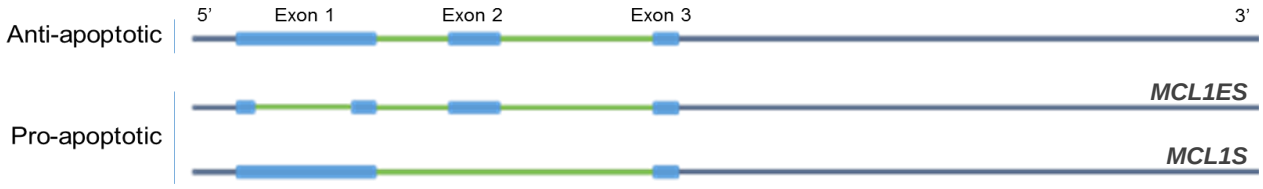


Figure 7 - MCL1 gene characterization. In this figure the light blue boxes correspond to exons, the dark blue lines correspond to 5'UTR (left) and 3'UTR (right), and the green lines correspond to introns. *MCL1* is transcribed in three mRNA isoforms; the longest mRNA produces an anti-apoptotic protein (Mcl-1) and the other two mRNA isoforms (MCL-1S and MCL-1ES) are generated by alternative splicing and encode pro-apoptotic proteins.

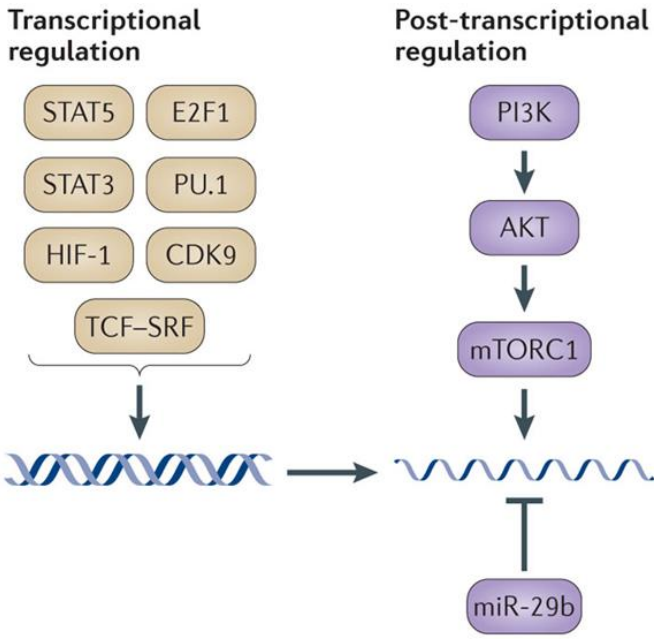


Figure 8 - MCL1 gene transcriptional and post-transcriptional regulation. *MCL1* is transcriptionally and post-transcriptionally regulated by a variety of transcription factors, by miRNAs, such as miRNA-29b and by signalling pathways, such as the PI3K-AKT-mTORC1 pathway. Juin *et al.*[24]

MicroRNAs

miRNAs are non-coding RNAs found in animals and plants representing a large family of post-transcriptional regulators of gene expression, whose mature products have 21 to 23 nucleotides in length.[43-47] Several studies have demonstrated the important role of miRNAs in a wide range of biological processes like development, proliferation and differentiation, metabolism, apoptosis and cancer.[45, 46, 48] Also, it is now known that a single miRNA might control numerous distinct targets and it is estimated that about 50% of all mammalian genes are under miRNA control.[43, 48]

The miRNAs are usually processed by RNA polymerase II from precursor molecules such as independent genes or introns of protein-coding genes. The product of RNA polymerase II (pri-miRNA) acts as a substrate for two members of RNase III family of enzymes, Drosha and Dicer. First, Drosha in complex with DGCR8, processes the pri-miRNA giving rise to a ~70-nucleotide precursor hairpin, the pre-miRNA. The pre-miRNA is then recognized by exportin 5 and thus exported to the cytoplasm where is processed by DICER, assisted by the transactivating response RNA-binding protein (TRBP). This leads to the formation of a ~20 nucleotide miRNA/miRNA duplex. One of the two strands of this complex, the mature miRNA, is then incorporated into an Argonaute (AGO) protein, essential for target recognition, giving rise to the miRNA-induced silencing complex (miRISC).[43, 45, 46, 48]

miRISC recognizes an mRNA to be silenced through the base pairing-interactions between the miRNA and its target mRNA. The seed region, a ~8-nucleotide motif at the 5' domain of the miRNA, is the predominant mechanism for target mRNA recognition. Along the mRNA sequence there is a plenitude of regions where seed matches may occur, however, they are more prone to silence an mRNA when they are in the 3'UTR[49, 50]. The targets to be silenced can contain a partially or fully complementary sequence to the corresponding miRNAs. According to the type of base pairing interactions, miRNA-mediated gene silencing can occur by mRNA cleavage, mRNA degradation or translational repression. If the miRNA contains a fully complementary sequence the target will be repressed by mRNA cleavage. In contrast, if the miRNA contains a partially complementary sequence the mechanism of action will be trough translational repression or mRNA degradation.[44-46]

According to a variety of studies that have been done in the past few years, mRNA degradation is responsible for ~75%-85% of the changes observed in protein synthesis, attributing a less important role of miRNAs in translational repression. [45, 47, 48] The mechanism of mRNA degradation by miRNAs is the following: once an mRNA is targeted by the miRISC complex to be silenced, GW182 interacts by its amino-terminal domain with AGO protein and by its carboxy-terminal domain with polyA-binding protein, cytoplasmic 1 (PABPC1) that is bound to the pA tail of the target mRNA. Then, the complex AGO-GW182-mRNA leads to the removal of the pA tail (deadenylation) by the CAF1-CCR4-NOT deadenylase complex. This mechanism, in turns, leads to the decapping of the mRNA by the DCP2 enzyme and mRNA degradation by the 5'-to-3' exoribonuclease 1 (XRN1).[43, 48, 51]

A general overview of the miRNAs biogenesis and function described above is illustrated in **figure 9**.

miRNAs in *MCL1* regulation

Several miRNAs were demonstrated to down-regulate *MCL1* expression in a variety of human cancers. Examples of this regulation are: miRNA-135a/b in a lung cancer cell line[52], miRNA-133a/b in human osteosarcoma[53, 54], miRNA-204 in pancreatic cancer[55], miRNA-100/99a in acute lymphoblastic leukaemia[56], miRNA-193a in epithelial ovarian cancer cells[57], miRNA-139 in glioma[58], miRNA-26a in breast cancer[59], and miRNA-29b in, for example, a glioblastoma multiforme cell line[60].

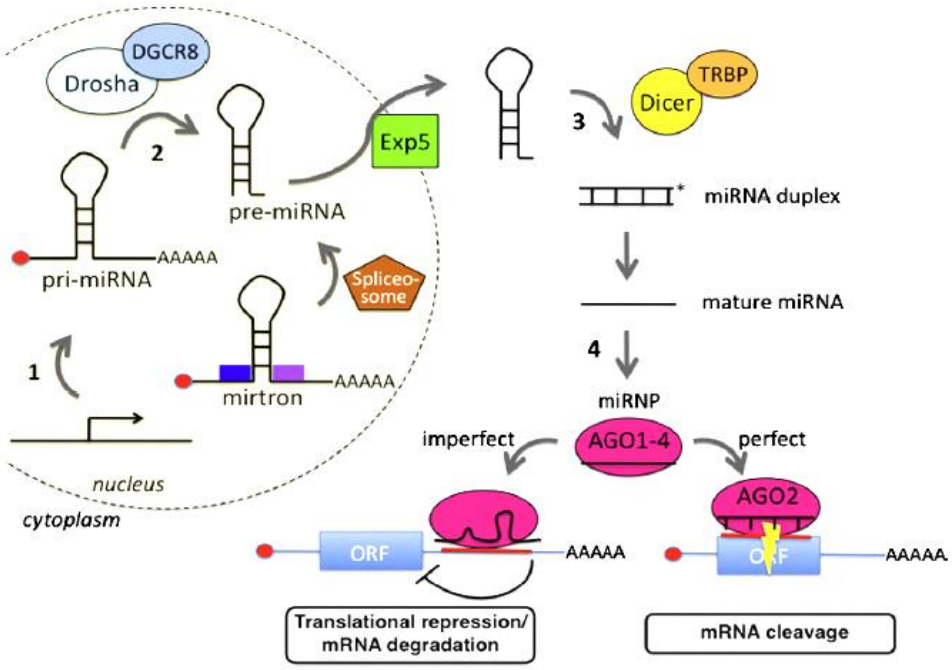


Figure 9 - miRNAs biogenesis and function. miRNAs are transcribed in the nucleus by RNA polymerase II and exert their function in the cytoplasm. miRNAs regulate their targets by translation repression, mRNA degradation or mRNA cleavage, depending on their complementary to their targets. There is strong evidence that target degradation provides a major contribution to silencing by animal miRNAs. Adapted from Breving *et al.*[45]

The most well-established miRNA that regulates *MCL1* expression is miRNA-29[42, 60]. Aldaz *et al.*[60] described that the overexpression of miRNA-29a and miRNA-29b in GN1C cells, a type of glioblastoma multiforme cell line, leads to the decrease of Mcl-1 protein expression (54% and 62%, respectively), resulting in an increase of apoptosis (about 1,6-fold and 1,56-fold increase, respectively).[60] In what concern cancers of the lymphoid lineage, Li *et al*.[56] demonstrated a role for miRNA-100 and miRNA-99a in down-regulating *MCL1* expression in ALL. In this study, they first demonstrated that both miRNAs had lower expression in acute lymphoblastic leukemia patients and that when they overexpressed these two miRNAs the levels of *MCL1* were decreased.[56]

Since *MCL1* plays a major role in suppressing apoptosis, characterization of the *MCL1* mRNA isoforms produced by APA and the miRNAs that regulate its expression may be a useful therapeutic tool in different clinical contexts in the future.

Aims of this thesis

Despite all the studies regarding *MCL1* regulation, nothing was known about the role of polyadenylation and APA on *MCL1* regulation. Also, post-transcriptional regulation of *MCL1* by miRNAs in T cells in resting and activated states had not been previously investigated. Therefore, the main goals of this thesis were to characterize the APA pattern of *MCL1* in primary human T cells in both resting and activated conditions and to identify miRNAs involved in *MCL1* post-transcriptional regulation.

Overall, the specific objectives of the experimental work were to:

- Map the mRNAs 3' ends of *MCL1* produced by APA;
- Determine the *MCL1* APA-derived mRNA isoforms expression both at mRNA and protein level.
- Identify the miRNAs that could influence *MCL1* expression in T cells;
- Quantify the expression of potential miRNAs in primary human T cells;
- Analyse the effect of mutations of the putative miRNA-binding sites on *MCL1* 3'UTR ;
- Study the consequences of miRNAs overexpression in the expression of Mcl-1 protein;
- Identify a miRNA that affects *MCL1* expression and that could be used as a potential therapeutic target in the future.

MATERIAL AND METHODS

IN SILICO ANALYSIS

In order to check for *MCL1* coding and 3'UTR sequences and for predicted APA pattern we used the UCSC genome browser. To assess the conservation of the 3'UTR, the pA signals and the putative-binding sites of the chosen miRNAs we performed a multiple sequence alignment of the mRNA or genomic sequence of ten mammalian species using the default settings of the MUSCLE 3.6 software running on Geneious program v4.8.

To search for miRNAs putative-binding sites on *MCL1*, TargetScan (*https://www.**targetscan**.org/*), microRNA.org (*https://www.**microRNA**.org/*) and miRTarBase (*https://www.**mirtarbase**.mbc.nctu.edu.tw/*) databases were assessed using the default settings. The microRNA.org database and miRNAmap (*https://www.**mirnamap**.mbc.nctu.edu.tw/*) databases were used to determine the expression of the miRNAs in different tissues.

EXPERIMENTAL MODELS

Cell culture

Jurkat E6.1 cell line was grown and maintained in culture at 37 ºC with 5% CO_2 atmosphere in complete medium (RPMI 1640 medium modified with GlutaMAX and phenol red, 10% fetal bovine serum (FBS) and 1% of a penicillin/streptomycin antibiotic solution). Cells were split every 3-4 days in order to be at a concentration around 2 x 10^6 cells/mL.

HeLa cell line was also grown and maintained in culture at 37 ºC with 5% CO_2 atmosphere in complete medium (DMEM with 10% FBS and 1% of a penicillin/streptomycin antibiotic solution). Cells were split in a 1:10 ratio every 3-4 days to maintain subconfluency.

All the reagents mentioned above are from Gibco®, Life technologies.

Human PBMCs and T cells isolation

Human blood buffy coats were obtained from volunteer blood-donors from Hospital de São João. Peripheral blood mononuclear cells (PBMCs) were isolated using Lympholyte®-H (Cedarlane Labs) according to the manufacturer's instructions.

In order to isolate T lymphocytes, previously isolated PBMCs were resuspended in 1X PBS (phosphate-buffered saline) at approximately $5x10^7$ cells/mL and the EasySep negative selection kit (STEMCELL technologies) was used. Using this kit the unwanted cells (using antibodies CD14, CD16, CD19,

CD20, CD36, CD56, CD66b and CD123) are retained using magnetic beads while T cells are enriched by separating the desire fraction into a new tube.

Both PBMCs and T cells were counted using an automated cell counter (Countess®, Life Technologies) and cultured at 1 x 10^6 cells/mL in RPMI complete medium.

Cell Activation

Both primary PBMCs and human T cells were activated with phytohemagglutinin (PHA) in a final concentration of 1 µg/mL for 48 hours. Cells in resting and activated states were harvested for further RNA extraction.

QUANTITATIVE MRNA EXPRESSION ANALYSIS

RNA fractionation

PBMCs were collected, washed twice with 1X PBS and resuspended in 1 mL of RSB buffer (10 mM Tris pH 7.4, 10 mM NaCl and 3 mM MgCl2). After 3 min incubation on ice, cells were centrifuged at 3200 g for 3 min at 4 ºC, and the supernatant was discarded. The pellet was resuspended in 150 µL of RSBG40 buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl2, 10% glycerol, 0.5% NP-40, 0.5 mM DTT (Invitrogen) and 40U/µL RiboLock RNase Inhibitor (Thermo Scientific)). Cells were centrifuged at 4500 g for 3 min at 4 ºC. Upon supernatant (the cytoplasmic fraction) had been transferred to a new eppendorf, it was added 1 mL of TRIzol to the supernatant and to the pellet (nuclear fraction). The RNA extraction was then performed as described bellow.

RNA extraction

Total RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer standard protocol. First we added 1 mL of TRIzol reagent and incubated 5 min at room temperature. Then 200 µL of chloroform was added and the samples were mixed and centrifuged for 15 min at 12000g at 4ºC. The upper aqueous phase was transferred to a fresh tube containing 1 µL of Glicoblue (15mg/mL, Life Technologies) and the same amount of isopropanol as the aqueous phase transferred. The samples were mixed and then frozen overnight at -80ºC. In the next day, after thawing, samples were centrifuged for 20 min at 12000g at 4ºC following by a pellet washing step with 500 µL of 75% cold ethanol centrifuged 10 min at 12000g at 4ºC . The pellet was air dried and resuspended in 11 µL of Nuclease-free water (Thermo Scientific).

Total RNA enriched with small RNAs extraction was performed using the mirVana™ isolation kit protocol (Ambion, Life Technologies). Primary T cells and/or Jurkat E6.1 cell line were counted as described above and 10^2 to 10^7 cells were pelleted and further washed using 1mL of cold PBS 1X. HeLa cells were trypsinized, counted and then 10^2 to 10^7 cells were pelleted and further washed using 1 mL cold PBS 1X. After cell suspension preparation the RNA extraction was performed according to the manufacturer's instructions with minor modifications. The first wash step using miRNA Wash Solution 1 was divided in two steps: instead of using 700 µL of this solution and centrifuged once, we added 350 µL twice and centrifuged each time performing the DNase (DNase I recombinant, Roche) treatment (final concentration 3 U/µL) between these two new steps.

RNA quantification was performed in a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and the RNA stored at – 80 ºC.

DNase treatment

DNase treatment for total RNA was performed in a final volume of 12 µL, the volume necessary to further perform cDNA synthesis. DNase recombinant I (Roche) was used at a final concentration of 1 U/µL and the samples were incubated at 37ºC for 90 min. Samples were then incubated 10 min at 75ºC for enzyme inactivation.

cDNA synthesis

After total RNA or fractionated RNA extraction and DNAse treatment, SuperScript III™ Reverse Transcriptase enzyme (Invitrogen, Life Technologies) was used to synthesise cDNA from 300 ng or 500 ng of DNase treated RNA from human primary T cells and PBMCs or HeLa and Jurkat E6.1 cell lines, respectively, following the manufacture's guidelines. Briefly, to the treated RNA was first added 1µL of dNTPs (10 mM) and 1 µL of random hexamers (50 µM) and the mixture was then incubated at 65ºC for 5 min in order to denature RNA, followed by 5 min at 4ºC. The mix for reverse transcription was prepared at a final volume of 6 µL containing 4 µL of cDNA synthesis buffer (5X), 1µL of DTT (0,1 M), 0,5 µL of RiboLock RNase Inhibitor (40 U/µL, Thermo Scientific) and 0,5 µL of SuperScript III reverse transcriptase enzyme (200 U/µL, Invitrogen). The samples were then incubated for 10 min at 25ºC, 60 min at 50ºC and finally 10 min at 70ºC to inactivate the enzyme, using a TPersonal thermocycler (Biometra). To discard genomic DNA contaminations, negative reactions were performed without SuperScript III.

To synthesise cDNA from total RNA enriched with small RNAs extraction the TaqMan®MiRNA Assays (Life Technologies) protocol was followed. For this reaction a total of 10 ng of RNA was used. The master mix for this reaction was composed by 1,5 µL of dNTPs (10 mM), 1 µL of Multiscribe™ reverse transcriptase (50U/µL), 1,5 µL of reverse transcription buffer (10X), 0,19 µL of RiboLock RNase Inhibitor (40 U/µL, Thermo Scientific) and Nuclease-free water (Thermo Scientific) up to 7 µL. To this reaction was then added 5 µL of RNA (10ng) and 3 µL of RT primer (5X) and the samples were then mixed. The mixture was incubated on ice for 5 min before proceeding to the thermal cycling at 16ºC for 30 min, 42ºC for 30 min followed by 5 min at 85ºC for enzyme inactivation.

All cDNA samples were stored at -20ºC.

Quantitative Real Time PCR (RT-qPCR)

For quantification of *MCL1* mRNA isoforms the primer pairs designed (table 1) were first optimized. The optimization consisted in performing the RT-qPCR reactions, as described below, using series of cDNA dilutions (1, 1:10, 1:100 and 1:1000) to create a standard curve. In this standard curve the slope should be around -3.3, in order to obtain an ideal efficiency (90% to 110%). The efficiency was obtained through the following equations: $E = 10^{(-1/slope)}$ and $\%E = (E-1) \times 100$.

Each RT-qPCR reaction was performed using 10 µL of SYBR® Select Master Mix (Applied Biosystems, Life technologies), 0,25 µL of each primer (10 µM), 1 µL of the cDNA sample and nuclease-free water up to 20 µL. Reactions were performed using the StepOne Real-time PCR System (Applied Biosystems) thermocycler following the program recommended by the company.

The reference gene chosen was the ribosomal gene 18S for all samples [61, 62]. The results were analyzed using the ΔCt method (relative expression=$2^{-(CT\ target\ gene\ -\ CT\ reference\ gene)}$), used to determine the expression of the target gene relative to the endogenous control, assuming the maximum efficiency (E=2), since the results do not suffered significant changes when the real efficiency was used.

For quantification of the miRNAs expression, it was prepared a master mix containing 10 µL of TaqMan® Universal PCR Master Mix II, no UNG (Applied Biosystems, Life Technologies), 1 µL of 20X TaqMan® Small RNA Assay (Applied Biosystems, Life Technologies) and Nuclease-free water (Thermo Scientific) up to 18,7 µL. To this mix it was added 1,3 µL of the prepared cDNA. Reactions were performed in the StepOne Real-time PCR System (Applied Biosystems) thermocycler using the recommended thermal cycling program (2 min at 50ºC; 10 min at 95ºC, and 40 cycles of 15 sec at 95ºC and 60 sec at 60ºC). The results were analysed with the previously

described ΔCT method using as reference gene the human U6 small nuclear RNA[63]. The codes of the TaqMan® probes used are described below (**table 2**).

3'RACE (RAPID AMPLIFICATION OF CDNA ENDS) AND NESTED PCR

The SMARTer™ RACE cDNA Amplification kit (Clontech) was used to synthesized 3´RACE cDNA, using SMARTScribe™ Reverse Transcriptase and 1 µg of total RNA, according to the manufacturer's protocol. The first and nested 3' RACE PCR reactions were done using the Phusion High-Fidelity DNA Polymerase (2U/µL – Thermo Scientific), following the manufacturer's protocols. The forward primers were designed according to SMARTer™ RACE kit protocol's specificities (table1). The products were then analysed by agarose gel electrophoresis (1,5%) stained with SybrSafe DNA gel stain (Invitrogen) and using the GeneRuler DNA ladder mix (Thermo Scientific). The desired bands from the agarose gel electrophoresis were cut and purified using the GRS PCR and Gel Band Purification Kit (Grisp).

CLONING INTO TOPO VECTOR

Previously to the TA-cloning procedure, we performed the addition of an adenine residue to the 3´-ends of the blunt gel purified PCR products by incubating 15 min at 72 ºC the following mixture: 4 µL of the purified band, 0,5 µl of 5X GoTaq Reaction Buffer (Promega), 0,3 µL of dATP (2.5 mM) and 0,2 µL of GoTaq® DNA Polymerase (5 U/µl - Promega). Cloning of the 3'RACE PCR purified bands was done using the TOPO TA Cloning Kit (Life technologies) according to the manufacturer's instructions with minor modifications. Briefly, 4 µL of the mixture described above was added to 1 µl of Salt Solution (Life Technologies) and 1 µL of pCR2.1-TOPO vector (Life Technologies) and incubated for 30 min at 22ºC. The total volume of this mixture was transformed in 100 µl of *Echerichia coli* TOP10 competent cells.

TRANSFORMATION OF COMPETENT BACTERIA

Ten µL (or 6 µL in the case of the TA-cloning) of the plasmid ligation product were added to 100 µL of TOP10 chemically competent *Echerichia coli* (Invitrogen, Life Technologies). Cells were incubated on ice for 15 min, followed by a heat shock at 42 ºC for 90 seconds and put back on ice for more 5 min. LB medium (400 µL) was added and cells were incubated for 60 min in an orbital shaker at 37 ºC. The cells were then plated in LB plates with kanamycin (25

µg/mL) or ampicillin (100 µg/mL), according to the antibiotic resistance present in the plasmid used, and put at 37ºC overnight (ON).

COLONY PCR

In order to determine the colonies that incorporated properly the insert a colony PCR was performed. The PCR reaction was performed using 0,15 µL of GoTaq® DNA Polymerase (5 U/µl - Promega); 2 µL of 5X Green GoTaq® Reaction Buffer (Promega); 0,75 µL of $MgCl_2$ (25 mM - Promega); 0,5 µL of each primer (10 µM); 0,5 µL dNTPs (10 mM); and Nuclease-free water up to 10 µL. The plasmid template was obtained by a stab of each colony with a tip that was then emerged in the reaction mix. The PCR reactions were carried out in TPersonal thermocycler using the following program: 5 min at 95ºC; 35 cycles of 1 minute at 95ºC, 30 seconds at 56ºC, 30 seconds at 72ºC; and a last extension time of 7 min at 72ºC. The reaction product was then analysed by a 1% agarose gel electrophoresis.

ACTINOMYCIN-D TREATMENT

In this experiment seven time-point conditions were used (0, 0.5, 1, 2, 4, 6 and 24 hours) and each condition was made in triplicates. Jurkat E6.1 cells were counted as described above and seed out at a confluence of $3x10^6$ cells/mL in 24 well-plates. To the time-point "0h" no Actimycine-D antibiotic (Sigma-Aldrich®) was added. To the other conditions 5 µg/mL of Actinomycin-D was added to the RPMI complete medium and the samples were then incubated at 37ºC. At each time-point, the samples were centrifuged at 300g for 5 min and, after removing the supernatant, the pellet was resuspended in 1 mL TRIzol. Posteriorly, the RNA extraction was performed as described above.

PLUC CONSTRUCTS/PMIRGLO CONSTRUCTS

Each *MCL1* 3'UTR isoforms were amplified using as template the sequences cloned into the TOPO vector and specific primers (**table 1**). The forward and reverse primers used to amplify all isoforms had at its 5' end a sequence for the *Sac*I and *Sal*I restriction enzymes (New England Biolabs), respectively. Using these primers all *MCL1* APA-derived mRNA isoforms were amplified using the Phusion High-Fidelity DNA Polymerase (2U/µL – Thermo Scientific). For this reaction it was used 4 µL of 5X Phusion HF buffer; 0,4 µL of dNTPs (10 mM); 1 µL of each primer (10 µM); 0,2 µL of Phusion High-Fidelity DNA

Polymerase (2 U/µL – Thermo Scientific); 1µL of the plasmid used as template (250 ng); and Nuclease free water up to 20 µL. The amplification was performed in the TPersonal thermocycler using the following program: 1 minute at 98ºC; 25 cycles of 10 seconds at 98ºC, 30 seconds at 56ºC, 2,5 min at 72ºC; and a final extension step of 7 min at 72ºC. The PCR products were analyzed by agarose gel electrophoresis (0,8%) as described above and then purified using QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's instruction. The purified PCR products and the plasmid "pLuc" were then digested with *Sac*I and *Sal*I restriction enzymes (New England Biolabs) and cloned as described below.

To clone the ~100-bp sequence of *MCL1* 3'UTR containing the putative binding sites for the selected miRNAs and those sites mutated two templates were used. To clone the wild type sequences it was used as template the pLuc constructs where *MCL1* pA4 3'UTR were cloned and as backbone vector pmiRGLO (Promega). To clone the sequences containing the putative-binding sites mutated, it was used the pLuc plasmid where pA4 *MCL1* 3'UTR was cloned and where all miRNAs putative-binding sites were mutated by directed mutagenesis (described bellow). Once again, the primers used to amplify these sequences contained at their 5'end the sequences for *Sac*I (primer forward) and *Sal*I (primer reverse). For the amplification reaction it was used 0,2µL of GoTaq® DNA Polymerase (5U/µl - Promega); 4 µL of 5X Green GoTaq® Reaction Buffer (Promega); 1,5 µL of $MgCl_2$ (25 mM - Promega); 1 µL of each primer (10 µM); 1 µL dNTPs (10 mM); 1 µL of the plasmid template and Nuclease-free water up to 20 µL. The PCR reactions were carried out in TPersonal thermocycler using the following program: 5 min at 95ºC; 25 cycles of 30 seconds at 95ºC, 30 seconds at 56ºC, 30 seconds at 72ºC; and a last extension time of 5 min at 72ºC.The PCR products were analyzed by agarose gel electrophoresis (0,8%) as described above and then purified using QIAquick PCR Purification Kit (QIAGEN) according to manufacturer's instructions. The PCR products and the plasmids used were then digested with *Sac*I and *Sal*I restriction enzymes (New England BioLabs).

To perform the cloning of the sequences in pLuc and pmirGLO it was used the T4 DNA ligase (Thermo Scientific) according to the manufacturer's guidelines. Posteriorly, the ligation product was transformed into competent cells as described above. Plasmid DNA extractions were performed using the PureLink™ Quick Plasmid Miniprep Kit (Life Technologies) and plasmids were sequenced in GATC Biotech company to confirm that they had the correct sequence.

DIRECTED MUTAGENESIS

This procedure was performed to mutate the proximal pA sinals in each APA isoform to obtain the *MCL1* 3'UTR isoforms containing only the distal pA signals and to mutate the putative-binding sites of the miRNAs selected. Using this procedure it was possible to generate: the pA2 isoform with pA1 pA signal mutated, pA3 isoform with the pA1 and pA2 pA signals mutated, and pA4 isoform with pA1, pA2 and pA3 isoforms mutated. The pA4 construct was used to mutate the miRNAs putative-binding sites and this construct was then used as a PCR template to clone the ~100-bp sequence of *MCL1* 3'UTR containing the putative binding sites for the selected miRNAs into pmirGLO vector.

This technique was performed according to the protocol described in Liu and Naismith 2008 [64], with minor alterations. The primers were designed so that their 5'ends were complementary to each other and contained at the complementary region the mutations to be inserted. These primers pairs amplified all the plasmid sequence inserting only the desired mutation. Due to the template length it was used the Phusion High-Fidelity DNA Polymerase (2 U/µL – Thermo Scientific). For this reaction it was used 1,5µL of the plasmid template (15ng), 10 µL of 5X Phusion HF buffer; 1 µL of dNTPs (10 mM); 2,5 µL of each primer (primer forward and primer reverse, 10 µM); 1,5 µL of Phusion High-Fidelity DNA Polymerase (2 U/µL – Thermo Scientific); and Nuclease free water up to 50 µL. After several optimizations, the PCR program used was the following: 1 minute at 98ºC; 3 cycles of 10 seconds at 98ºC, 30 seconds at 70ºC, 5 min at 72ºC; 3 cycles of 10 seconds at 98ºC, 30 seconds at 68ºC, 5 min at 72ºC; 18 cycles of 10 seconds at 98ºC, 30 seconds at 65ºC, 5 min at 72ºC; and a last extension step of 10 min at 72ºC. The PCR products were analyzed by agarose gel electrophoresis (0,8%) as described above and then purified using QIAquick PCR Purification Kit (QIAGEN) according to the manufacturers' instructions. To eliminate any remains of the parental plasmid, the purified PCR products were digested by using 2 µL of *Dpn*I enzyme (New England Biolabs), 10 µL of 10X Cut Smart buffer, 49µL of the purified PCR product and Nuclease-free water up to 100 µL, for 2 hours at 37ºC followed by an inactivation time of 20 min at 80ºC. After, the PCR-generated mutant plasmids were transformed into competent cells as described above. All plasmids were sequenced in GATC Biotech company to confirm that they had the correct mutations.

TRANSFECTION ASSAYS

These procedures were performed in order to transfect the cells with the pLuc constructs containing the *MCL1* 3'UTR isoforms, the pmirGLO constructs containing the ~100bp sequences around the putative-binding sites for the selected miRNA, or to perform the overexpression of the selected miRNAs (Addgene Plasmid 21113: pcDNA3-miR17; Addgene Plasmid 21121: pcDNA3-miR29b; Addgene Plasmid 46672: pcDNA3.2 miR-1-1 reporter hsa-miR-92a-1).

HeLa cells were transfected using the Lipofectamine® 3000 reagent (Life Technologies) according to the manufacturer's guidelines. Cells were placed on 24-well plates at a concentration of $2x10^5$ cells/mL and transfected when reached 70%-90% confluency. Transfections were performed using in total 0,5 µg of plasmids. In the experiments to measure the Luciferase activity produced by the *MCL1* 3'UTRs isoforms two plasmids had to be transfected: pLuc (0,35 µg) and pCMV-Renilla (0,15 µg), once pLuc didn't have the *Renilla* gene in its backbone. For pmiRGLO (has luciferase and renilla genes in its backbone) and miRNA overexpression experiments, 0,5 µg of plasmid were used.

LUCIFERASE ASSAY

To perform the Luciferase assays the Dual-Luciferase® Reporter Assay (Promega) was used according to the manufacturer's guidelines. The lysis process used in these experiments was the Passive Lysis procedure. Briefly, the cells were harvested and rinsed once with 1X PBS. Then, 100 µL of 1X Passive Lysis Buffer was added followed by 15 min shaking at room temperature. Next, 10 µL of each sample was added to a 96-well plate and 50 µL of LAR II was added to perform the *firefly* luciferase activity measurement. After, 50 µL of Stop & Glo® Reagent was added to perform the *Renilla* luciferase activity measurement. For the measurements of both firefly and renilla luciferase activities a Synergy 2 Multi-Mode Reader (Bio-Tek) was used. To achieve the final luciferase activity, the values obtained for luciferase were normalized to renilla. Final luciferase activity values represent the mean of three independent experiments. Each experiment was performed using duplicates.

WESTERN BLOT

To obtain the protein lysates needed to perform the western blot the medium from HeLa cells was removed and cells washed with PBS 1X. In the PBMCs and Jurkat

E6.1 cell line cells were first centrifuged for 5 min at 300 g, the supernatant was removed and cells washed with PBS 1X. 100 µL of Lysis buffer (10mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) with 1X proteases inhibitors (Roche) was added to the cells and incubated on ice for 15 min. The lysates were then transferred to an eppendorf and centrifuge for 10 min at 14,000g. The supernatant (containing the protein lysates) was then transferred to a new eppendorf. In order to perform the protein quantification the Bradford reagent (Bio-Rad) was used and absorvancy was read at 595 nm in a microplate spectrophotometer (µQuant™ - BioTek). After, 25 µg of protein (in a final volume of 10 µL) of each sample to which was added 10 µL of 2X loading buffer (with 5% β-mercaptoethanol) was boiled at 95ºC for 5 min. Then, the samples were loaded on a 12% SDS–PAGE gel and after 45 min at 200V, transferred onto a nitrocellulose membrane (Life Technologies) and subjected to western blot analysis. Briefly, membrane was blocked for 45 min with 5% non-fat milk in TBS with 0.2% tween and incubated overnight at 4ºC with a mouse monoclonal anti-human Mcl-1 antibody (eBioscience; 1:500 dilution). The horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:20000 dilution; Santa Cruz Biotechnology) was added for 45 min. Between antibody incubations membrane was washed with TBS with tween 0,2% for 10 min four times. After the final wash, membrane was incubated with ECL for 5 min on dark and then revealed using a ChemiDoc™ XRS$^+$ System instrument (Bio-Rad).

FLOW CYTOMETRY

Flow cytometry experiences were done using 1 x 10^6 cells per condition. Briefly, cells were centrifuged at 300g for 5 min at 4ºC and the supernatant was removed. Pellet was washed with 4 mL of 1X PBS and centrifuged at 300g for 5 min at 4ºC. After supernatant has been removed, the cells were fixed with 2mL of 2% paraformaldehyde during 20 min at room temperature in the dark. Cells were washed and resuspended in FACS buffer (1X PBS, 0.2% BSA, 0.1% NaN3). Afterwards, cells were incubated with anti-CD69-APC and anti-CD3-PE (Pharmingen) during 20 min at 4ºC in the dark. Following antibody incubation, cells were washed twice with FACS buffer and resuspended in 200 µL of FACS buffer for flow cytometry analysis. All experiments were then performed on the FACS CALIBUR (Becton Dickinson).

STATISTICAL ANALYSIS

To perform all statistical analysis the student t-test (for paired samples) was used. A *p*-value lower than 0,05 (*), 0,01 (**) or 0,001 (***) denoted the presence of a statistically significant difference. The analysis was performed using the GraphPad Prism® 6 software.

Table 1 - List of primers used in this study

Primers name	*Primers Sequence (5'-3')*	*Application*
MCL1-E2-RACE	CTCGTAAGGACAAAACGGGACTGGCTAG	3'RACE PCR
MCL1-E3-RACE	AGGTGGCATCAGGAATGTGCTGCTG	Nested RACE PCR
cod-F-new	AGGACCTAGAAGGTGGCATCAG	RT-qPCR
cod-R-new	TAGATATGCCAAACCAGCTCCT	RT-qPCR
pA4	GCTTGGGGCAGTGAGGGCT	RT-qPCR
pA4	ACCACCTGCCTCCTCCTCC	RT-qPCR
MCL1-UTR-F -SacI	TATGAGCTCCCTTACTGTAAGTGCAATAG	Cloning
MCL1-UTR-R-SalI	CGAGTCGACGAATAAAAGATTTATTTTTTTTTCTC	Cloning
MCL1-pA1.2-R-SalI	CGAGTCGACATAGAAACAAAACAGTAAGTATT	Cloning
MCL1-pA1.1-R-SalI	CGAGTCGACCCAAGCCATCATTTTATAATTTATTAAG	Cloning
MCL1-pA0-R-SalI	CGAGTCGACGGAGCACTCTTCCCATGTATT	Cloning
pA1mut-F	CAAGAACTAGTACATGGGAAGAGTGCTCCC	Directed mutagenesis
pA1mut-R	CATGTACTAGTTCTTGTTAGCCATAATCCTCTTGC	Directed Mutagenesis
pA2mut-F	TGCTTAACTAGTTATGGAATGATGGCTTGGAAAAGC	Directed Mutagenesis
pA2mut-R	TTCCATAACTAGTTAAGCAAACAAGGGATCAAATGTC	Directed Mutagenesis
pA3mut-F	GTATGTCAACTAGGCAAATACTTACTGTTTTGTTTC	Directed Mutagenesis
pA3mut-R	ATTTGCCTAGTTGACATACTAGGCTTAGACCTGT	Directed Mutagenesis
miR-92a mut-F	TAAACTCCGTCGTTGACTTTTAACCAACCACC	Directed Mutagenesis
miR-92a mut-R	GTCAACGACGGAGTTTACAGTAAGGGAGCTCG	Directed Mutagenesis
miR-29b mut-F	CTAACCCTGATTCGATTATTAGGCTTGCTTGTTAC	Directed Mutagenesis
miR-29b mut-R	ATAATCGAATCAGGGTTAGACTAGCCTGCTTTTC	Directed Mutagenesis
miR-320 mut-F	TTCCTACGGAGTTCCCCTGCCATCCCTGAACTC	Directed Mutagenesis
miR-320 mut-R	CAGGGGAACTCCGTAGGAATTGATATAAATATCTGTAG	Directed Mutagenesis
miR-17mut-F3	GGACCTAGAATCAAGCTATGTAGCTCTTCATTAGTC	Directed Mutagenesis
miR-17mut-R3	GAGCTACATAGCTTGATTCTAGGTCCTGAGAGATAC	Directed Mutagenesis
F_Luc_F	AGGTCTTCCCGACGATGA	RT-qPCR
F_Luc_R	GTCTTTCCGTGCTCCAAAAC	RT-qPCR
RL-F	GCAGAAGTTGGTCGTGAGG	RT-qPCR
RL-R	TCATCCGTTTCCTTTGTTCTG	RT-qPCR
92-f	CTCGAGCTCCCTTACTGTAA	Cloning
92-R	CGAGTCGACGAAGTTACAGCTTGGAGTCC	Cloning
29-f	TATGAGCTCGGAGAGACATTTGATCCCTT	Cloning

Primers name	*Primers Sequence (5'-3')*	*Application*
29-R	CGAGTCGACCTAGGCTTAGACCTGTGTGT	Cloning
320-F	TATGAGCTCGTTCTGCTCCCTCTACAGA	Cloning
320-R	CGAGTCGACCAGTGCCAA AATCTAAAAGGG	Cloning
17-F	TATGAGCTCCTTGATCATAAGCCGCTTA	Cloning
17-R	CGAGTCGACCACTGGATTTGGCAGACA	Cloning
pmiRGlo_seq-F	CATGACCGAGAAGGAGATCG	Sequencing
M13 F	CCCAGTCACGACGTTGTAAAACG	Sequencing
M13 R	CAGGAAACAGCTATGAC	Sequencing

Note: Some of the sequencing and RT-qPCR primers were also used to perform the colony PCRs, according to the sequence used.

Table 2 - List of the TaqMan probes used for the quantification of miRNAs expression

Target	*Code*
miRNA-92a	hsa-miR-29 - 000431
miRNA-29b	hsa-miR-29 - 000413
miRNA-320	hsa-miR-320 - 002277
miRNA-17	hsa-miR-17 - 002308
U6	U6 - 001973

RESULTS AND DISCUSSION

Characterization of the MCL1 alternative polyadenylation pattern in human T cells

MCL1 mRNA 3' end mapping identified four APA-derived isoforms

Using high throughput RNA-sequencing data, a collaborator of the Gene Regulation group at IBMC (Joel Neilson) identified *MCL1* as a good candidate for studying APA in T cells as: 1) its expression is altered upon T cell activation originating transcripts with shorter 3'UTRs, 2) this event is conserved between primary human and mouse T cells and Jurkat E6.1 cell line and 3) this gene is important for T cell homeostasis and therefore *MCL1* is a good model gene for studying APA in T cells.

To attest the APA pattern of *MCL1* identified by RNA-sequencing we first inspected the UCSC Genome browser (*https://www.**genome**.ucsc.edu/*). We observed that the previously described three *MCL1* mRNA isoforms produced by alternative splicing contain the same 3'UTR sequence. In this browser four putative pA sites are predicted, all located in tandem in the 3'UTR of *MCL1* (**figure 10**). However, at the beginning of this study in 2013, only the two most distal pA sites were described in the UCSC Genome browser.

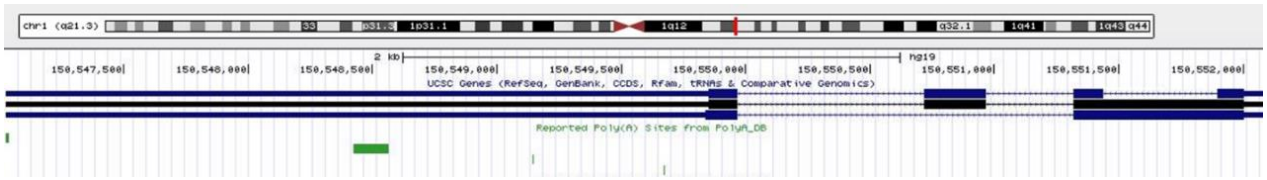


Figure 10 - MCL1 gene characterization by in silico analysis. This scheme illustrates the position of *MCL1* gene in chromosome one (red bar) and its genomic organization (under chromosome scheme). Blue or black boxes represent exons and the dashed lines between them represent introns. The thin blue or black boxes represent the 5' UTR (at right) and the 3'UTR (at left). *MCL1* produces three mRNA isoforms by the exclusion of part of exon one (upper scheme), inclusion of the three exons (middle scheme), or skipping of exon two (lower scheme). All mRNA isoforms present the same 3'UTR, which contain four regions that are predicted pA sites (green bars below genomic sequence).

We extended our *MCL1 in silico* analysis to 3'end mapping by collecting all the deposited ESTs (expressed sequence tags) of human *MCL1* available from multiple tissues at the UniGene database (ncbi.nlm.nih.gov/unigene/). From these, we selected the sequences (n = 350) that contained a pA signal or a pA tail for ESTs analysis in order to guarantee mRNAs with 3' ends, and aligned these sequences against the *MCL1* reference gene sequence (ENSG00000143384) to map their location. By this analysis (**figure 11**), we identified four APA-derived mRNA isoforms. For simplicity, we

decided to number these isoforms according to the position of the pA site in the *MCL1* 3'UTR. Thereby we named pA1, pA2, pA3 and pA4 the mRNA isoforms produced by the recognition of the first, second, third and fourth pA signals, respectively. However, the pA2 pA site described by the human EST analysis does not match the one currently reported in the UCSC genome browser (green boxes in **figure 10**). Instead, the pA2 pA site identified by human EST analysis corresponds to the pA3 region reported in UCSC genome browser that also comprises pA3 of the EST analysis. The pA2 reported in UCSC genome browser was not identified in the EST analysis. These differences in reported mRNA isoforms may be due to differential expression of *MCL1* APA-derived mRNA isoforms in different tissues. What we observed from the human EST analysis is that pA4 mRNA is the isoform with more ESTs deposited sequences (**Figure 11**).

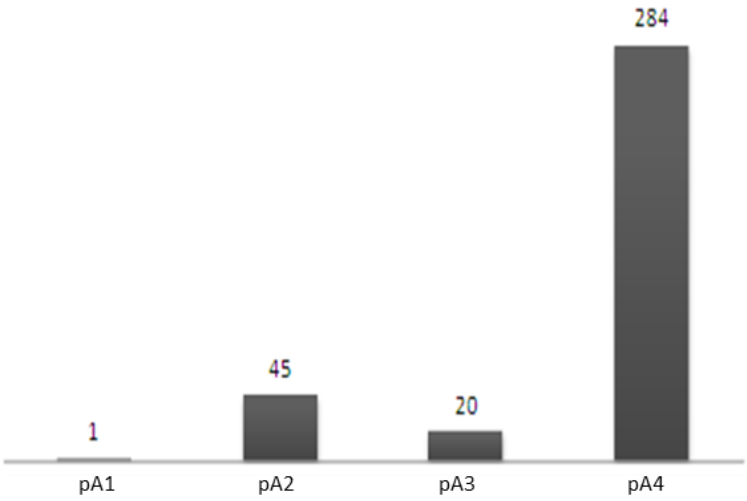


Figure 11 - Human MCL1 EST analysis. The pA4 is the mRNA isoform that has more deposited ESTs, followed by pA2, pA3 and pA1 isoforms.

To experimentally validate the results obtained with the *in silico* analysis, we performed 3'RACE and nested PCR (**figure 12A** and **12B**, respectively) using primary human T cells (in a resting state and activated with PHA), Jurkat E6.1 (human cell line derived from an acute T cell leukemia) and HeLa cells (human cervical cancer cell line). Besides human primary T cells, we included HeLa cells in our analyses because as a cell line is easier to work and good to perform optimizations and Jurkat E6.1 cell line as its a good T cell model widely used. We observed that the APA pattern is the same in all the cell types analyzed (**figure 12**). Although 3'RACE is not a quantitative method, in **figure 12A**, in primary T cells, there is an increase in the intensity of the band correspondent to the *MCL1* pA3 APA-derived isoform upon T cells activation.

Similarly to activated T cells (a proliferative condition), the intensity of the band correspondent to the pA3 mRNA isoform is also higher in Jurkat E6.1 and HeLa cell lines (oncological cell lines), in comparison to resting T cells (**figure 12B**). These evidences are in agreement to Sandberg *et al*.[18] and Mayr *et al*.[19] where they have shown an increase of transcripts with shorter 3'UTRs under proliferative conditions, such as activated T lymphocytes, and oncological conditions, respectively.

For further experiences we decided to investigate the four APA-derived mRNA isoforms: pA1, pA3 and pA4, identified both in the EST analysis and in 3'RACE, and the pA2 isoform, detected only by EST analysis. For that, we cloned the PCR products of the Jurkat E6.1 cell line produced by 3'RACE and obtained three of the four mRNA isoforms produced by APA represented in **figure 11** (pA1, pA3 and pA4). The construct containing pA2 was generated by PCR from pA3. All construts were sequenced in order to map their pA signals and cleavage sites. **Figure 13** summarizes the results obtained by the *in silico* analysis, 3'RACE and sequencing: all *MCL1* APA-derived mRNA isoforms are represented as well as the sequence of the *MCL1* 3'UTR, where all four pA signals are highlighted and the mRNA cleavage sites are indicated by an arrow.

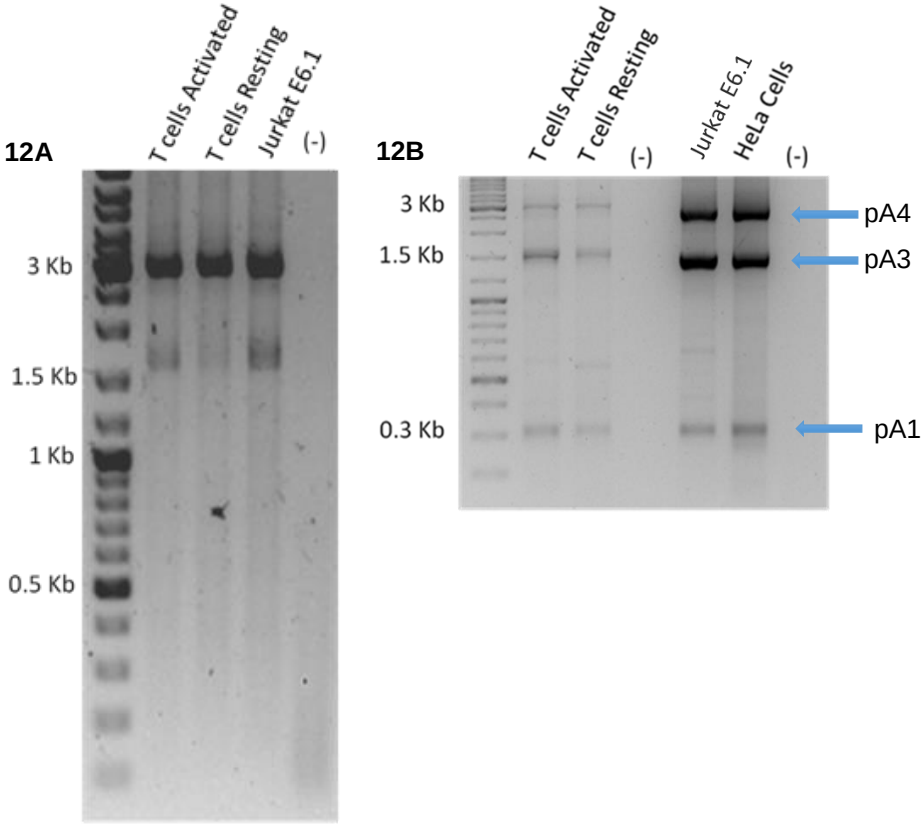


Figure 12 - mRNA isoforms of MCL1 produced by APA (blue arrows) by 3' RACE and Nested PCR. As observed in figure **12A** APA gives rise to a two mRNA isoforms with about 3Kb and 1,5 Kb in length. By Nested PCR (figure **12B**) it was possible to identify one more mRNA isoform (the shortest one) with about 0,3 Kb. (-) stands for no template negative control.

As described above the recognition of one pA signal over another is dependent of a variety of factors. However, one important factor to take into account in this mechanism is the strength of the pA signal. The most distal pA signals are usually described to be stronger, i.e., have the canonical AAUAAA pA signal, and thus tend to be more used[65]. Having this in consideration, our results from the EST analysis are in agreement with this concept, since pA4 has more ESTs deposited in comparison with the other pA signals. However, by sequencing the PCR bands of the 3'RACE we observed that all the four *MCL1* pA signals have the AAUAAA canonical hexamer (**figure 13**). The presence of four strong canonical pA signals in the same gene is not a common event[65]. This particularity may impact the regulation of *MCL1* APA-isoforms since it cannot be the strength of the pA signal by itself that dictates the choice of one pA signal over another. It is therefore possible that in this case there are more intervenients in the choice of the pA sites.

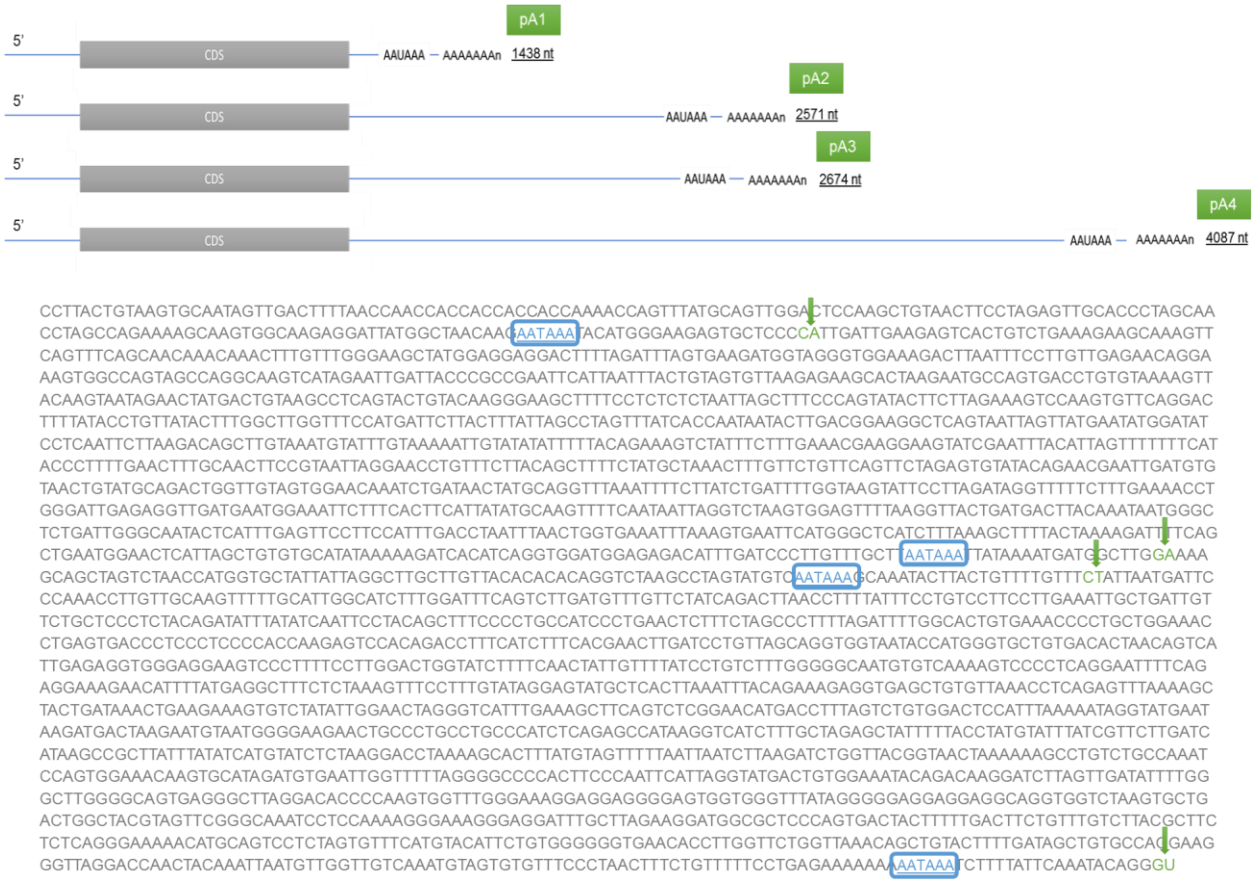


Figure 13 - Schematic illustration of MCL1 APA-derived mRNA isoforms, pA signals and cleavage sites used. The blue boxes highlight the four pA signals and the green arrows represent the sites where the mRNA cleavage takes place in order to produce each *MCL1* APA-derived mRNA isoform.

MCL1 pA signals are highly conserved in mammals

Given that the human *MCL1* has four canonical pA signals, we went on to verify if this is the case for other mammals, revealing their importance throughout evolution. By the alignment of the *MCL1* sequence in 10 representative mammalian species we observed a high degree of conservation of the *MCL1* 3'UTR with an overall pairwise identity of 65.5 % (**figure 14**). This degree of conservation on a non-coding region is striking, indicating a strong selective pressure throughout evolution to maintain sequences important for *MCL1* function and regulation in different species. In accordance, one of the most important *cis*-regulatory elements present in the 3'UTR, the pA signals, are also highly conserved in these mammals: 96% for pA1, 90% for pA2, 97% for pA3 and 100% for pA4 (**figure 14**). Curiously, all species analysed have four pA signals on its 3'UTR. The majority of the species display 4 canonical pA signals and the ones that do not display the canonical pA signal present at least three canonical pA signals and a non-canonical one (mouse and Tasmanian devil) or two canonical and two non-canonical pA signals (elephant). These results suggest that the *MCL1* 3'UTR is important for *MCL1* functioning and regulation due to its high degree of conservation in different species. Moreover, it seems that the human *MCL1* orthologues also possess four pA signals possibly undergoing alternative polyadenylation, indicating that this mechanism is conserved for *MCL1* in many mammalian species.

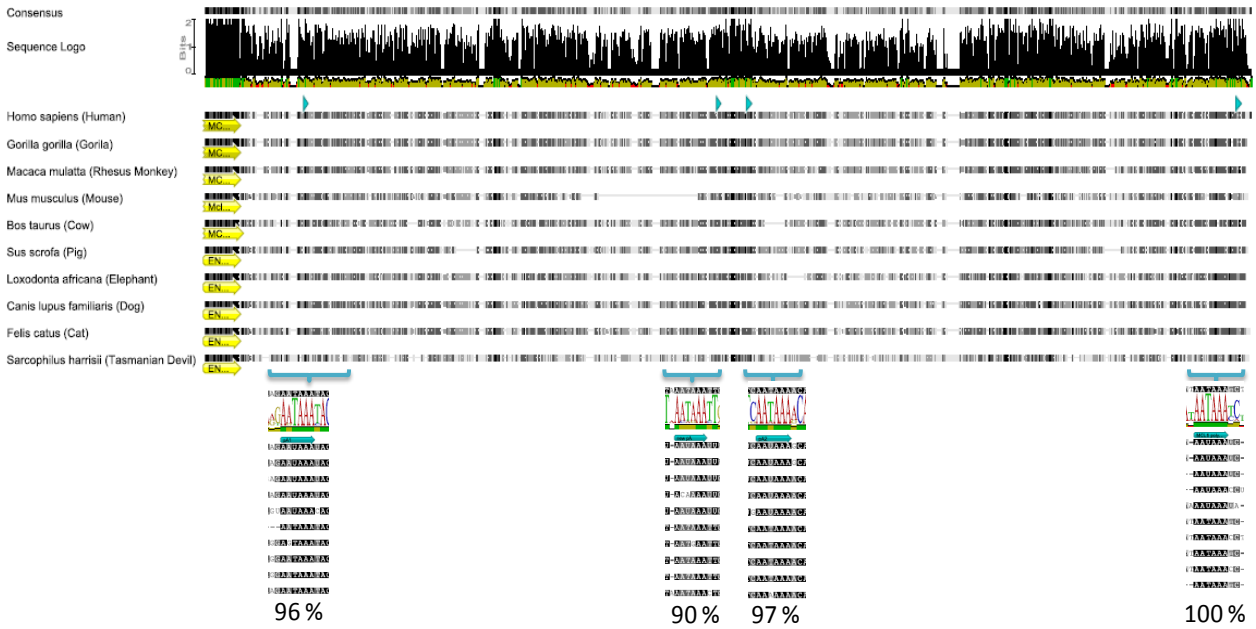


Figure 14 - The MCL1 3'UTR and pA signals are highly conserved in mammals. The multiple sequence alignment was made using Geneious v4.8 software. The pA signals are indicated by blue arrows above the alignment and a snapshot of the pA signal alignment, sequence logos and pairwise identities is represent bellow the overall alignment. All four pA signals are highly conserved among a variety of mammals, being pA4 the most conserved one (100%).

pA4 *MCL1* mRNA isoform is highly regulated at the post-transcriptional level

As it was described above, Sandberg *et al*.[18] described that in proliferative conditions, specifically upon T cell activation, there is an increase in the expression of mRNAs with shorter 3'UTRs. This could be due through the usage of proximal pA sites or by down-regulation of the longest isoforms. Also, by RNA-sequencing our collaborator Joel Neilson identified *MCL1* as one of the genes that undergoes 3'UTR shortening upon T cell activation, so we decided to investigate what is the process behind this mechanism. We started by quantifying the expression of *MCL1* APA-derived mRNA isoforms by RT-qPCR in primary T cells and PBMCs in resting and activated conditions (with PHA for 48 hours). We performed this experiment in PBMCs to check if this working model could be used instead of primary T cells, since PBMCs are cheaper to work and are very enriched in T lymphocytes.

In order to confirm that the cells were efficiently activated we first analysed the phenotype of both T cells and PBMCs and then performed flow cytometry in PBMCs. As it can be observed in **figure 15A,** T cells and PBMCs were activated using PHA for 48 hours since both produced large clusters of aggregated cells. We then quantified the activation in PBMCs using CD69 as a marker of activation and two time points: 24 hours and 48 hours. As observed in **figure 15B**, the time point of 48 hours was the one that demonstrated to activate PBMCs at a higher level (~72% of CD69+ cells). From this figure it is also clear that the activation of T cells has a great contribution for the overall activation (68.4% of CD3+CD69+ cells). Also by analyzing these results we verified that from PBMCs isolation about 77% of the cells are CD3+, and thus T cells (**figure 15 C**). For that reason we decided to activate PBMCs using PHA for 48 hours.

To measure the expression of *MCL1* APA-derived mRNA isoforms by RT-qPCR two different primer sets were used: one that measures the expression of all APA-derived isoforms (total RNA) that is localized in the coding region (named *coding*) and another primer pair that only measures the expression levels of the longest isoform (named *pA4*) (**figure 16**). Using these two primer pairs we could investigate how the *pA4 MCL1* mRNA isoform expression is regulated in resting and activated conditions.

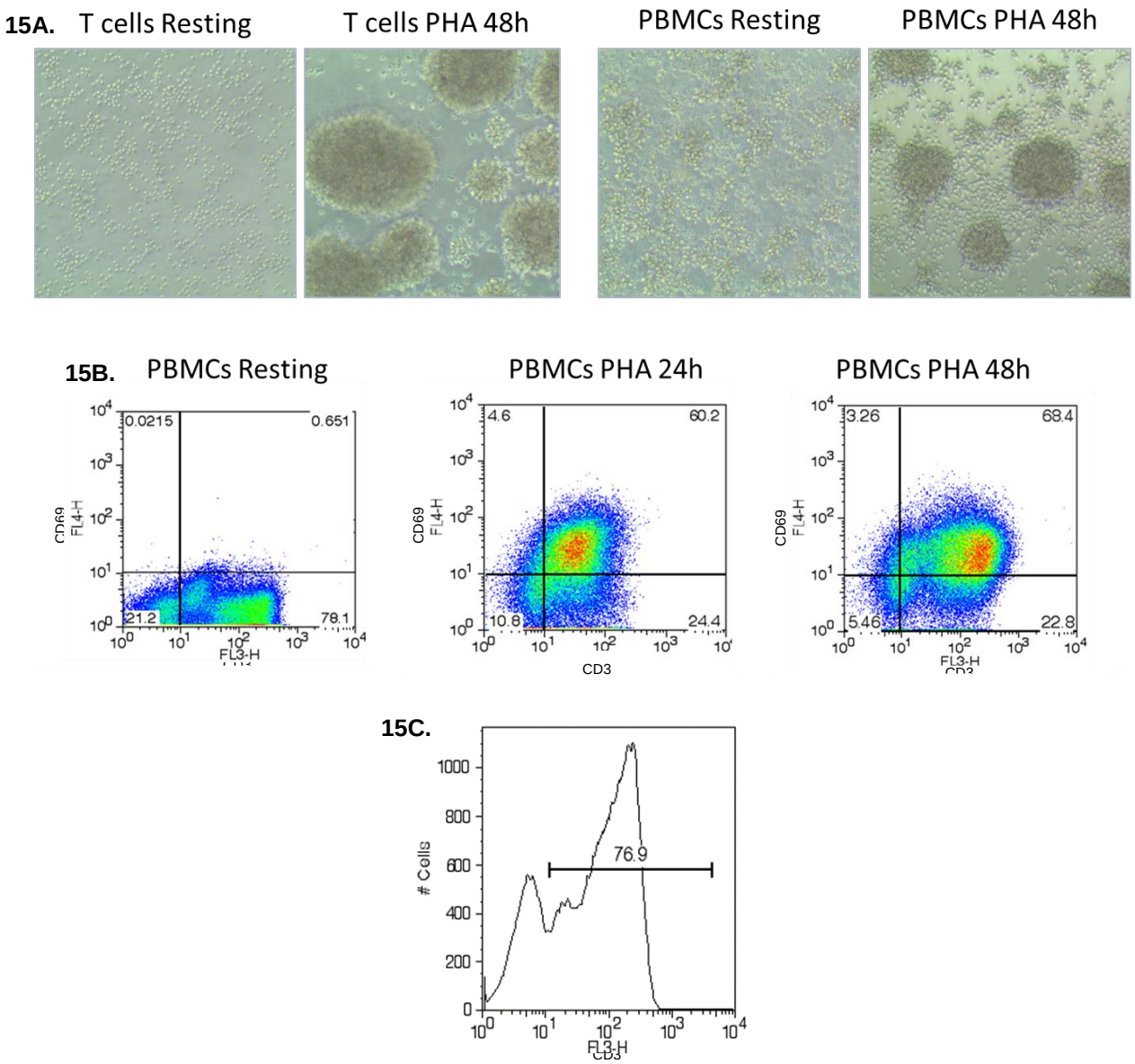


Figure 15 - Optimization of T cells and PBMCs activation using PHA. A) Cells phenotype in both resting and activated conditions. The formation of cell clusters upon T cells/PBMCs activation with PHA for 48h is observed. **B)** Representative flow cytometry analysis showing that PBMCs present an increase in activated cells (CD69+ cells) when PHA was used for 48 hours. **C)** PBMCs contain approximately 77% of CD3+ cells demonstrating that they are very enriched in T cells.

Figure 16 - Schematic illustration of the primer pairs used to measure the expression of MCL1 APA-derived mRNA isoforms. The primer pair named *coding* measures the expression of all *MCL1* isoforms since is located on exon three, which is present in all isoforms. The primer pair named *pA4* only measures the expression of the longest isoform being located immediately upstream of the pA4 pA signal.

Figure 17 shows the expression levels of *MCL1* in primary T cells and PBMCs, in both resting and activated conditions, using the *coding* and *pA4* primer pairs. We observe that although the expression of the measured isoforms in T cells being 10 times smaller than in PBMCs, the pattern of expression is the same in these two cell types, which allowed us to use PBMCs in the following studies. Comparing the expression levels between resting and activated states, it is evident a statistically significant increase in the mRNA expression levels of all *MCL1* isoforms upon T cells (**17A**) and PBMCs (**17B**) activation. On the contrary, in both cell types there are no differences in the expression of the *MCL1* longest mRNA isoform (pA4) between the two cell states. Given that the expression of the *pA4* isoform does not change but there is an increase in the expression of the *coding* region, this indicates that upon activation there is an increase in the shorter *MCL1* APA-derived mRNA isoforms, confirming the previous results obtained for *MCL1* by RNA-sequencing.

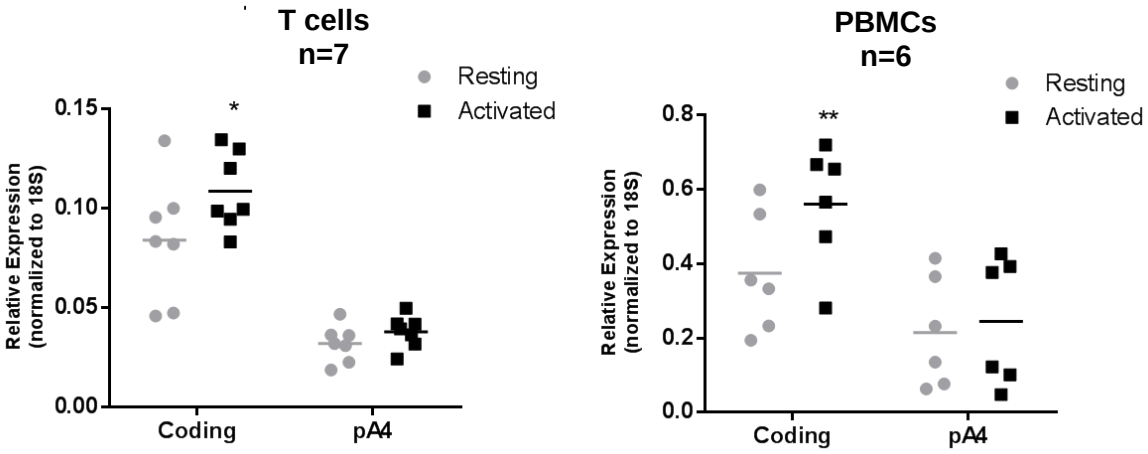


Figure 17 - MCL1 APA-derived mRNA isoforms relative expression. Relative expression of both *coding* and *pA4* isoforms in resting (grey) and activated conditions (black) in primary T cells (left) and PBMCs (right), quantified by RT-qPCR; n=7 or 6 donors, respectively. Asterisks indicate significant values (p-value < 0.05* and p-value < 0,01 **).

Given that this experience was performed using total RNA (that includes both nuclear and cytoplasmic fractions), it is not possible to discriminate if the pattern of expression observed in **figure 17** is due to APA, by more usage of proximal pA signals (mRNA regulation at the nuclear level), or to post-transcriptional regulation at the cytoplasmic level. To investigate this we performed a fractionation of nuclear and cytoplasmic RNA isolated from PBMCs.

As it can be observed in **figure 18A,** the relative expression of pA4 mRNA comprises half of the *coding* relative expression in the nuclear fraction, suggesting that pA4 is the isoform that contributes more for the total *MCL1* expression. This is in

agreement with the EST analyses performed above. Also, it is observed in the nuclear fraction a decrease in the expression measured by *coding* primer pair upon PBMCs activation. Besides not statistically significant, this decrease is also seen in *pA4* expression in the nucleus, and thus, once *pA4* seems to be the isoform more expressed, the decrease in *pA4* expression may have a major contribution in the overall decrease of *MCL1* mRNA under PBMCs activation. Also, the decrease verified in *pA4* expression upon PBMCs activation is higher than the decrease of the *coding* expression. This may indicate that upon cell activation the pA signals of the shorter isoforms are being recognized and more isoforms with shorter 3'UTRs are being produced.

In the cytoplasmic fraction of activated PBMCs a decrease in *pA4* relative expression is observed, suggesting that this isoform is regulated at the post-transcriptional level in the cytoplasm of activated cells. In contrast, although not statistical significant, it seems that the total *MCL1* expression (measured by *coding* primers pair) is higher in activated PBMCs than PBMCs in a resting state. As it has been described a role for *MCL1* in T cells activation[29], this could be a mechanism of to compensate the decreased levels of *MCL1* mRNA seen in nucleus upon PBMCs activation. In order to understand in which cellular state the *MCL1* APA-derived mRNA isoforms are more regulated in the cytoplasm, we measured the fold induction (**figure 18B**) of cytoplasmic fraction in relation to total fractions. From this result we can conclude that *MCL1* total mRNA (*coding*) levels are increased in the activated state in comparison to the resting state. In contrast, *pA4* mRNA levels seems to be more prone to suffer down-regulation upon PBMCs activation.

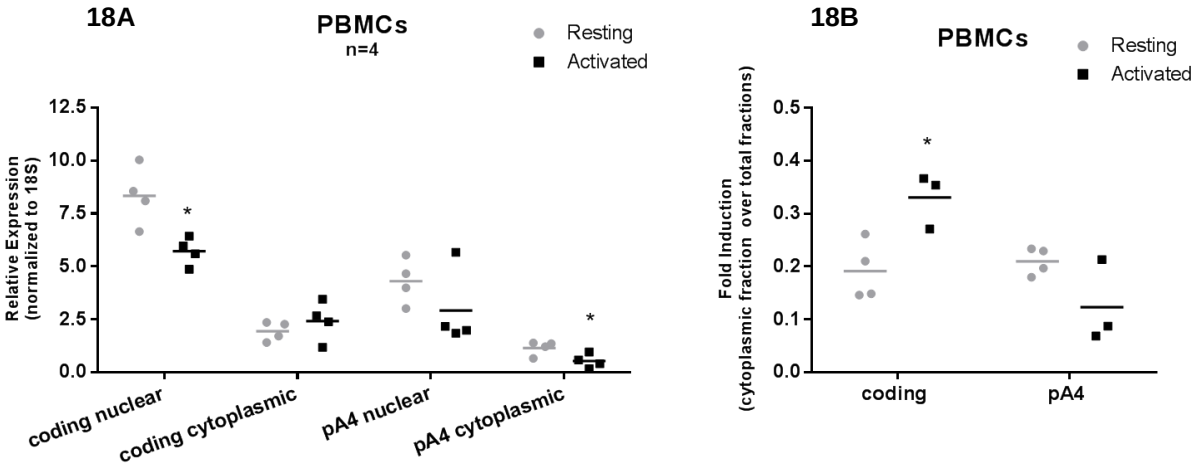


Figure 18 - Relative expression of fractionated MCL1 APA-derived mRNA isoforms. **A.**Relative expression of *MCL1* APA-derived mRNA isoforms measured in the nucleus and cytoplasmic fractions. The expression was measured both in resting (grey) and activated (black) states. **B.** Fold induction of cytoplasmic fraction over total fractions (nuclear and cytoplasmic). A decrease in the *coding* and an increase in *pA4* is observed upon PBMCs activation. Asterisks indicate significant values (p-value < 0.05*). For the fold induction (**figure 18B**) a donor from activated condition was eliminated due to its discrepancy.

Taken together the results shown in **figure 18** indicate that upon T cell activation there is a shortening of the 3'UTR of *MCL1* and that both APA at the nucleus, by the choice of proximal pA signals, and cytoplasmic post-transcriptional regulation mostly by the down-regulation of the *pA4* longest isoform, have a role in *MCL1* mRNA metabolism.

Mcl-1 protein levels increase upon PBMCs activation

To quantify Mcl-1 protein upon T cell activation, we performed western blot using protein extracts prepared from both Jurkat E6.1 and PBMCs (**figure 19A** and **19B**, respectively). As described in Dzhagalov et al.[29], we observe an increase in Mcl-1 protein levels upon T cell activation. In accordance to what Mayr *et al.*[19] described for oncogenes that isoforms with shorter 3'UTRs produce more protein due to its higher stability since these 3'UTRs have less binding-sites for negative regulators, a possible scenario is that the longest mRNA isoform of *MCL1* is down-regulated in activated T cells in order to increase the production of Mcl-1 protein by translation of mRNAs with shorter 3'UTRs.

This result together with the results obtained from RNA frationation assays may indicate that down-regulation of pA4 *MCL1* APA-derived mRNA occurs in order to increase Mcl-1 protein levels in T cells states dependent of Mcl-1 expression, such as the activated state[29].

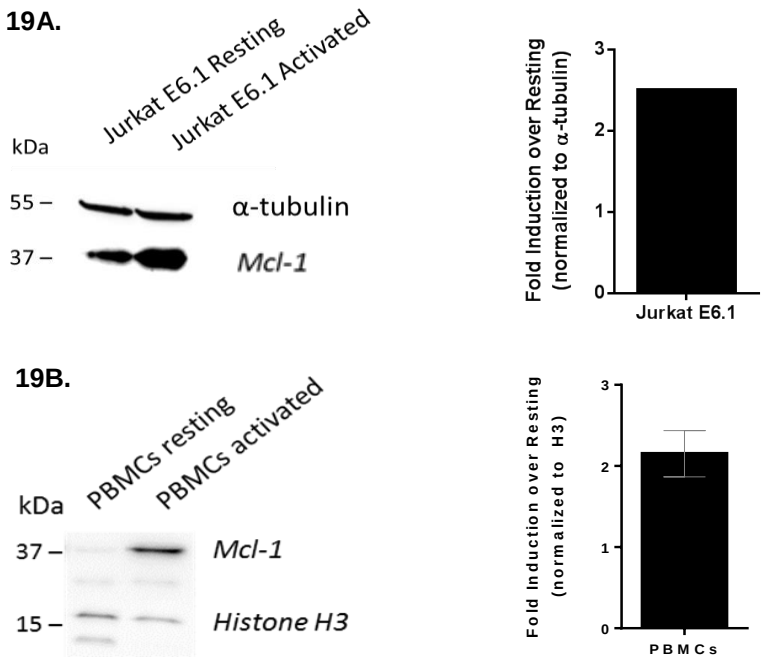


Figure 19 – Mcl-1 protein levels in both Jurkat E6.1 cell line and PBMCs in resting and activated states. **A**) An increase in Mcl-1 protein expression upon PBMCs and Jurkat E6.1 activation is observed by western blot. **B)** Quantification of the western blot bands showing a ~2-fold increase of Mcl-1 in activated over resting states in the two cell types analysed.

MCL1 APA-derived mRNA isoforms have a half-life of four hours

To determine *MCL1* APA-derived mRNA isoforms stabilities, which could explain the differences observed in the relative expression of these isoforms, we performed an Actinomycin-D assay using the Jurkat E6.1 cell line. Actinomycin-D inhibits RNA transcription by interfering with the action of RNA polymerase II and thus with the elongation process[66]. Therefore, this experiment is used to measure the stability of the mRNAs once the transcription was blocked. From this experiment (**figure 20**) we observed that there are no differences in the stability of the mRNAs as the half-life of all isoforms is approximately four hours. It has been described that Mcl-1 protein and *MCL1* mRNA have a very short half-life and that this depends on cell-type and cellular conditions[23]. Thus, we conclude that the stability of each isoform does not seem to have an impact in the relative expression of the *MCL1* APA-derived mRNA isoforms in Jurkat E6.1 cells.

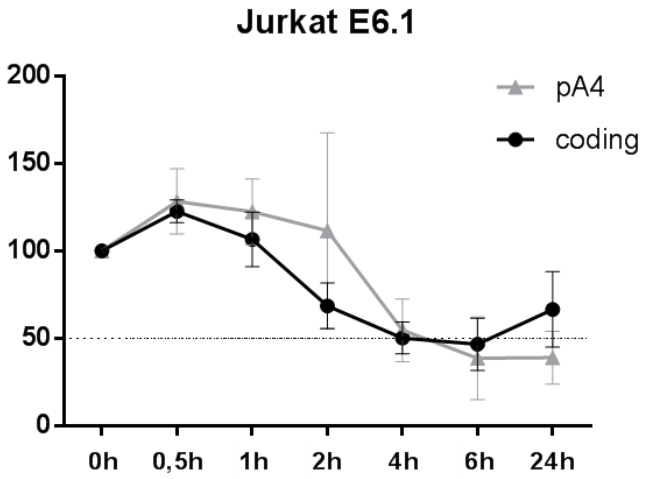


Figure 20 – MCL1 mRNA stability in Jurkat E6.1 cells after Actinomycin-D treatment. There are no differences in the stability of the different mRNA isoforms measured, being the half-life of all isoforms 4 hours. N=3 independent experiments.

pA3 *MCL1* mRNA is the isoform that produces higher Mcl-1 protein levels

In order to investigate the function of the different 3'UTRs of *MCL1* APA-derived mRNAs in protein production we cloned the 3'UTRs from each isoform downstream of a luciferase reporter gene. To assure that each isoform was correctly expressed and used the corresponding pA signal at the end of the 3'UTR, all the proximal pA signals were mutated. By Luciferase assay (**figure 21A**) we show that pA4 is in fact the isoform that gives rise to less amount of luciferase activity and that the shortest isoforms are the ones originating more luciferase protein, in particular the pA3 isoform. We also performed a ratio between protein and mRNA levels in order to infer about the

translation efficiency for each mRNA (**figure 21B**). From this result it seems that pA3 isoform seems to be the one more efficiently translated. In contrast, pA2 and pA4 isoforms are the ones less efficiently translated.

The decrease of luciferase protein production by pA4 could be also due to post-transcriptional regulation of this long isoform. This result is in accordance with the results of the RNA fractionation (**figure 18**) and the western blots (**figure 19**) providing evidence that pA4 mRNA is down-regulated at post-transcriptional level to produce more protein by the shortest isoforms, which is once again in accordance to the Mayr *et al.*[19] study.

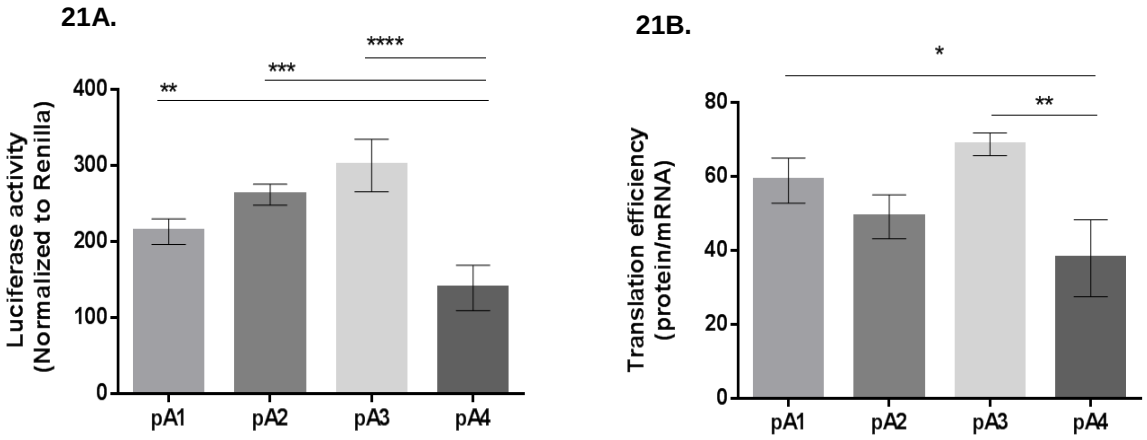


Figure 21 - Luciferase assay for MCL1 APA-derived mRNA isoforms. A) Luciferase activity produced by the use of the 3'UTRs of each mRNA isoform of *MCL1* generated by APA. Cells were transfected with luciferase reporter vectors containing the 3'UTRs of the four different APA-derived isoforms. In each construct the upstream pA signal was mutated. B) Translation efficiency measured by dividing protein levels by mRNA levels. Asterisks indicate significant values (p-value < 0.05*, p-value < 0.01**, p-value < 0.001***, p-value < 0.0001****). This procedure was performed in three independent experiments using duplicate replicas.

Characterization of post-transcriptional regulation of MCL1 APA-derived mRNAs by miRNAs

MCL1 has a variety of putative-binding sites for miRNAs on its 3'UTR

As previously observed in **figure 18,** we observed that pA4 *MCL1* mRNA isoform is down-regulated at the post-transcriptional level in the cytoplasm, given that its expression significatively decreases under PBMCs activation. As described above, miRNAs are good candidates to be regulators in this process and for that reason we decided to study this possibility. By *in silico* analysis, we searched for putative-binding sites for miRNAs that could target *MCL1*. As we obtained a large number of putative miRNAs-binding sites on *MCL1* 3'UTR we decided to restrict our analysis using the following criteria: a) study the miRNAs that were predicted to target *MCL1* in at least two of the databases used (*https://www.**targetscan**.org/; https://www.**miRNA.org**/miRNA/home.do; https://www.**mirtarbase**.mbc.nctu.edu.tw/*); b) study those that target a conserved sequence in mammals and c) choose the ones that were described to be expressed in T cells. According to these criteria, we selected three miRNAs to be studied: miRNA-17 that only targets the *MCL1* longest APA-derived isoform; miRNA-29b that binds pA3 and pA4 and that was previously described to regulate *MCL1* in other cell types*;* and miRNA-92a that can target all *MCL1* APA-derived isoforms. We decided to also study miRNA-320 that, although its target site was not very conserved among the mammalian species analysed, is described to be expressed in T cells and is predicted to target *MCL1* in two of the databases used. **Figure 22** represents the location and sequence of each miRNA putative-binding site on *MCL1* 3'UTR.

The conservation of the miRNAs binding sites on *MCL1* 3'UTR was verified by multiple sequence alignment of the 3'UTR of the ten mammalian species used for the *in silico* analysis shown in **figure 14**. From this analysis it is clear that, with the exception of the putative-binding site for miRNA-320, all the other putative-binding sites are 100% conserved (**figure 23**). This suggests that these sequences have been important for *MCL1* regulation throughout evolution and that the miRNAs that putatively bind these sequences could regulate *MCL1* in other mammalian species as well.

Later on, we used the miRNAMap database (*https://www.**mirnamap**.mbc.nctu.edu.tw/*) to search for the reported expression of the selected miRNAs. We search for the expression in thymus since this tissue has high amounts of T lymphocytes. From **figure 24** we can observe that all selected miRNAs are expressed in thymus. Also, it is clear that miRNA-92a is the one that is more expressed in thymus, followed by miRNA-17, miRNA-320 and miRNA-29b,

respectively. Thus, we decided to investigate the possible role of these miRNAs in *MCL1* regulation.

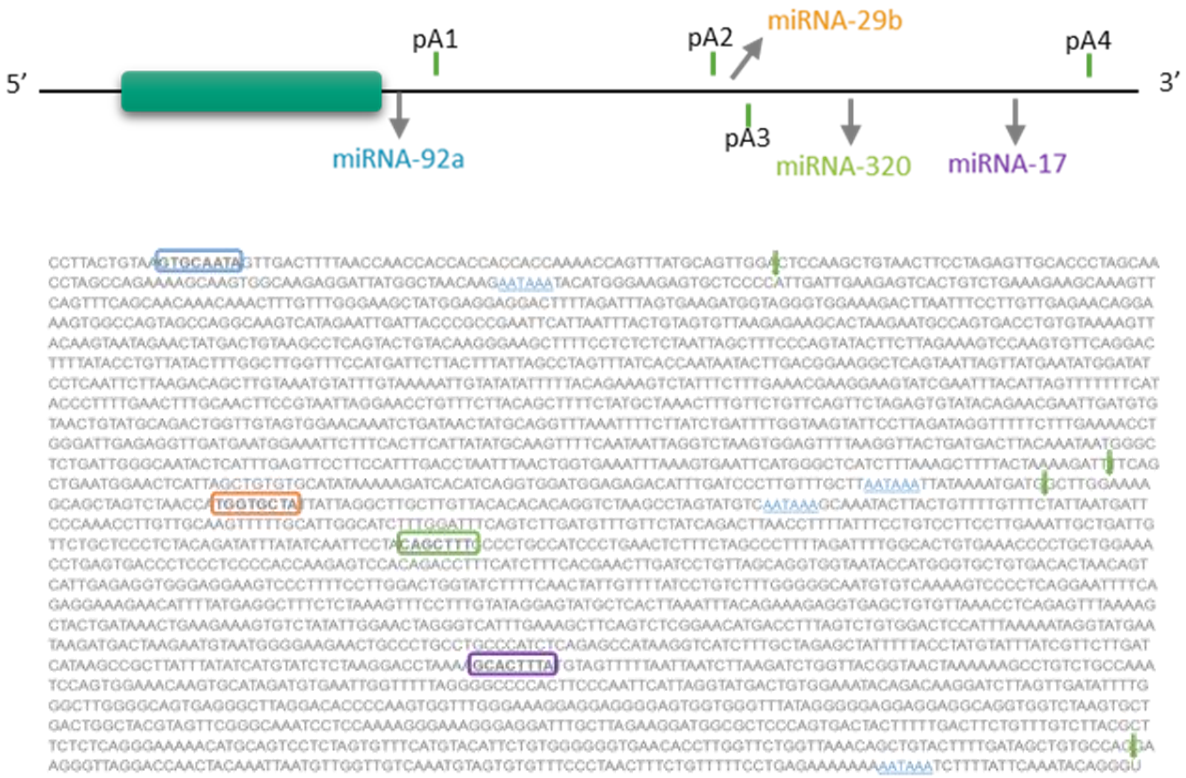


Figure 22 - miRNAs putative-binding sites on MCL1 3'UTR. 3' UTR sequence with the pA signals (AAUAAA sequence in blue) and the cleavage sites for each isoform (green arrows). The miRNAs putative-binding sites are represented by the outlined sequences: miRNA-92a in blue, miRNA-29b in orange, miRNA-320 in green and miRNA-17 in purple.

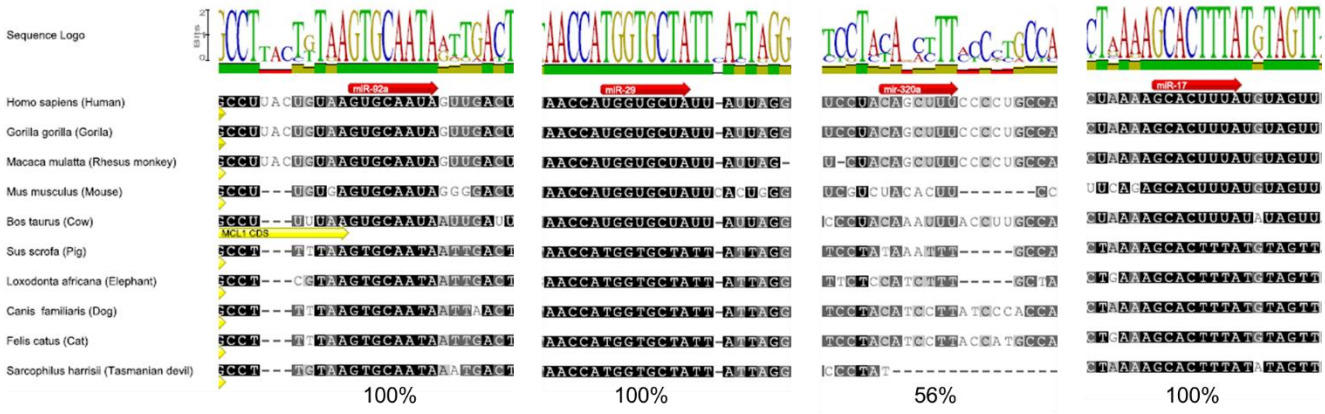


Figure 23 – Conservation of the miRNAs putative-binding sites on MCL1 3'UTR conservation. Red arrows represent the putative-binding sites of each miRNA. The putative-binding sites for miRNA-92a, miRNA-29b and miRNA-17 are 100% conserved among a variety of mammalian species. The putative-binding site for miRNA-320 presents a 56% of conservation among the studied species.

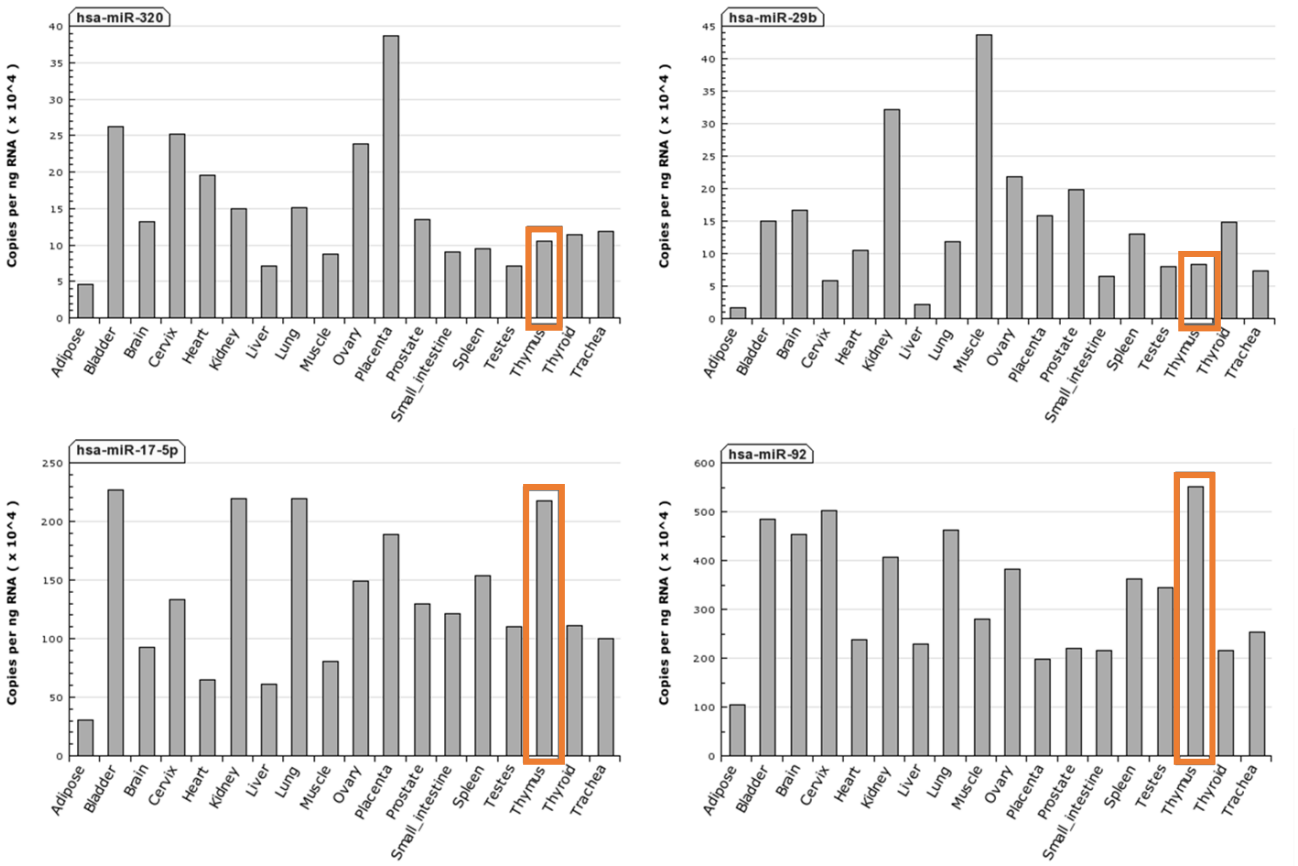


Figure 24 - miRNAs expression in miRNAMap database. The highlighted bar demonstrates the expression of each miRNA in the thymus, the tissue where T lymphocytes mature. From these graphs we observed that miRNA-92a is the one more expressed, followed by miRNA-17, miRNA-320 and miRNA-29b.

miRNA-17 and miRNA-320 expression increase upon T cell activation

To experimentally determine the expression levels of the four selected miRNAs in our cell model, we measured their expression by RT-qPCR using TaqMan probes. Since we observed that *MCL1* mRNA isoforms are differential regulated between resting and activated conditions (**figure 18**), we decided to measure miRNAs expression in these two cell conditions in human primary T cells. Moreover, we also performed this experiment in Jurkat E6.1 and HeLa cells given that they are suitable testing models. As Jurkat E6.1 is a cancer cell line, the proliferation rate and the miRNAs expression are themselves already altered, therefore in these cells, we did not quantify miRNAs expression in resting and activated conditions.

As it can be seen in **figure 25**, all chosen miRNAs are expressed in T cells. However they are differential expressed: miRNA-92a is the one more expressed, followed by miRNA-320, miRNA-17 and miRNA-29b. These results are very similar to those obtained with the *in silico* analysis. In **figure 25**, it is also clear that both miRNA-92a and miRNA-29b, are not differentially expressed between resting and activated

conditions. On the contrary, miRNA-17 and miRNA-320, that presumably target the longest *MCL1* mRNA (pA4), are more expressed upon T cell activation. In accordance with the fractionation experience, where a decrease of pA4 expression is seen in activated conditions in the cytoplasmic fraction (**figure18A**), the increase of these two miRNAs upon PBMCs activation could be an indirect evidence that miRNA-17 and/or miRNA-320 may be involved in pA4 isoform down-regulation.

The high expression levels of miRNA-92a and the increase in the expression of miRNA-17 during T cell activation are in accordance with previous studies. It has been previously shown that the cluster miRNA-17-92 is strongly induced after T cell activation being an important regulator of the rapid proliferation of these cells and also important in a variety of immune diseases[67 68].

In **figure 25** it is also represented the expression of miRNA-92a, miRNA-29b and miRNA-17 in Jurkat E6.1 and HeLa cell lines. We verified that both miRNA-92a and miRNA-17 are highly expressed, which is in accordance with studies that described these miRNAs as being frequently amplified in some cancers types, since they belong to the miRNA-17-92 cluster described to be an oncomir, i.e., a miRNA associated with oncological conditions[67, 68]. From these results it can be concluded that the expression pattern of miRNA- 92a, miRNA-29b and miRNA-17 is the same in both cell lines.

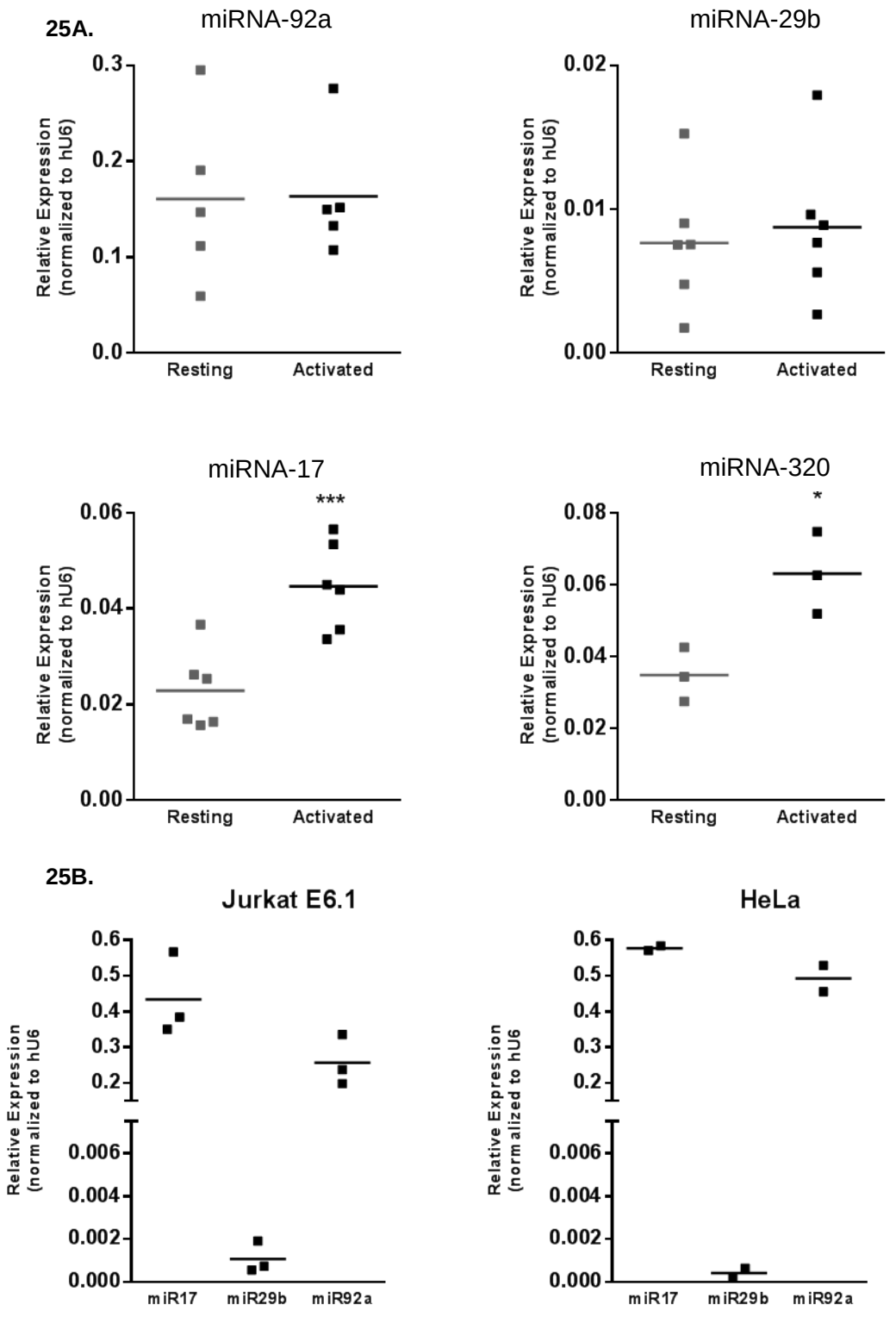


Figure 25 - Relative expression of the selected miRNAs. **A**) The miRNAs that potentially target the longest *MCL1* mRNA isoform (pA4), miRNA-17 and miR-320a, are the ones that increase their expression upon T cell activation. **B**) Expression of each miRNA in the two cell lines used in this study. It is clear that the expression pattern of these miRNAs is the same between Jurkat E6.1 and HeLa cells, two cancer cell lines. Asterisks indicate p value<0,05 * and p value<0,001 ***.

miRNA-17 and miRNA-29b have a role in regulating *MCL1* 3'UTR

To investigate the function of the chosen miRNAs in *MCL1* regulation we cloned a fragment of *MCL1* 3'UTR containing the putative-binding sites of each miRNA (wild type) and the putative-binding sites of each miRNA mutated, using the luciferase reporter gene. After transfection of HeLa cells we compared their effect by luciferase assays. In **figure 26** it is seen a statistical significant increase in luciferase activity when putative-binding sites of miRNA-17 and miRNA-29b are mutated. This indicates that they both target *MCL1* mRNA at those specific sites (described in **figure 22)** and regulate *MCL1* 3'UTR once when the putative-binding sites of these two miRNAs were mutated an increase in luciferase activity was seen compared with the luciferase activity produced by the wild type construct. The increase of luciferase activity seen when the putative-binding site for miRNA-29b was mutated is in accordance with previous studies that have demonstrated a role for miRNA-29b in regulating *MCL1*, as previously described in the introduction[42].

On the contrary, when the putative-binding sites of miRNA-320 and miRNA-92a were mutated no differences were observed in luciferase activity comparing with the wild type. From this result we can infer that miRNA-320 and miRNA-92a do not have a role in regulating *MCL1* 3'UTR or another possibly is that its putative-binding site is not the one described above. In fact, MiRTarBase database (*https://www.**mirtarbase**.mbc.nctu.edu.tw*) described another putative-binding site for miRNA-320 in the beginning of the *MCL1* 3'UTR.

In the future we will focus on miRNA-17 as a good candidate to down-regulate the longest isoform to support the results observed in the RNA fractionation.

The luciferase assays were also performed in the Jurkat E6.1 cell line (data not shown). Although being only preliminary results (n=1), we observed the same pattern of luciferase activity depicted on figure 26 for HeLa cells.

One interesting finding from these experiments was that the overall luciferase activity values produced by the fragment of *MCL1* 3'UTR where miRNA-320 was supposed to bind was always very diminished (about five-fold decrease) when comparing with the other constructs. This may indicate that other *cis*-regulatory elements may be present in this region of *MCL1* 3'UTR and are somehow down-regulating it. For that reason we searched for putative-binding sites for miRNAs and/or RBPs in that sequence (**figure 27**). We have identified four sequences where three RBPs can bind (*https://www.**rbpdb**.ccbr.utoronto.ca/*): RBMX, MBNL1 and ELAVL1; and one sequence where two miRNAs can bind: miRNA-16 and miRNA-15a (*https://www.**mirtarbase**.mbc.nctu.edu.tw/*). RBMX is a RBP known to be implicated in tissue-specific regulation of gene transcription and alternative splicing of several pre-

mRNAs[69]. Also, MBNL-1 was described to regulate alternative splicing events and mRNA decay[70, 71]. ELAVL1 has been described to regulate APA in the brain and also to stabilize the mRNA with longer 3'UTRs[72]. Thus, this RBP does not seem to be the one that is exercising the down-regulation of *MCL1* in this sequence in HeLa cells. We then searched for the predicted expression of miRNA-16 and miRNA-15a. From the graphics depicted on **figure 27** it is observed that miRNA-16 is highly expressed in the thymus. Although miRNA-15a is expressed, its expression levels are lower than those presented by miRNA-16. Also, some studies have demonstrated the role of these two miRNAs in down-regulating *MCL1*[73, 74]. For that reason these two post-transcriptional regulators may be the ones more responsible for the decrease in luciferase activity produced by this specific sequence of *MCL1* 3'UTR. In this way it seems that all of these putative regulators, except ELAVL1, could be involved in the regulation of *MCL1* 3'UTR through binding to this sequence and therefore explaining the lower values in the luciferase assays.

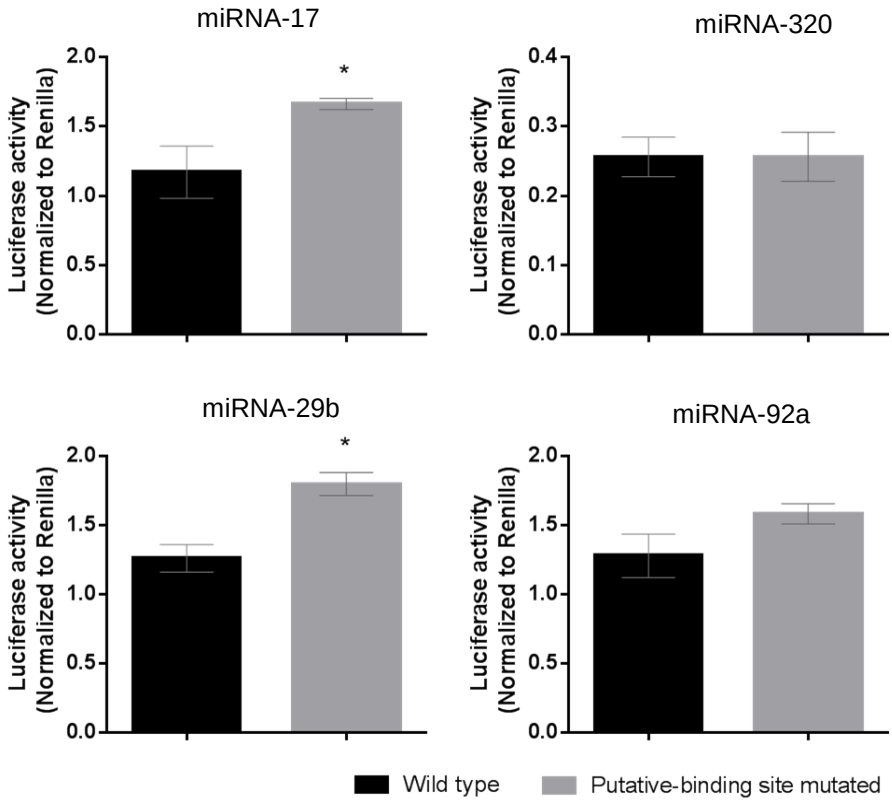
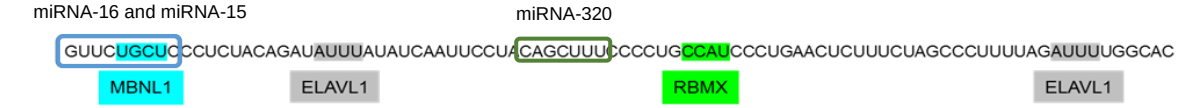


Figure 26 - Role of the four selected miRNAs on MCL1 3'UTR regulation. Luciferase activity produced by the wild type sequence of *MCL1* 3'UTR (black) and the Luciferase activity produced by the miRNA target sequence mutated (grey). Mutations in miRNA-17 and miRNA-29b target sites increases luciferase activity. Asterisk indicate p value < 0,05*. N=3 independent experiments.

27A.



27B.

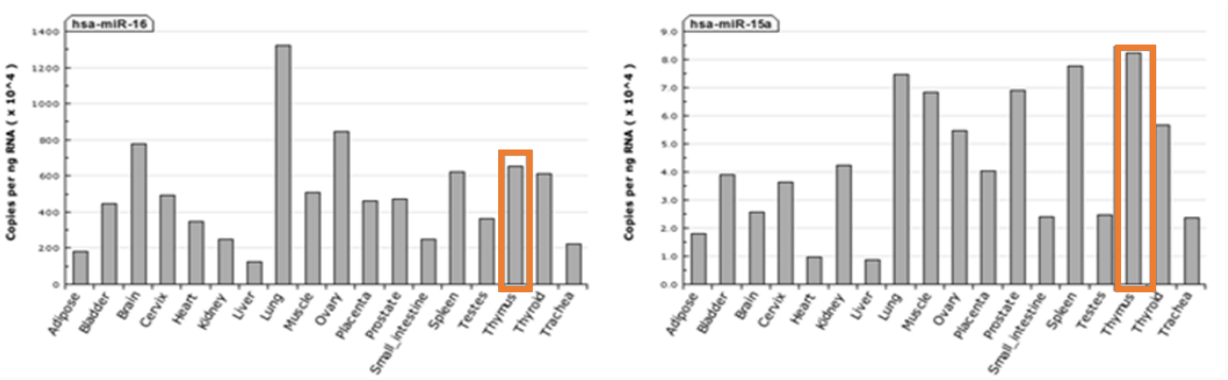


Figure 27 - In silico analysis to identify RBPs and miRNAs in the MCL1 3'UTR sequence used to test the role of miRNA-320. **A.** Three RBPs are described to have putative-binding sites in this sequence, indicated by coloured boxes. Also, two miRNAs (miRNA-16 and miRNA-15) were predicted to target this sequence, blue line. **B**. Predicted expression of miRNA-16 and miRNA-15 in thymus (highlighted bars).

miRNA-17, miRNA-29b and miRNA-92a down-regulate Mcl-1 protein

We then performed the overexpression of miRNA-17, miRNA-92a and miRNA-29b in HeLa cells to study the effect of these miRNAs on endogenous Mcl-1 protein expression. We were particularly interested in testing miRNA-17, because it targets the *MCL1* longest mRNA. However, we also tested miRNA-29b as a positive control in our experiment, because is well established that it down-regulates Mcl-1, and miRNA-92a to investigate its function in overall down-regulation of Mcl-1 expression. If this is the case, then miRNA-92a could be used in the future as a strategy to down-regulate Mcl-1 in conditions where this protein is overexpressed, such as in some cancers as previously described.

As it can been observed in **figure 28** it is clear that miRNA-29b down-regulates Mcl-1 expression as previously described[42]. Also, another finding from this experience is that miRNA-92a also down-regulates Mcl-1 in similar levels as miRNA-29b. From the western blot (**figure 28A and 28B**), Mcl-1 expression is decreased by approximately 30% in miRNA-17 and 60% in miRNA-92a and miRNA-29b overexpression conditions, in comparison to the control. However, when the overexpression of miRNA-17 was measured by RT-qPCR (**figure 29**) no differences were observed in relation to pcDNA3.1 (the backbone of the plasmids, used as a control). This experiment was repeated twice and the same result was obtained (data not shown). This could be due

to the fact that this miRNA is already highly expressed in HeLa cells (**figure 18**) so it is possible that miRNA-17 levels are autoregulating themselves.

From these results, we conclude that overexpression of miRNA-17, miRNA-29b and miRNA-92a down-regulate endogenous Mcl-1 protein expression. and thus all of them could be good candidates to regulate Mcl-1 expression. So, miRNA-17 could be used by the cell as a strategy to down-regulate the longest *MCL1* mRNA and promote the translation of the shortest isoforms that produce more protein, in situations when more Mcl-1 protein is required, such as during T cell activation. However, in **figure 28A** it is clear that miRNA-17 down-regulates endogenous Mcl-1 protein. A possible explanation for this is that HeLa cell is a tumor cell line and since Mcl-1 is already highly expressed in this cell line maybe the shortest isoforms are not being efficiently translated. Instead, in a situation where more Mcl-1 is needed, the down-regulation of the longest isoform by miRNA-17 overexpression could lead to an activation of the shortest isoforms translation, since in this case Mcl-1 is not highly expressed. To investigate this possibility, in the future, this experience has to be performed in PBMCs in resting and activated conditions.

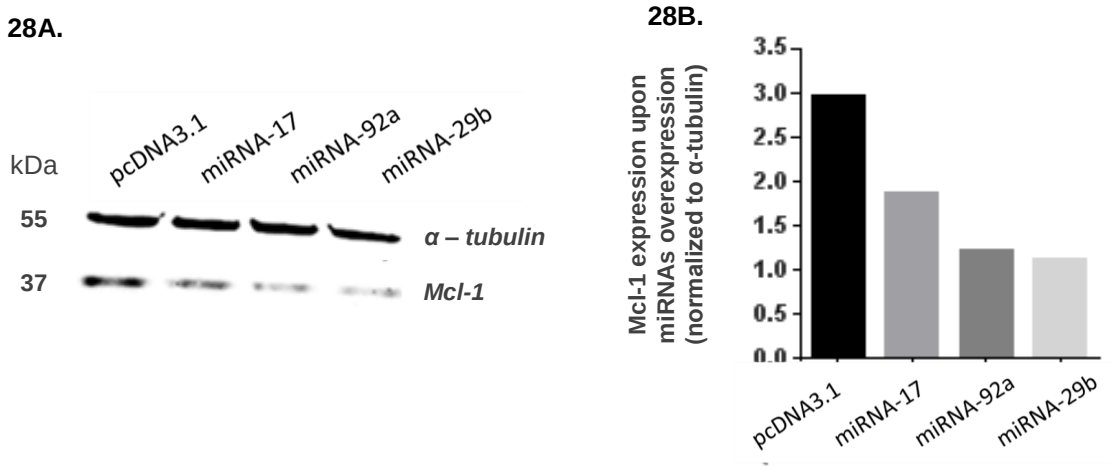


Figure 28 - Role of the selected miRNAs on endogenous Mcl-1 protein expression. **A)** Western blot showing that miRNA-17, miRNA-92a and miRNA-29b overexpression in HeLa cells lead to a decrease in the endogenous Mcl-1 protein expression. **B)** Quantification of Mcl-1 protein expression upon miRNAs overexpression by densitometry of the western blot bands demonstrating that miRNA-29b and miRNA-92a are the ones that lead to a higher downregulation of Mcl-1, followed by miRNA-17.

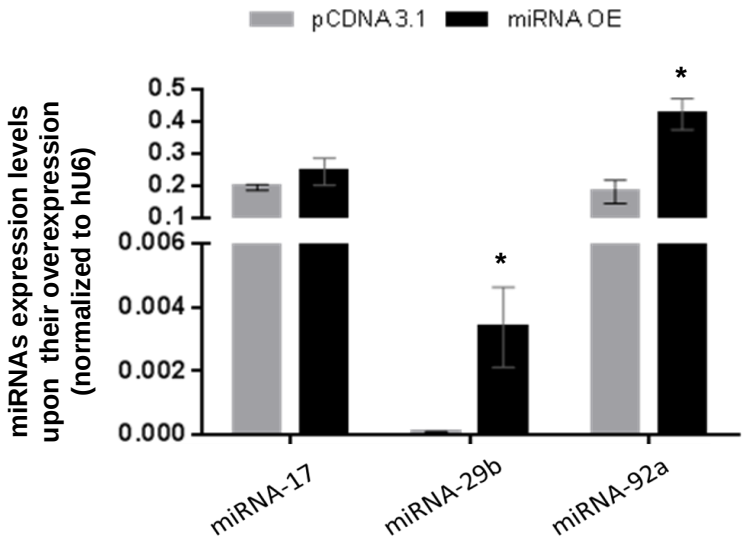


Figure 29 - miRNAs expression quantification after overexpression in HeLa cells. miRNAs expression was quantified by RT-qPCR after overexpression in HeLa cells. Black bars – miRNAs; grey bar – control. Asterisks indicate p value < 0,05*. N= 3 experiments.

CONCLUSION

Characterization of MCL1 alternative polyadenylation pattern in human T cells

From the results of this study we conclude that *MCL1* undergoes APA generating four mRNA isoforms with different 3'UTR lengths. From the RNA fractionation results it is observed a decrease in the expression of both *coding* and *pA4* mRNA in the nucleus, upon PBMCs activation. However, this decrease is more evident in the longest isoform (pA4) which is in agreement with our previous results and may indicate that proximal pA signals are preferentially used by APA, to produce shorter mRNAs, in this cell state. Another evidence from this result is that *MCL1* is regulated in the cytoplasm at a post-transcriptional level given that a decrease in the pA4 isoform upon PBMCs activation is observed. This result suggests that this mRNA is silenced in the cytoplasm by the action of miRNAs. Also, although not statistical significant, there is an increase in total mRNA levels in the cytoplasm upon PBMCs activation demonstrating that the shorter isoforms account for this increase by possibly escaping regulation in the cytoplasm. From these evidences we can conclude that there is an increase of the shorter 3'UTRs upon cell activation both due to a switch in the pA signal recognition from the distal to a proximal one, and to the post-transcriptional regulation of the longest isoform (pA4) by miRNAs.

Dzhagalov *et al*. [29] described that upon T cell activation more Mcl-1 protein is needed, and in fact we show that there is an increase in Mcl-1 protein upon activation of PBMCs and Jurkat cells. Also, Mayr *et al*.[19] have demonstrated that the shortest mRNA isoforms of oncogenes give rise to more protein. According to these facts, one possible explanation for the RT-qPCR results is that two mechanisms occur simultaneously in order to increase the Mcl-1 protein levels upon cell activation. First, the pA4 isoform is silenced in the cytoplasm by miRNAs upon PBMCs activation and second the shortest isoforms, the ones demonstrated to be more effyciently translated by luciferase assays, specially pA3, are less prone to regulation and thus more *coding* mRNA is measured upon cell activation.

Taken together our results indicate that *MCL1* mRNA levels produced by the longest isoform are regulated in the cytoplasm by miRNAs upon PBMCs activation. As it has been previously described that Mcl-1 is needed to promote activation of T cells, we propose that this is a mechanism used by the activated cells to promote the translation of the shortest mRNA isoforms, and consequently, produce higher amounts of Mcl-1 protein.

Characterization of the post-transcriptional regulation of MCL1 APA-derived mRNA isoforms by miRNAs

As it was clear that *MCL1* undergoes APA and that the four mRNA isoforms produced are differentially regulated under resting and activated cell conditions, we decided to identify the miRNAs that could be exerting a function in this control. All the four miRNAs chosen in this study are expressed in T cells and miRNA-17 and miRNA-320, the ones that target the longest mRNA isoform, increase their expression upon T cell activation. For that reason these two miRNAs were used as two potential key regulators of the *MCL1* longest mRNA upon T cell activation. From the selected miRNAs, miRNA-92a was the one more expressed in T cells both in resting and activated conditions. This result, together with the increase of miRNA-17 upon T cell activation is in accordance to what is already described for the miRNA-17-92a cluster that is known to be involved in proliferative conditions and highly expressed upon T cells activation[67, 68].

Using reporter assays, we identified miRNA-17 and miRNA-29b as targeting *MCL1* 3'UTR since when the putative-binding sites for those miRNAs where mutated an increase in luciferase activity was observed. On the other hand no statistical significant changes were seen when the same analysis was performed for miRNA-92a and miRNA-320. Since we have shown a decrease in Mcl-1 expression upon miRNA-92a overexpression (**figure 28**), it is possible it is targeting *MCL1* 3'UTR in another site, different from the one we mutated in our experiments. It is worth noting that there is a general decrease in luciferase activity when the fragment surrounding the miR-320 putative-binding site was used. This indicates that this fragment could have important *cis*-elements to which regulators of *MCL1* 3'UTR may bind. It will be interesting to further investigate this possibility in future works.

At the endogenous Mcl-1 protein level it is clear that miRNA-17, miRNA-29b and miRNA-92a overexpression induced protein downregulation. miRNA-29b was already described to down-regulate Mcl-1 expression, however, we showed that both miRNA-17 and miRNA-92a also down-regulate endogenous Mcl-1. As in some pathologies Mcl-1 is overexpressed, such as in cancers and since the miRNA-17-92a cluster is described to be important in T cells biology the use of these miRNAs could be a therapeutic strategy in some hematopoietic cancers in order to decrease cancer cell survival.

Overall, in this thesis we dissected some of the molecular mechanisms involved in *MCL1* regulation and illustrated the interplay between APA and miRNA silencing that regulates Mcl-1 expression in human T cells.

Working Model

According with the results presented in this thesis we proposed a working model for *MCL1* APA-derived isoforms regulation in T cells. In this model we propose that in the nucleus, in T cells resting, there is a preferential usage of the distal pA signal (pA4). In activated T cells there is a decrease in the recognition of the pA4 signal and thus the *MCL1* expression is predominantly due to the transcription of the shortest isoforms. In the cytoplasm, the longest isoforms are post-transcriptional down-regulated by miRNA-17 in activated T cells. Therefore the shortest isoforms, the ones more efficiently translated, are the ones more expressed in this cell condition leading to an increase of Mcl-1 protein expression upon T cell activation.

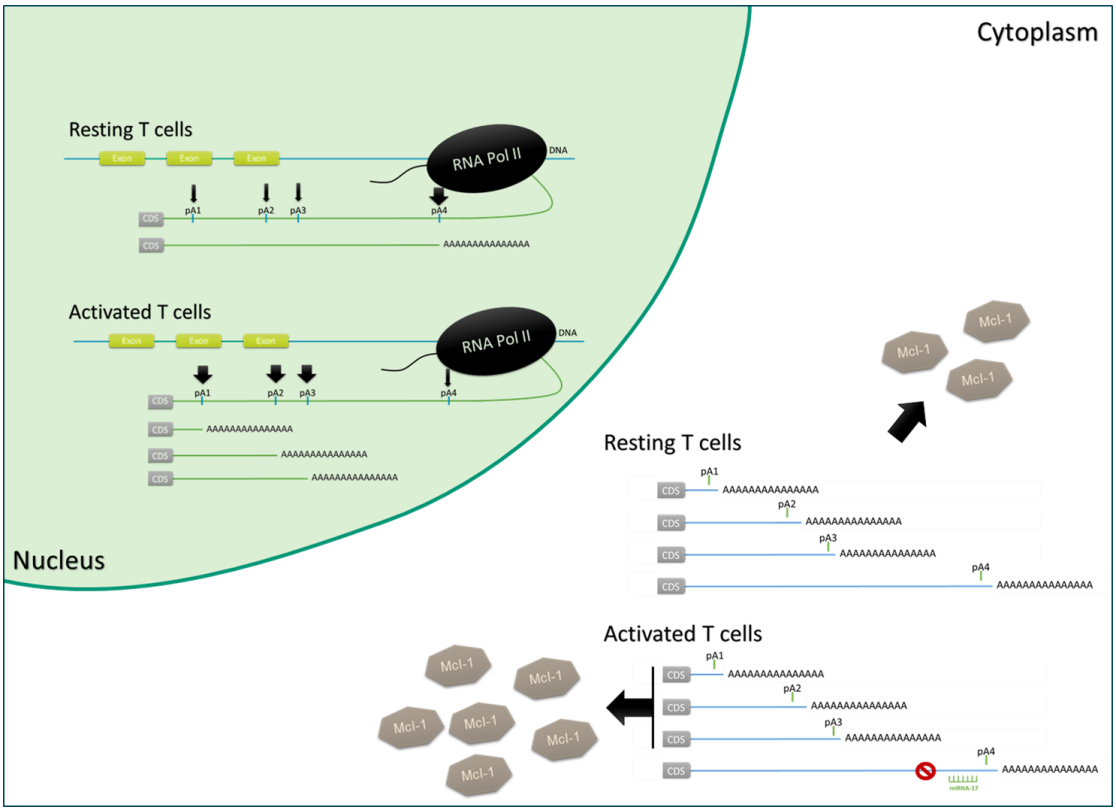


Figure 30 - Working model for MCL1 APA-derived isoforms regulation in T cells. Schematic representation of APA and post-transcriptional regulation role in MCL1 and Mcl-1 expression in T cells.

Future perspectives

In future work it will be necessary to repeat the luciferases assays in resting and activated PBMCs, to characterize both the protein levels that each *MCL1* APA-derived mRNA isoforms produces and also to study the miRNAs, in a more physiological context. The overexpression of the selected miRNAs should be also performed in PBMCs in both resting and activated states in order to understand if, in fact, the down-regulation of the longest isoform could lead to an increase in Mcl-1 protein levels. Simultaneously, it will be important to quantify *MCL1* APA-derived mRNA isoforms by RT-qPCR in the cells where the overexpression of miRNA-17 was performed. This would further confirm if miRNA-17 down-regulates the mRNA levels of *MCL1* longest isoform. Also, it would be important to perform luciferase assays with overexpression of miRNA-17 and inhibit miRNA-17. With these experiments we could further validate our idea that miRNA-17 is involved in Mcl-1 biology.

It will be important to understand how *MCL1* APA impacts on T cell biology, by investigating its effect in proliferation, cellular viability and apoptosis, and also to disclose what happens to these cellular processes under miRNA-17 overexpression and inhibiton.

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