El oncogen MYC como activador transcripcional del receptor del virus de Epstein-Barr (CR2/CD21)

The *MYC* oncogen as a transcriptional activator of the Epstein-Barr virus receptor (CR2/CD21)

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2019

UNIVERSIDAD DE CANTABRIA

FACULTAD DE MEDICINA

INSTITUTO DE BIOLOGÍA MOLECULAR Y BIOMEDICINA

(IBBTEC)

El oncogen MYC como activador transcripcional del receptor del virus de Epstein-Barr (CR2/CD21)

Programa de Doctorado en Biología molecular y Biomedicina

Tesis Doctoral

Ester Molina Hoyo

Por la presente informo que ESTER MOLINA HOYO ha completado su tesis doctoral, titulada “El oncogen MYC como activador transcripcional del receptor del virus de Epstein-Barr (CR2/CD21)”.

Este trabajo identifica un nuevo y relevante gen diana del oncogén MYC, CR2/CD21, que es el receptor del virus de Epstein-Barr. Considero que el trabajo de Ester aporta información relevante en cuanto a la patogénesis del linfoma de Burkitt y otras neoplasias relacionadas con este virus. Estos resultados son originales y obtenidos con la metodología apropiada.

Considero que esta Tesis está finalizada y lista para ser depositada y defendida, dentro del programa de doctorado de Biología Molecular y Biomedicina de la Universidad de Cantabria

Santander, 13 de junio de 2019

Firmado: Javier León Serrano

Catedrático de Bioquímica y Biología Molecular de la Universidad de Cantabria

Esta Tesis ha sido realizada en el Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) perteneciente a la Universidad de Cantabria (UC) y al Consejo Superior de Investigaciones Científicas (CSIC).

La financiación para la realización de esta Tesis doctoral ha sido proporcionada por el Ministerio de Educación y Ciencia (SAF2014-53526-R) (MINECO/FEDER,UE) (SAF2017-88026-R) (AEI/FEDER,UE).

He disfrutado de varios contratos de investigación financiados por los proyectos anteriormente mencionados, la Universidad de Cantabria y SODERCAN.

AGRADECIMIENTOS

A mi modo de ver, cualquier trabajo de esta envergadura no es posible realizarlo sin el apoyo de mucha gente. Ya sea a nivel técnico, intelectual o emocional, requerimos de continuos estímulos para seguir adelante y sacar lo mejor de nosotros. Por ese motivo y porque a mí me enseñaron que es de "bien nacido ser agradecido", esta tesis está dedicada a mucha más gente de la que siquiera puedo nombrar. Por tanto, haré mención de las personas más relevantes que han intervenido en este trabajo, dejando probablemente en el tintero, otras muchas que de forma indirecta también tienen su parte en él.

Por supuesto, el primero es Javier, que me dio la oportunidad de volver al mundo científico. Nunca he querido nada más que poder vivir de hacer lo que más me gusta. Y aunque a mucha gente le desconcierte, la investigación me hace feliz, así que eternamente agradecida por esta oportunidad. Ha sido un placer y un privilegio aprender estos años contigo. Por corto o largo que sea mi camino en la ciencia dudo muchísimo que vuelva a tener un jefe mejor que tú. Al final has conseguido hacer de mí una científica bastante decente, a pesar de tu úlcera.

A Dolo, que siempre está pendiente de todas nuestras necesidades tanto científicas como personales. Es muy fácil trabajar cuando sabes que tienes a quien recurrir, que va ayudarte y enseñarte dónde está el camino si te desvías.

A los miembros de mi laboratorio, presentes y pasados. A María, Rosa y Lorena. A Mari Car que tuvo una paciencia infinita conmigo y me enseñó lo poco que sé de microscopia y sobretodo los ChIPs. A Alex, que estuvo muy poco tiempo, pero nos marcó con su entusiasmo y sus ganas de aprender.

Muy especialmente quiero agradecer a Lucía y a Judit. Lucía ha estado en todos los tropiezos, cuando nada sale. Ella hace que este trabajo parezca fácil, viendo con claridad cristalina lo que, para mí, la mayor parte de las veces, es opaco. Y a Judit que tiene una paciencia infinita y una capacidad de trabajo y tesón, realmente admirables. Ella me ha aguantado estos cuatro años, ayudándome en lo grande y lo pequeño. Este trabajo es en gran parte también suyo.

Trabajamos en un centro pequeño, pero bien avenido. Es un placer trabajar en un sitio donde cuando necesitas una ayuda, un aparato o un reactivo, siempre va a haber alguien que te lo proporcione. Quiero agradecer a todos los miembros del grupo de Piero Crespo, especialmente a Lorena que incluso me ha proporcionado su poyata para hacer experimentos. Estos años han estado siempre disponibles para cualquier duda o necesidad.

Agradecer también a todos los miembros del grupo de Nacho Varela que han estado siempre disponibles. No hay mayor ayuda que ofrecer respuestas a nuestras preguntas y eso es lo que nos habéis proporcionado con los Lab Meetings. Además, debo agradecer especialmente a Laura González por introducirme en el mundo de la citometría. Muchas gracias por tu paciencia.

Una mención para la gente del café, que perteneciendo a otras ramas de la ciencia o incluso a otras disciplinas puedes conversar a diario y plantear dudas cuyas respuestas pueden venir, quizá, de quién menos esperas.

Estoy igualmente agradecida al personal técnico y de administración de este instituto porque sin ellos nuestro trabajo sería imposible de realizar. Especialmente quiero dar las gracias a Sandra, por su colaboración en la purificación de DNA y a Pepi por su ayuda en la realización de pedidos. Si el tiempo es oro, me habéis ahorrado millones.

Por supuesto no puedo olvidarme de mis compañeros de carrera que me introdujeron en la investigación, sobre todo a Paloma, Ana, Vero y Jose. Ellos lo empezaron todo. Y también a Susana que fue mi primera “jefa”, aunque a ella no le gustara.

Y finalmente a mi familia, sobre todo a mi madre, que siempre nos empujó a mis hermanos y a mí a que hiciéramos lo que más nos gustara.

Termino citando a mi jefe y diciendo…HAVE FUN!

AGRADECIMIENTOS A COLABORADORES

A lo largo de estos años hemos recibido ayuda y colaboración de varios grupos científicos que en mayor o menor medida han aportado conocimientos y medios para llevar a cabo este trabajo.

El grupo de Daniela Ulgiati en la Universidad de Western Australia. Nos proporcionó los vectores luciferasa que contenían las secuencias del promotor del CR2 para hacer los estudios funcionales llevados a cabo en esta tesis.

El grupo de Ramón y Jesús Merino del Departamento de Inmunología de la Universidad de Cantabria. Especialmente me gustaría agradecer a Pilar Álvarez y María Aramburu. Colaboramos en un proyecto para estudiar la interacción de MYC y CR2 en un modelo de ratón con inmunodeficiencia (Lupus eritematoso sistémico).

El grupo de Jose Ramón Regueiro de la Universidad Complutense de Madrid. Nos han proporcionado la línea celular productora del virus de Epstein-Barr (B95.8), así como los protocolos de producción del virus.

El grupo de Almudena Ramiro del CNIC y de Ignacio Moreno del CNB. Están probando la regulación de CR2 por MYC en linfocitos B de ratón con expresión de MYC condicional. Especialmente agradecer a Virginia Yébenes que ya ha obtenido los primeros resultados en ratones wild type.

El grupo de Nacho Varela del IBBTEC. Están trabajando en la construcción de un plásmido con expresión de MYC condicional para usarlo en modelos de ratón. Especialmente me gustaría agradecer a Laura Quevedo que está trabajando en esta construcción.

Muchas gracias a todos.

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Abbreviations

7-AAD	7- Aminoactinomycin D
BET	Bromodomain and extraterminal
BCR	B cell receptor
b-HLH-LZ	Basic-Helix-loop-Helix Leucine zipper
BL	Burkitt´s lymphoma
bp	Base pair
BRD	Bromodomain
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CFU	Colony forming units
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation sequencing
CKI	Cyclin-dependent kinase inhibitor
CML	Chronic myeloid leukaemia
CSR	Class switch recombination
Ct	Cycle threshold
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
eBL	Endemic Burkitt´s lymphoma
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetracetic acid
Eμ	Immunoglobulin intron enhancer μ
ER	Estrogen receptor
ET	Extraterminal
FBS	Fetal Bovine serum
GC	Germinal centre
HDACs	Histone deacetylase

HIV	Human immunodefiency virus
Ig	Immunoglobulin
Kb	Kilobase
KDa	Kilodalton
LiCl	Lithium chloride
LCLs	Lymphoblastoid cell lines
miRNA	Micro-RNA
MOI	Multiplicity of infection
mRNA	Messenger RNA
NP40	Nonidet-P40 or octyl phenoxylpolyethoxylethanol
o/n	overnight
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pol	Polymerase
qPCR	Quantitative PCR
R.L.U	Relative light units
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxygen especies
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
rRNA	Ribosomal RNA
RT	Room temperature
RT-qPCR	Reverse transcription and quantitative PCR
sBL	Sporadic Burkitt´s lymphoma
SCR	Short consensus repeats
SDS	Sodium dodecyl sulfate
SHM	Somatic hypermutations
shRNA	Short hairpin RNA
TAD	Transcriptional activator domain

Abbreviations

T-ALL	T-cell acute lymphoblastic leukaemia
TBS-T	Tris buffer saline-Tween 20
TE	Tris-EDTA buffer
tRNA	Transfer RNA
TSS	Transcription start site
Wt	Wild type

Introduction

1 INTRODUCTION

1.1 MYC

1.1.1 MYC gene and protein

In mammalian cells MYC protein arise from three distinct gene family members (*MYC*, *MYCN* and *MYCL1*) that function in a similar manner but display notable differences in potency and patterns of expression. *MYC* (also called *c-MYC)* is overexpressed in either hematopoietic or solid tumours, while *MYCN* (also called N-MYC) is most frequently overexpressed in solid cancers of neuronal origin, such as neuroblastoma and glioma (Strieder and Lutz, 2002). *MYCL1* (also called L-MYC) is most often overexpressed in small cell lung carcinomas (Nesbit et al., 1999). However, it is important to note that most of the work performed in *MYC* involved in tumorigenesis focused on *c-MYC* referred to herein as *MYC.*

In humans, *MYC* is located in chromosome 8q24. *MYC* is a relatively small gene comprised by three exons. Exon 1 is non-coding and exon 2 and 3 encoded MYC protein with translation initiation in nucleotide 16 of exon 2 (Boxer and Dang, 2001). *MYC* occupies almost all the band 24 and it is surrounded by 3 Mb of non-coding genome in

which is often called a “gene desert”. This is an exceptional feature of *MYC* gene, moreover for a gene only 5 Kb long (Cole, 2014).

MYC is regulated by complex mechanisms either transcriptional or post-transcriptionally. Until now, four transcriptional promoters have been identified, but RNA initiated at the P2 promoter **(Figure 1.1)** contributes to 80-90% of total *MYC* steady-state RNA in most cells (Taub et al., 1984).

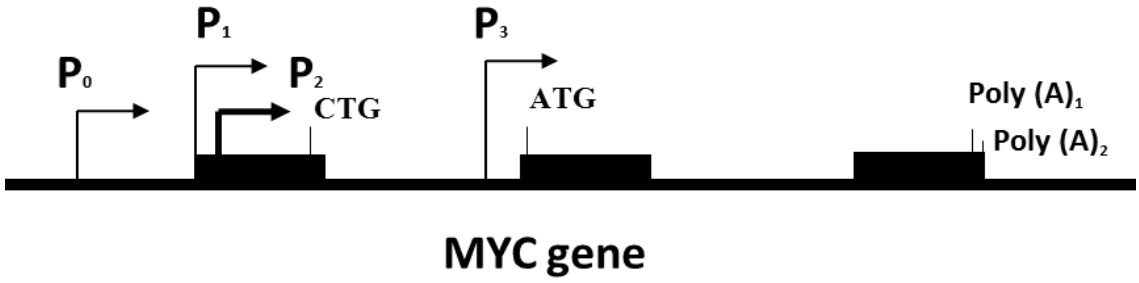


Figure 1.1. Schematic representation of different promoters along *MYC* gene. P_2 promoter contributes to the majority of MYC protein in steady state conditions. Adapted from Yang and Okamoto (2010).

The N-terminal region of MYC contains the transactivation domain or transcriptional activator domain (TAD) which is susceptible to be bound and activated by other proteins (Kato et al., 1990). The C-terminal domain is comprised by around 100 aminoacids that forms the basic-helix-loop-helix leucine zipper (b-HLH-LZ) region that function as a DNA binding domain (Jones, 2004) **(Figure 1.2)**. Transcription factors with this kind of structure have to dimerise, as homo or heterodimers, to exert its function. MYC binds to MAX protein (another b-HLH-LZ protein) through their leucine zipper domain forming an extended coiled coil between the two proteins and to DNA through their basic domain that insert in a scissor-like fashion in the major grove of the DNA (Blackwood and Eisenman, 1991). MYC archaic form appears in early metazoans and bind to E-Boxes (CAYGTG *consensus* motif) that are present in all target genes induced by MYC (Dang, 1999; Grandori et al., 2000; Nilsson and Cleveland, 2003).

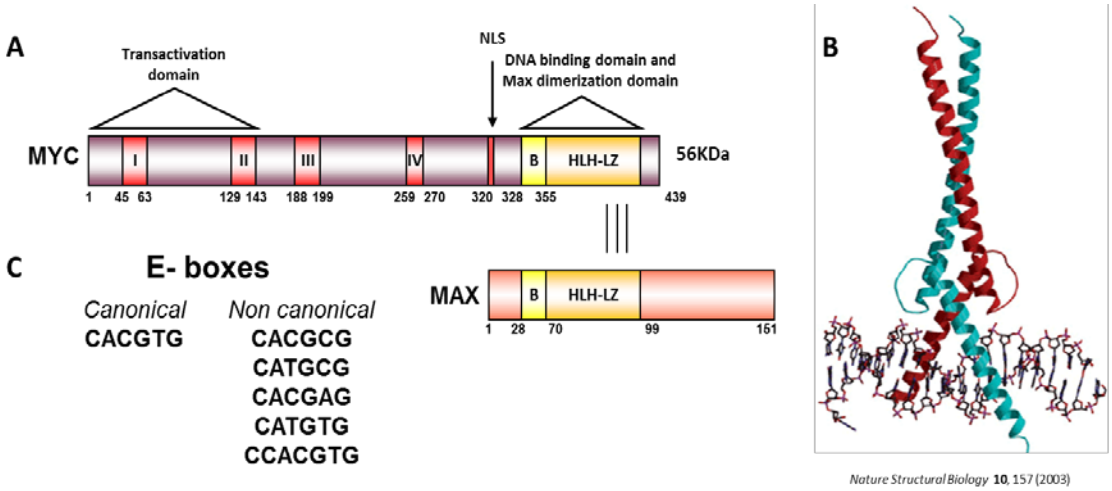


Figure 1.2. **A**. Schematic representation of the c-MYC protein. The N-terminal region contains the transactivation domain and the C-terminal region possess the DNA binding and the dimerisation domains. MAX protein dimerises with MYC through the leucine zipper domain. **B**. MYC-MAX heterodimer binds to DNA through their basic domain in the major groove of the DNA. **C.** MYC binds to DNA in consensus sequences called E-boxes that could be canonical or non-canonical.

In normal cells, expression of the endogenous *MYC* gene is upregulated upon diverse mitogenic and developmental signals (Schaub et al., 2018). Upon *MYC* activation, it could bind directly to its target genes that are estimated in two to four thousand genes along human genome (Sabo et al., 2014), which comprehend approximately 15% of all human genes. MYC direct target genes are involved in almost all functions within the cell including metabolism, cell cycle progression, immune surveillance, proliferation and differentiation (Chen et al., 2018).

ChIP-seq analyses of MYC occupancy *in vitro* define approximately 20,000 binding sites for endogenous MYC while after *MYC* overexpression MYC binding to DNA increases to about 45,000. From these, 15,000 of them were localized in promoters (Walz et al., 2014) of its target genes. It is estimated that approximately half of these genes are upregulated and well known and described in the literature. On the other hand, *MYC* function as a transcriptional repressor is less known (Allevato et al., 2017; Fernandez et al., 2003; Zeller et al., 2006).

The first clue that *MYC* could act as a transcriptional repressor came from different studies in the 80s. Some studies demonstrated that the ectopic expression of *v-Myc* (c-Myc homologous transforming gene of avian myelocytomatosis virus (MC29)) (Dalla Favera et al., 1982) was able to downregulate endogenous *MYC* and further observations showed that ectopic expression of *MYC* could downregulate the endogenous *MYC* in a dose-dependent manner (Walz et al., 2014). Furthermore, structural-function analysis of *MYC* showed that the same regions, which are required for transformation, are also necessary for negative autoregulation (Cleveland et al., 1988; Penn et al., 1990). Thus, there is a negative feed-back loop within *MYC* gene in which enforced expression of *MYC* induces the silencing of its own endogenous expression.

MYC exerts a broad activity over many pathways within the cell performing a holistic approach toward transformation. These are only some examples of MYC regulating network **(Figure 1.3).**

Cell cycle: MYC has a double role in cell cycle regulation. It can both activate cell cycle progression genes and inhibit cell cycle inhibitors what is a powerful combination (Karn et al., 1989). Indeed, forced MYC expression is sufficient to allow quiescent cells re-enter cell cycle (Eilers et al., 1989). The majority of MYC target genes are involved in the control of cell proliferation in some way. The activity of these genes is related to cell-matrix interaction, DNA synthesis and transition from G_1 to S phase (Schuhmacher et al., 2001). MYC expression is sufficient to overcome the restriction point and pass through S phase to G_1 in the absence of growth factors. MYC is able to perform its activity regulating the cyclin/CDK complexes directly (activating cyclin D2, D1 or CDK4) (Bouchard et al., 2001) or suppressing cell cycle inhibitors such as *Ink4b* gene which codifies for cyclin-dependent inhibitor (CKI) $p15^{INK4b}$ (Seoane et al., 2001). It has been also described that MYC regulates directly *SKP2* coding the ubiquitin ligase that marks

p27 cell cycle inhibitor for its degradation (Bretones et al., 2011; Garcia-Gutierrez et al., 2019).

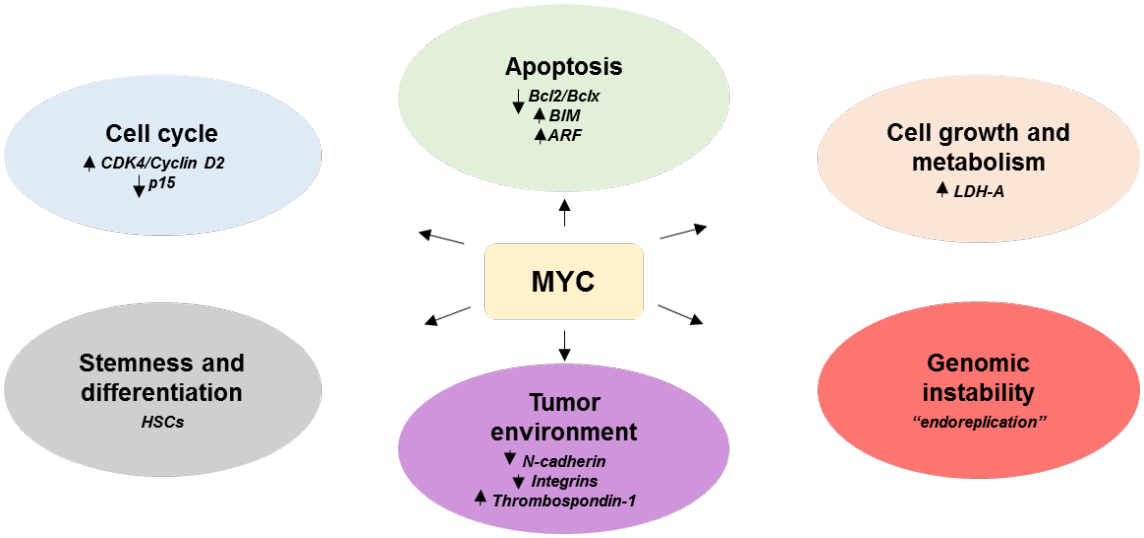


Figure 1.3. Schematic representation of MYC functions within the cell. MYC performs a broad activity within the cell. MYC is able to induce cell cycle progression concomitantly with cell growth through its direct target genes. MYC upregulation induces apoptosis through different pathways. MYC upregulation is also associated with genomic instability and lose of adhesion, which confer these cells tumorigenic properties and metastatic potential. MYC *doses* is also important during cell differentiation and to maintain cell stemness.

Apoptosis: in the absence of survival factors, enhanced MYC expression in normal cells leads to apoptosis, a paradoxical effect since MYC overexpression is present in tumour cells. It has been proposed that this response to MYC overexpression is related with a kind of safeguard mechanism that all cells have, to prevent tumorigenesis. This ability of MYC to induce apoptosis is not exclusive of MYC oncogene but shared with other oncogenes. Again, MYC promotes apoptosis in a dual fashion way either suppressing the anti-apoptotic genes BCL2 and BCL-X or activating the expression of pro-apoptotic genes such as BIM (BCL2 antagonist) (Youle and Strasser, 2008). However, MYC strongly induces apoptosis activating the ARF-MDM2-TP53 pathway. MYC activates ARF which inhibits MDM2 (TP53 inhibitor) and eventually leads to a tumour-suppressive

apoptotic response. Data collected throughout years in broad screenings of many cancer types showed that MYC overexpression correlates in almost all cases with p53 deregulation. This underline the importance of this pathway to be switched off in cancer to allow MYC driven transformation (Eischen et al., 2001; Eischen et al., 1999).

Cell growth and metabolism: as one of the most important functions of *MYC* is to promote cell cycle progression, it is not surprising that MYC regulates not only cell cycle genes but also genes involved in metabolism and cell growth, given that biomass accumulation is an essential need to allow proliferation. MYC can induce cell growth through different mechanism. On one hand, MYC is known to increase the synthesis of rRNA and tRNA, as well as ribosomal proteins, leading to higher rates of protein synthesis in MYC-overexpressing cells (Cole and Cowling, 2009; Grandori et al., 2005). Additionally, MYC overexpression in tumour cells offers an advantage for tumour transformation, owe to MYC function in glycolysis and glutamine metabolism and is able to reprogram the metabolic capacity of the cells to support a rapid expansion in oxygen deprivation. MYC performs this function regulating many genes involved in cellular metabolism, including a key glycolytic regulator such as LDH-A (Kroemer and Pouyssegur, 2008). Therefore, MYC contributes to the Warburg effect typical of tumour cells.

Genomic instability: uncontrolled proliferation is a general characteristic of the majority of tumour cells. This feature causes problems in DNA replication; a process, which is normally tightly controlled but is commonly deregulated in tumour cells. Replication stress (the collapse of the DNA replication forks at fragile sites in the genome), loss of heterozygosity and other abnormalities often occurs in tumour cells (Halazonetis et al., 2008). It is known that MYC deregulation provokes amplification, rearrangements and other chromosomal abnormalities. Additionally, MYC overexpression induces DNA damage dependent or independently of reactive oxygen species (ROS) (Ray et al., 2006;

Vafa et al., 2002). Furthermore, MYC enforced expression uncoupled the events occurring in S-phase from those in mitosis and eventually induce a process called "endoreplication" and thus aneuploidy (Li and Dang, 1999; Srinivasan et al., 2013).

Tumour environment: malignant tumours usually metastasize and escape from primary tumours due to the high demand of oxygen and nutrients necessary to maintain the tumour mass. MYC overexpression is often correlated with metastasis and poor prognosis on tumour development. This makes sense if we take into account that MYC is involved in the regulation of some cytoskeleton proteins reducing cell adhesion (integrins, calveolin-*1* or *N*-cadherin) (Hann, 2014). Additionally, MYC regulates some miRNAs involved in epithelial and mesenchymal transition. MYC also downregulates thrombospondin-1 facilitating the "angiogenic switch" releasing cancer cells from the tumour bulk to vascular tissue and finally colonize other tissues or organs (Baudino et al., 2002; Claassen and Hann, 1999).

Stemness and differentiation: *MYC* was described by Takahashi and Yamanaka (2006) as one of the genes necessary to reprogram stem cells together with other genes such as *NANOG*, *SOX2* or *KLF4* (Takahashi and Yamanaka, 2006). Many studies confirm the importance of *MYC* in pluripotency of different cell lineages but it is particularly relevant during haematopoiesis. Haematopoiesis is the process that generate all the different lineages of blood cells from hematopoietic stem cells (HSCs). The study of genetically modified mice with overexpression or deletion of *MYC* has shown that *MYC* is required for the correct balance between self-renewal and differentiation of HSCs. Enforced *MYC* expression in mice leads to reduced HSC pools owing to loss of self-renewal activity at the expense of increased proliferation of progenitor cells and differentiation (Delgado and Leon, 2010b).

In summary, we can conclude that MYC affects different pathways within the cell and is able to switch from physiological to aberrant behaviour dramatically fast, which increases

the need to tightly control its expression as at many levels as possible. Indeed, MYC is necessary for cell survival and all the cells have developed safety mechanisms to maintain MYC expression between certain levels to avoid cell transformation.

1.1.2 Novel MYC target genes

Describing a novel MYC direct target gene has never been an easy task. First, we have to take into account that E-boxes are found on average once every 4 Kb across human genome (Eisenman, 2008) but there are more than 50 b-HLH-LZ proteins encoded by the human genome (Jones, 2004) with the ability to bind these consensus sequences. Thus, knowing the primary sequence of a given gene could not be enough to ensure MYC binding. Additionally, some studies demonstrated that the presence of an E-box is not sufficient to attract MYC to this site. Apparently, MYC has strong preferences for binding sites surrounded by CpG islands, which are commonly known as regions of open and active chromatin (Zeller et al., 2006). However, MYC presence in certain chromatin regions does not ensure transcriptional activation (Gaubatz et al., 1994). Finally, we have to take into account the amount of MYC necessary to regulate all these binding sites.

However, it has been shown that MYC at physiological (low) levels occupies less active E-boxes promoters than MYC at pathophysiological (high) levels. High MYC levels within the cell also provoke an increase in MYC binding to enhancers and segments with non-canonical E-boxes (Lin et al., 2012).

Taken all these into account, MYC binding into DNA would be different if we compare different cells lines. More importantly, the same cell line could not share MYC binding sites upon different treatments or genetic modifications. Therefore, we should expect different MYC binding sites depending on the *status* of the cell, which increases again the difficulty of finding novel MYC direct target genes.

1.1.3 MYC in cancer

MYC performs many different functions within the cell affecting the main pathways that determine cell survival, growth and differentiation. Alterations in MYC regulating network induces tumorigenesis. For instance, MYC family of proteins have been found altered in more than half of human tumours of all types (Schaub et al., 2018) **(Figure 1.4)**.

There are four major mechanisms by which MYC levels could be increased in tumours. At the gene level, *MYC* could be either amplified or translocated enhancing *MYC* expression. Additionally, the increase in mRNA production due to *MYC* enhanced transcription could increase (but not necessarily) MYC protein amount. Finally, at the protein level, different mutations in MYC protein could lead to increase protein stability and thus, MYC enhanced availability.

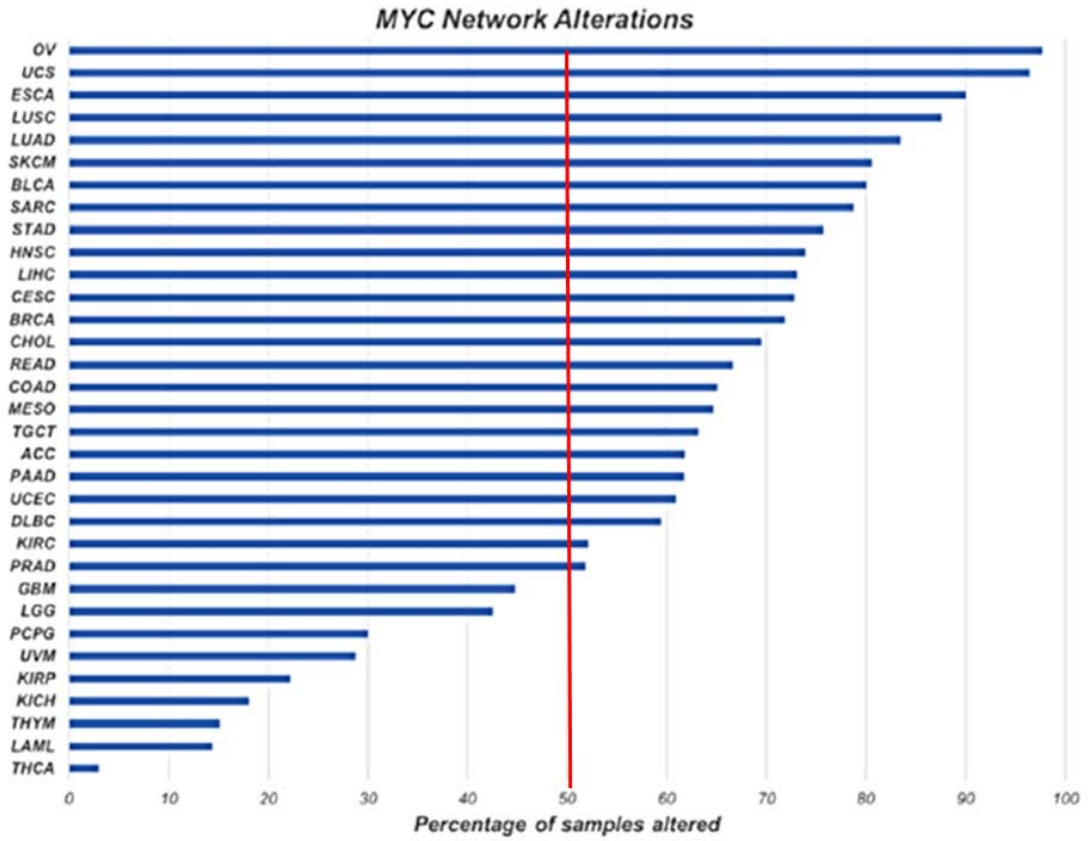


Figure 1.4. Percentage of MYC family protein alterations in different tumor sample types. The graph shows that MYC family of proteins is altered in more than 50% of the samples in 24 of 33 tumor types. The abbreviations corresponded to: ACC Adrenocortical carcinoma; BLCA Bladder Urothelial Carcinoma; BRCA Breast invasive carcinoma; CHOL Cholangiocarcinoma; COAD Colon adenocarcinoma; DLBC Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA Esophageal carcinoma; GBM Glioblastoma multiforme; HNSC Head and Neck squamous cell carcinoma; KICH Kidney Chromophobe; KIRP Kidney renal papillary cell carcinoma; LAML Acute Myeloid Leukemia; LGG Brain Lower Grade Glioma; LIHC Liver hepatocellular carcinoma; LUAD Lung adenocarcinoma; LUSC Lung squamous cell carcinoma; MESO Mesothelioma; OV Ovarian serous cystadenocarcinoma; PAAD Pancreatic adenocarcinoma; PCPG Pheochromocytoma and Paraganglioma; PRAD Prostate adenocarcinoma; READ Rectum adenocarcinoma; SARC Sarcoma; SKCM Skin Cutaneous Melanoma; STAD Stomach adenocarcinoma; THCA Thyroid carcinoma; THYM Thymoma; TGCT Testicular Germ Cell Tumors; UCEC Uterine Corpus Endometrial Carcinoma;UCS Uterine Carcinosarcoma; UVM Uveal Melanoma; CESC Cervical squamous cell carcinoma and endocervical; adenocarcinoma KIRC Kidney renal clear cell carcinoma (Grandori et al., *Cell systems*. 2018)

1.1.3.1 MYC gene increased transcription

There are different mechanisms of *MYC* transcriptional regulation. As mentioned before, MYC *locus* is surrounded by 3 Mb empty of gene codifying regions. Thus, it is not surprising that many regulation sites have been found, either upstream or downstream MYC *locus*. Additionally, many distal regulation mechanisms have been described as well as long non-coding RNAs codified in these regions, which in turn regulate *MYC* itself (Swier et al., 2019). For instance, 335 Kb upstream MYC *locus* there is an enhancer that could perform its function through a loop in the DNA that allows WNT/TCF4-β-catenin pathway to induce MYC expression (Wright et al., 2010). Other regions comprised 600-200 Kb upstream MYC *locus* have been identified as cancer risk regions in prostate cancer (Gurel et al., 2008). Furthermore, the lncRNAs PVT1 (located 3´side MYC *locus*) and PRNCR1 (transcribed from a distal region 5´ of *MYC*) are also related to different malignancies. This regulation is not necessarily linked to an increase in MYC expression but it has been described its role in cancer cell growth (Huppi et al., 2012).

1.1.3.2 MYC gene amplification

MYC family of proteins is one of the most frequently amplified across all cancer types (Schaub et al., 2018). In general, cancers with high copy-number gains of *MYC* correlate with increased levels of MYC mRNA and protein levels. Gene duplication occurs through genome doubling or tandem duplications, giving rise to copy number alterations. The first evidence of *MYC* amplification was discovered in human leukaemia HL60 cell line (Collins and Groudine, 1982). Since next generation sequencing (NGS) techniques were introduced, MYC amplification has been detected at high frequencies and it is often

associated with aggressive diseases, metastatic potential, therapeutic resistance and poor patient outcomes (Kalkat et al., 2017). However, MYC amplification requires additional events to drive tumorigenesis, like disarming apoptotic pathways.

1.1.3.3 MYC gene translocation

MYC expression is tightly controlled at the transcriptional level *via* cell type-specific enhancer surrounded *MYC locus.* However, in some cancer types, *MYC* is translocated into high active lineage-specific enhancers. This process leaves *MYC* under control of active gene enhancers, such as immunoglobulins in lymphoma cells, increasing MYC expression.

MYC translocation was firstly discovered in BL (Dalla-Favera et al., 1982). *MYC locus* was found translocated from chromosome 8 to the immunoglobulin heavy chain enhancer in chromosome 14, with a frequency of 70-80% in BL cases. Later on, MYC was also found translocated into the light chain enhancers of κ and λ enhancer with less frequency (10-15%) (Molyneux et al., 2012).

MYC could be also found translocated into one of T cell receptor *loci* in some cases of T cell acute leukaemia (T-ALL) and in multiple myeloma (MM) (Shou et al., 2000) **(Figure 1.5).**

1.1.3.4 MYC enhanced protein stability

MYC protein has a very short half-life around 20-30 minutes (Luscher, 2001) and is tightly regulated at the post-transcriptional level. Deregulation in this process enhance MYC

protein stability, which means an increased amount of MYC available for the cell. Of particular interest are two phosphorylation residues often mutated in cancer, Thr-58 and Ser-62. MYC phosphorylation at these sites increased MYC stability avoiding its degradation *via* proteasome (Henriksson et al., 1993; Sears et al., 1999).

In conclusion, MYC deregulation often leads to increased levels of this oncoprotein. MYC levels have to be tightly controlled because they cannot pass the threshold beyond which a tumoural process could be triggered (Nilsson and Cleveland, 2003).

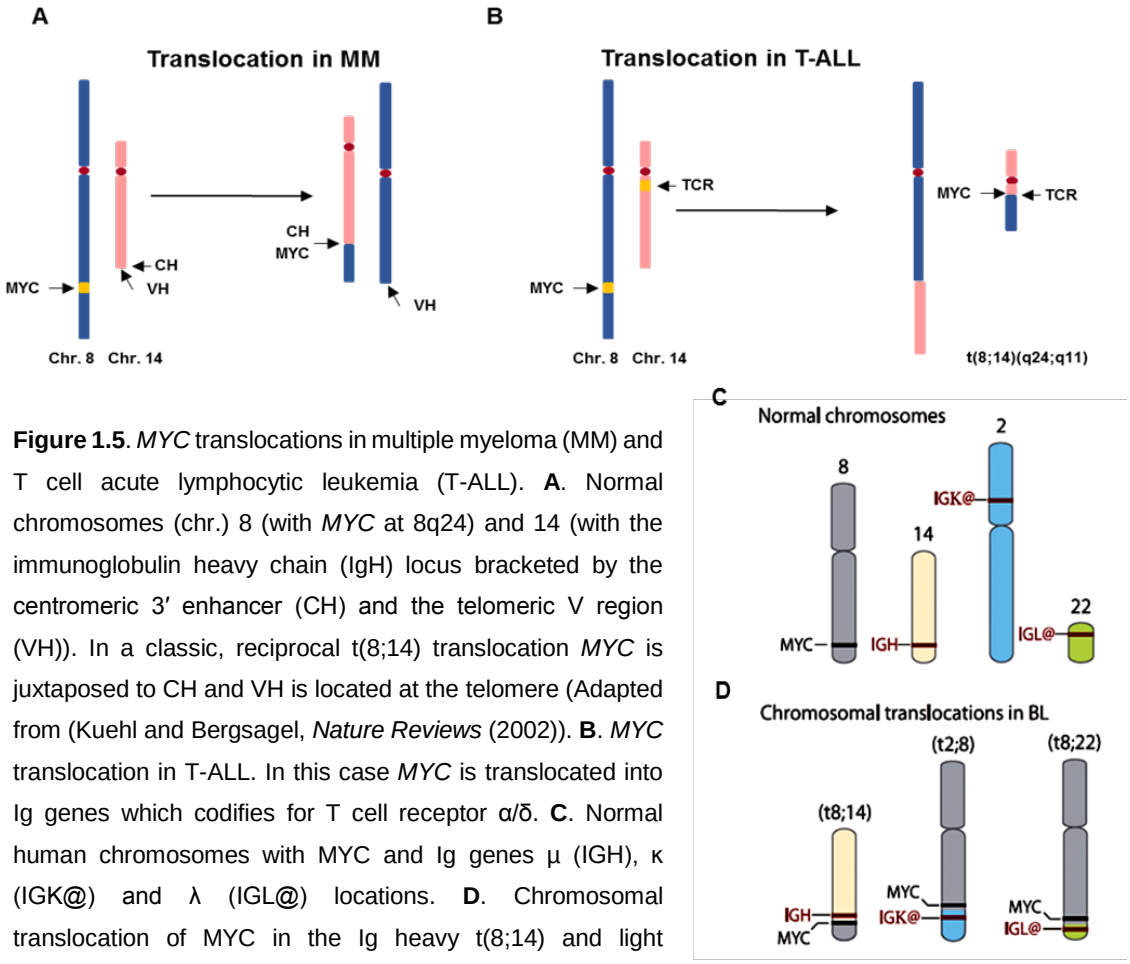


Figure 1.5. *MYC* translocations in multiple myeloma (MM) and T cell acute lymphocytic leukemia (T-ALL). **A**. Normal chromosomes (chr.) 8 (with *MYC* at 8q24) and 14 (with the immunoglobulin heavy chain (IgH) locus bracketed by the centromeric 3′ enhancer (CH) and the telomeric V region (VH)). In a classic, reciprocal t(8;14) translocation *MYC* is juxtaposed to CH and VH is located at the telomere (Adapted from (Kuehl and Bergsagel, *Nature Reviews* (2002)). **B**. *MYC* translocation in T-ALL. In this case *MYC* is translocated into Ig genes which codifies for T cell receptor α/δ. **C**. Normal human chromosomes with MYC and Ig genes μ (IGH), κ (IGK@) and λ (IGL@) locations. **D**. Chromosomal translocation of MYC in the Ig heavy t(8;14) and light t(2;8)t(8;22) chains. Adapted from Klapproth, Br *J Haematologica* (2010).

1.1.4 Targeting oncogenic MYC

It seems clear for many authors that a good strategy in cancer therapy would be the inhibition or downregulation of MYC. However, many strategies have been developed throughout years to target MYC expression in cancer and it has turned out to be challenge. Probably, one of the main issues is that under normal physiological conditions, MYC is required for many processes within the cell to maintain cell homeostasis. Thus, MYC total inhibition could cause many side effects and also undesirable drug resistance after prolonged application (Hartl, 2016).

Despite all the problems, multiple strategies have been developed to inhibit MYC through direct or indirect mechanisms. Two main strategies have been employed to decrease MYC aberrant expression.

The first one focused on impairing MYC-MAX dimerisation. As MYC is unable to exert its function without MAX, preventing MYC-MAX dimerisation emerged as an appealing mechanism to impair MYC aberrant expression. 10058-F4 is a small molecule developed to avoid MYC-MAX heterodimerisation and is able to enter cells with low non-specific toxicity (Wang et al., 2007). This compound has been demonstrated high efficiency *in vitro* but not *in vivo*.

A similar approach is based on sequestering MYC with the small molecule Omomyc which is a b-HLH mutant that acts as a dominant negative molecule interfering with MAX interaction. As opposed to 10058-F4 inhibitor, Omomyc has proved some successful in *in vivo* models, like human glioma in mouse models and glioblastoma *in vitro* and *in vivo* (Annibali et al., 2014; Beaulieu et al., 2019).

Because most of the strategies aimed at inhibiting MYC directly have not worked as effectively as expected, new approaches have arisen in the past years. One of them is based on the theory of MYC as a global transcriptional amplifier. This model proposed

that MYC is bound to virtually all active promoters and enhancer transcriptional elongation sites. Some studies proposed a possible correlation between MYC levels and phosphorylation of Ser-2 in the CTD carboxy-terminal region of RNA Pol II. This phosphorylation occurs through CDK9 that belongs to P-TEFb complex (Yang et al., 2005).

Histone lysine acetylation is read by proteins containing specific interacting domains termed bromodomains (BRD). This motif of 110 amino acids binds the epsilon-aminoacetyl groups of lysines in nucleosomal histones. The BRD and extraterminal proteins (BRD2, 3, 4 and BRDT) contain a double BRD in the N-terminal region and an extraterminal (ET) protein-protein interaction domain in the C-terminal region. BRD4 and bromodomain family of proteins recognize the acetylated tails of histones and recruit P-TEFb complex to the DNA (Fowler et al., 2014). It has been described that BRD4 interacts directly with both P-TEFb and MYC (Bres et al., 2008).

JQ1, an inhibitor of BRD4, competes for binding the acetylated lysines and displaces BRD4 from the super-enhancers within active gene *loci* (Chen et al., 2018) **(Figure 1.6)**. MYC transcription, as many active genes, is under BRD4 regulation. The partial JQ1 specificity over MYC could be explained owe to MYC is an active transcription factor bound to many different promoters and enhancers, even more when it is upregulated (Donato et al., 2017). BET family protein inhibitors have been proved as a good strategy to downregulate MYC levels in different tumour types, with more success in lymphomas and leukaemias (Fowler et al., 2014; Mertz et al., 2011). Of course, different combined drug strategies have also been performed, showing a synergistically effect with other inhibitors such as rapamycin (mTOR) or histone deacetylate (HDACs) inhibitors. These combined strategies results in tumour regression in T cell acute lymphoblastic leukaemia (T-ALL), pancreatic ductal carcinoma or osteosarcoma (Lee et al., 2015; Manzotti et al.,

2019). Also in osteosarcoma BET inhibitors have a synergistic effect when combined with CDK inhibitors (Posternak and Cole, 2016).

In summary, the strategy of targeting directly MYC expression as anti-tumoural therapy have demonstrated poor success so far. Thus, the different approaches employed to achieve MYC specific downregulation have a mild effect in tumour regression. However, combination of different strategies have demonstrated to be a promising therapy for MYC-driven tumours. For this reason, the search of new potential MYC target genes as well as different critical pathways susceptible of being used as therapeutic targets should be an important aim in MYC-driven tumorigenesis or MYC addicted tumours.

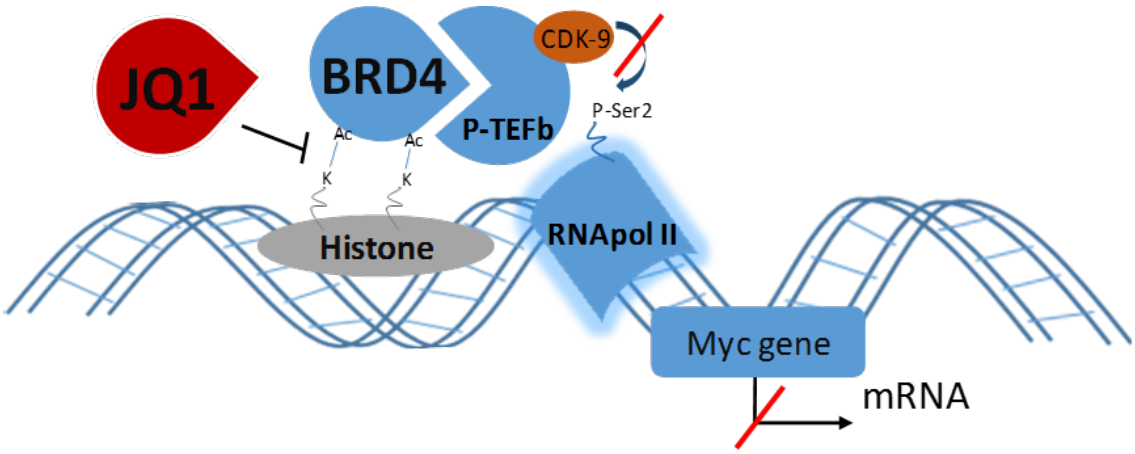


Figure 1.6. BRD4 family proteins recognize the acetylated lysine tails and recruit P-TEFb protein complex. This complex when recruited phosphorylate the CTD from RNA Pol II, releasing it from paused state and allowing gene transcription. JQ1 belongs to bromodomain inhibitors family of compounds that compete for the binding to these histone marks, avoiding P-TEFb complex recruitment mediated by BRD family proteins.

1.1.5 MYC in Lymphoma

MYC alteration was initially described in lymphoid neoplasm and, as we previously mentioned, 8q24 translocation in BL was the first MYC alteration found in human haematological malignancies. Since then, different amplifications, mutations and gene rearrangements have been described throughout years in different lymphomas and leukaemias. Furthermore, MYC rearrangement is often correlated with aggressive profiles and poor prognosis (Delgado and Leon, 2010b; María G. Cortiguera, 2015; Vaque et al., 2014).

The Eμ-MYC mice come from a transgenic mouse model that expressed *MYC* under control of the immunoglobulin intron enhancer (Eμ), modelling the chromosomal translocation found in BL. These mice develop pre-B and B cell lymphomas after a latency period of 4-6 months. MYC-induced lymphomagenesis was evident since this mouse model could rapidly develop aggressive B cell lymphomas with high penetrance (Adams et al., 1985; Conacci-Sorrell et al., 2014b).

MYC plays a critical role in the proliferation of many human B cell lymphomas, the vast majority of which originate from germinal centre (GC) or post-GC B cells as demonstrated by their somatically mutated Ig genes (Klein et al., 2003; Victora et al., 2012). GCs are sites within secondary lymphoid organs, such as lymph nodes or spleen, where mature B cells proliferate, differentiate, and mutate their antibody genes. These mutations could occur mainly through two mechanisms: somatic hypermutation (SHM) to achieve higher affinity of the immunoglobulins (Ig) light chain and class switch recombination (CSR) of their Ig (for example from IgM to IgG) during a normal immune response to an infection.

Indeed, in the GC microenvironment rapidly dividing B cells undergo SHM and CSR in their Ig genes, both of them involving DNA strand breaks. Infidelity in these processes

increases the probability of oncogenic events such as chromosomal rearrangements (Calado et al., 2012). In human GC-derived B cell lymphomas *MYC* is frequently involved in chromosomal translocations. Many mature B cell malignancies, including BL, shares the gene expression markers of their normal GC B cells counterparts. One of the genes overexpressed in these cells is Activation Induced Cytidine Deaminase (*AID*), which mediates CSR and SHM introducing mutations and consequently double strand breaks in the DNA. Experimental procedures have shown that AID can also induced Ig/MYC translocation similar to those found in BL (Ramiro et al., 2006). Other types of lymphomas such as diffuse large B cell lymphoma (DLBCL) also showed *MYC* translocation into the immunoglobulin gene *loci* and also present translocation of either *BCL2* or *BCL6* (called “double-hit” aggressive B cell lymphomas) (Nguyen et al., 2017; Sanchez-Beato et al., 2003)

MYC is initially expressed in B cells, after the encounter of the antigen and it is essential for GC formation. After GC formation, MYC expression is gradually downregulated by BCL6, which is a master regulator of GC reaction and a transcriptional repressor. Switching MYC and BCL6 expression causes formation of GC Dark zone. When MYC expression in Dark Zone is absent, TCF3 (E2A) acts as transcription factor. TCF3 together with CCDN3 acts in the Dark Zone to make these cells highly proliferative (Cai et al., 2015; Schmitz et al., 2012) **(Figure 1.7)**.

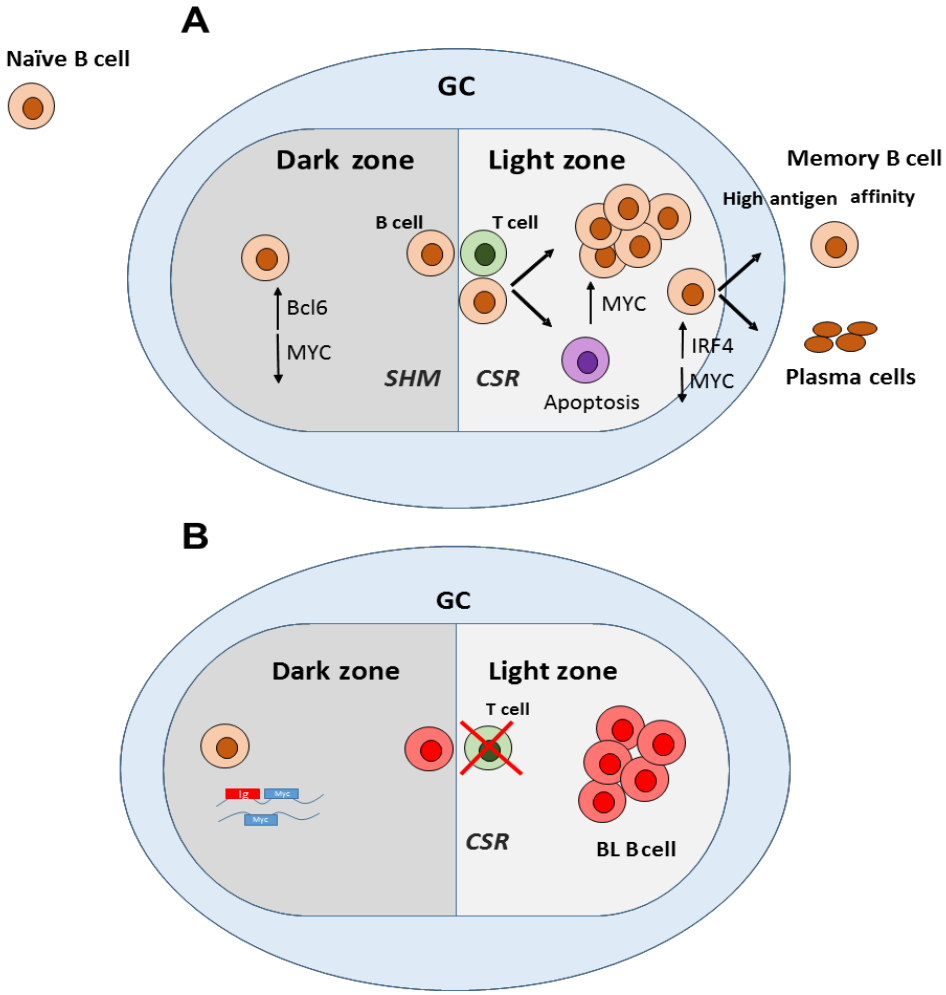


Figure 1.7. MYC is involved in GC reaction. **A**. BCL6 is highly expressed in GC Dark zone where B cells proliferate and suffered SHM and clonal expansion to give rise B cells with different receptor affinity. These B cells could encounter T cells and they are selected for high antigen affinity and divide rapidly. This B cell activation downregulates BCL6 expression, which releases MYC from BCL6 repression. B cells with low antigen affinity are selected to die by apoptosis. Finally, B cells could leave GC compartment to differentiate toward either memory B cells or plasma cells. **B**. *MYC* translocation in B cells removes BCL6 binding site from *MYC* promoter allowing *MYC* upregulation. These B cells are no longer under control of GC reaction and they are independent of T cell encounter to pass GC and move to memory B cell compartment. These B cells also avoid negative selection by apoptosis.

The mechanisms that undergo these processes make B cells from GC susceptible to malignant transformation mainly from genomic instability (Liu et al., 2008; Schlissel et al., 2006). High affinity immunoglobulin selection is an important event in immune system. Nevertheless, mistakes occurs and the complete knowledge of every step is necessary to improve therapeutic targets.

1.1.6 MYC in Germinal Centre

As mentioned before, GC are transient structures that form within secondary lymphoid organs. In GCs, B cells are selected based on their ability to produce high-affinity antibodies. The GC reaction is triggered by T cell-dependent antigens, in response to which B cells initiate vigorous proliferation coupled with SHM of their immunoglobulin (Ig) genes. These events take place in the Dark Zone where different B cell populations with different B-cell receptor (BCR) affinities coexist (Victora et al., 2012). The BCR is a transmembrane receptor protein, located in the outer membrane of lymphocytes that it is composed mainly by immunoglobulin molecules. BCR is able to capture different antigens and activate B cells.

B cells with high affinity BCRs could move forward to the Light zone and they are selected based on their affinity for the antigen. The ones with high affinity could differentiate towards either memory B cells or plasma cells (Calado et al., 2012; Victora and Nussenzweig, 2012; Victora et al., 2010).

Upon selection, B cells can also re-enter the Dark Zone for additional cycles of SHM an iterative process known as “cycle re-entry”. Thus, GC development requires coordinated signals dictating the induction of proliferation, cell cycle exit, cell cycle re-entry, and differentiation, as well as the elimination of non-selected B cells by apoptosis (Domínguez-Sola et al., 2012).

MYC expression is gradually downregulated by BCL6, binding directly *MYC* promoter (Saito et al., 2005). The signals that initiate GC reaction are known to negatively regulate BCL6 activity, allowing MYC to escape BCL6 repression (Domínguez-Sola et al., 2012). The gene expression profile of isolated MYC-expressing Light Zone GC-B cells do not reveal signatures of plasma cell commitment. Thus, MYC induction could be a way to induce Dark Zone re-entry as opposed to plasma cell differentiation (Calado et al., 2012; Dominguez-Sola et al., 2012). The Light Zone MYC-positive cells have high affinity BCRs and they can re-enter the Dark Zone, proliferate and further acquire SHM perpetuating the GC reaction. MYC-negative cells in the Light Zone exit GC, become memory B cells or early plasmablast cells. Induction of PRDM1 (BLIMP-1) reduces MYC expression binding to its promoter and gives rise to plasma cell differentiation (Cai et al., 2015; Victora et al., 2012).

Although MYC expression have received little attention during the years (Klein et al., 2003) recent publications have confirmed that MYC is one of the key genes that rule GC formation and B cell antigen high affinity selection (Calado et al., 2012; Dominguez-Sola et al., 2012; Scheller et al., 2010). More importantly, together with BCL2 and BCL6, MYC rearrangement is one of the main events in lymphoma development and in the case of BL is the driver oncogene.

1.2 Burkitt lymphoma

In 1958 the surgeon Dennis Burkitt described a tumour mainly affecting facial bones and abdomen of African children (Burkitt, 1958). Initially classified as a form of sarcoma, the disease was later identified as a worldwide occurring highly aggressive non-Hodking lymphoma, named Burkitt´s lymphoma (BL) (O'Conor and Davies, 1960). Later on, several pathologists described a lymphoma that resembles this African lymphoma in Europe and USA. Histologically these lymphomas were indistinguishable. However, the World Health Organization (WHO, 1969) created two variants: the African lymphoma, was called endemic (eBL) because its high incidence in this region whereas BL occurring elsewhere were called sporadic (sBL).

eBL affects principally children, more often boys and has a frequency range 3-6 per 100,000 in children aged 0-14 per year. This means 30-50% of all childhood cancers in equatorial Africa (Magrath, 2012). On the other hand, sBL is typical from USA and Europe where the incidence is 1-3 per million, considerably lower than in eBL. sBL comprises 1-2% of all lymphomas in USA and 30-50% of all childhood lymphomas. The median age of diagnosis is 30 years (Said et al., 2014). A major difference between eBL and sBL is the differential involvement of Epstein-Barr virus (EBV) infection, a virus originally described in eBL samples (Schmitz et al., 2014).

In 1961 the microbiologist Epstein discovered a type of herpesvirus in BL tissue culture samples (Epstein et al., 1964). Lately it was shown that almost all samples from eBL carried this virus while in sBL the presence of the virus is observed only in nearly 30% of cases (Said et al., 2014). In 1982, the observation that patients with human immunodeficiency virus (HIV) developed aggressive non-Hodking lymphoma (including

BL) lead to the inclusion of a new category in this illness, the immunodefiency-related BL (Magrath, 2012; Ziegler et al., 1982)

MYC translocation was firstly observed in BL cells (Dalla-Favera et al., 1982). This translocation renders *MYC* expression under control of the immunoglobulin enhancers, which often occurs into the immunoglobulin heavy chain t(8;14)(q24;q32) but it could also be found in the light chain *loci* via t(2;8)(p11;q24) and t(8;22)(q24;q11) (Dalla-Favera et al., 1982) **(Figure 1.5)**. Transcription of the translocated *MYC* is greater than in resting B cells, but in some cases the increase in protein amount is no more than that observed in actively dividing, but not-malignant B cells. It could be likely because the normal *MYC* allele is almost transcriptionally silenced and most of MYC protein in BL comes from the translocated *MYC* allele (Lopez et al., 2019).

BL expression profile showed that these cells comes from the Light Zone of GC (Calado et al., 2012). Altogether these observations suggest that MYC translocation provokes an aberrant MYC overexpression no longer under control of physiological GC reaction. Indeed, 8q24 translocation often removes the BCL6 binding site at 5´ *MYC locus*, avoiding BCL6-mediated *MYC* repression (Schmitz et al., 2014).

MYC translocation in BL contributes to maintain the GC-like gene expression program (Scheller et al., 2010). However, Eμ-MYC mouse models demonstrated that MYC overexpression is not sufficient to induce lymphomagenesis mainly because MYC increased levels provoke TP53 activation and consequently induce apoptosis. Thus, it is predictable that this pathway should be knockdown in these tumours. In agreement with this, 30% of BL tumours carry TP53 mutations while BL tumours that retain wild-type TP53, present mutation in other genes involve in the same pathway like p19/ARF or MDM2 (Lindstrom and Wiman, 2002).

An interesting feature about BL oncogenic mechanism is that the translocation of *MYC* occurs exclusively into the non-productively rearranged Ig heavy-chain *locus*, sparing the *allele* that produces the productive Ig heavy-chain that is used to construct the BCR. That implies that BL cells are selected to have a BCR signalling pathway active. Enhancing BCR signalling could affect either PI3K or NF-kB pathway activation. As opposed to other types of lymphomas from GC origin, BL tumours show inactive NF-kB signalling (Schmitz et al., 2012). Thus, maintaining an active PI3K pathway is an important mechanism in BL pathogenesis.

There is another important factor that plays a main role in BL development, especially in eBL. EBV infection and *MYC* translocation co-occur in almost all cases of eBL while in the sBL EBV infection is observed in less percentage, as mentioned above. Since its discovery more than sixty years ago, BL cell of origin and the mechanism underneath the process of tumorigenesis remain elusive. Thus, several theories have come to be developed regarding *MYC* and EBV correlation in BL that we will discuss below.

1.3 Epstein-Barr Virus (EBV)

EBV is a ubiquitous gamma herpesvirus that establishes a seemingly harmless latent infection in B cells in over 95% of human population, but is also involved in several types of cancer (Brady et al., 2007). It is estimated that more than 200,000 cases of cancer each year and 1.8% of all cancer deaths are due to EBV-attributable malignancies (Young et al., 2016). EBV was the first virus described with oncogenic potential due to its ability to transform naïve B cells into lymphoblastic cell lines (LCLs) *in vitro*. EBV infection has been also found being involved in other types of cancer such as nasopharyngeal carcinoma, gastric cancer and different haematological malignancies (Lin et al., 2014; Rowe et al., 1986; Wang et al., 2014).

Primary infection occurs by the oral route and leads to local virus replication in the oropharynx, involving lytic infection of mucosal epithelium and possibly local B cells. The virus-driven growth transformation of B cells in pharyngeal lymphoid tissues, is followed by a switch to a truly latent infection of the generalized memory B cell pool (Thorley-Lawson et al., 2013) **(Figure 1.8)**. After primary infection, virus could be reactivated from B cell *reservoir* leading to low levels of shedding infectious virus and a new growth-transforming B cells infection. There are evidence that suggest that these lytic and growth-transforming latent infections are subject to T cell-mediated immune control both during primary infection and throughout life (Hislop et al., 2005). In this way, individuals with severe impairment of T cell response (as in patients from post-transplant organs or HIV-positive patients) are in high risk to develop acute EBV-positive B lymphoproliferative diseases (Shannon-Lowe et al., 2017).

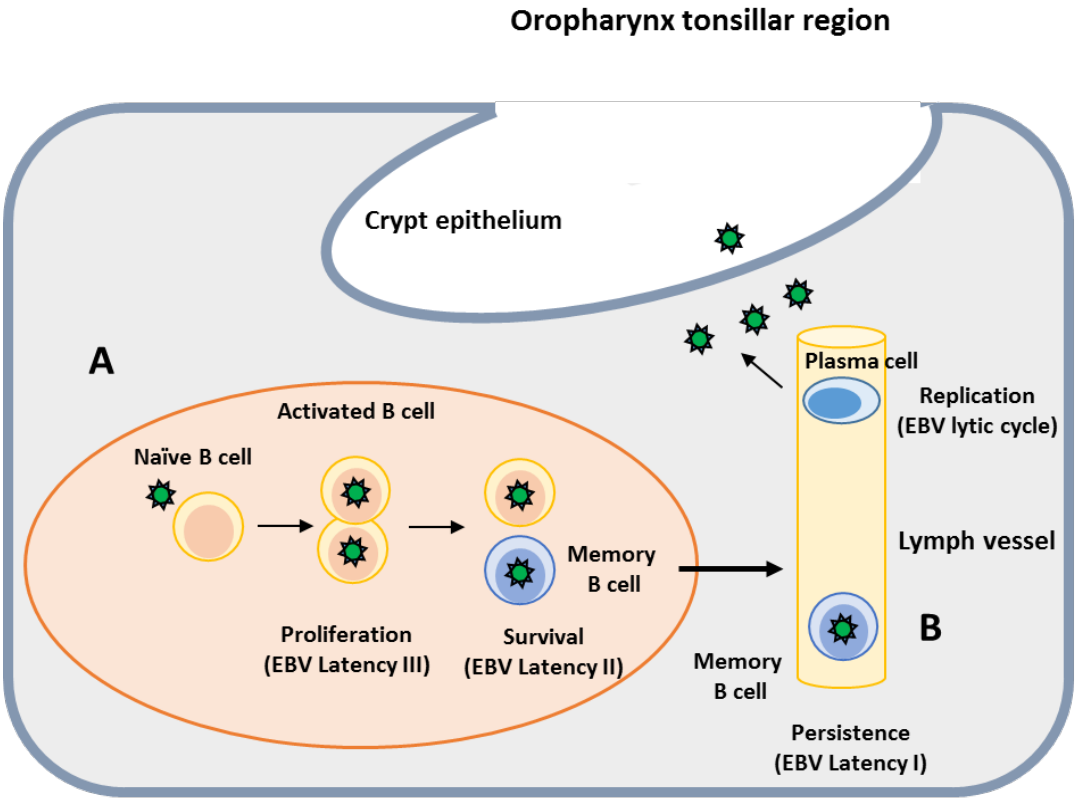


Figure 1.8. A model of EBV infection and latency gene program expression during different stages of EBV persistent and lytic cycle in B cells (Adapted from Vetsika and Callan, 2004). **A**. EBV enters human body through the oropharynx cavity, encounter naïve B cells surrounded this area and infected them. These B cells begin to express the growth program (Latency III) and that activates B cells, which can enter into lymphoid tissue and form germinal centers. Concomitantly, these B cells change to a more restrictive latency program, leaving EBNA1 and the LMPs to allow these cells to pass germinal center reaction and enter in memory B cell compartment. **B**. In memory B cell compartment all the viral latent genes are downregulated, sparing EBNA1 expression, in order to avoid host immune response (Latency program I). Meanwhile, in memory B cell compartment, EBV lytic cycle might occur, allowing virus replication, shedding into saliva and thus, facilitating transmission from host to host.

There are three major types of EBV-associated B cell malignancies: BL, Hodking lymphoma and diffuse large B cell lymphoma. These lymphomas could be either positive or negative for EBV infection and are characterised for emanate from GC or post-GC B cells.

EBV can display three different types of gene expression programs:

Latency I or latency programme: persistent infection *in vivo* presents expression of EBNA1 plus the EBER RNAs. This is the more restricted expression programme and is typical from BL cells. BL cells almost exclusively express EBNA1, two small RNAs EBER1 and EBER2 and certain clusters of miRNAs (Brady et al., 2007). Indeed, BL cells are the only EBV-infected cells showing this suppressed expression and entirely lacking viral antigens required for immortalization (Rowe et al., 1986). The most accepted hypothesis proposes that this restricted pattern of EBV genes expression confers these cells an advantage allowing them to bypass immune surveillance from the host.

Latency II or default programme: aside from EBNA1 these infected B cells also express the latent membrane proteins LMP1 and LMP2. Latency II is expressed in Hodking´s disease and nasopharyngeal carcinoma. LMP1 mimics CD40, activating NF-κB pathway and inducing anti-apoptotic signalling (Mosialos et al., 1995) and LMP2 mimics IgG receptor activating PI3K-AKT pathway promoting B cell differentiation, survival and cell growth (Merchant et al., 2000).

Latency III or growth programme: is characterised by the expression of all latent genes and occurs in primary infection of B cells. EBV in LCLs shows specific growth phenotype imposed of viral replication programme latency III. The gene expression profile comprehended the EBV nuclear antigens (EBNA1, EBNA2, EBNA-LP, EBNA3, EBNA3B, EBNA3C) and the latent membrane proteins (LMP1, LMP2A and LMP2B) (Klapproth and Wirth, 2010). Latency III programme is very immunogenic and cells displaying this pattern of EBV latent genes are rapidly erased by cytotoxic T cell responses. This allows positive selection of B cells expressing a more restricted pattern of EBV latent genes (Thorley-Lawson, 2001).

EBV is widespread in all human communities, but only a very small minority of infected individuals develop BL or any of the other tumour types associated with EBV infection. Additionally, as already pointed out, not all the cases of BL are EBV-associated (Rowe et al., 2014). Altogether, these evidences indicate that there is not a clear role of EBV in BL or any other tumour type.

However, it has been hypothesised that the main function of EBV in BL might be to block apoptosis in B cells with *MYC* translocation. This could be done through either the EBNA1 protein, EBER transcripts, or epigenetic modification and subsequent repression of the pro-apoptotic BIM1 protein by the latent transcript LMP1 (Molyneux et al., 2012; Rowe et al., 2014; Schmitz et al., 2014). Additionally, the expression of LMP2A contains an immunoreceptor tyrosine-based activation motif that can replace BCR signalling (Mancao and Hammerschmidt, 2007) and LMP1 mimics CD40 receptor which is able to maintain pro-survival signalling pathways (Henderson et al., 1991). According to this hypothesis, EBV is a coadyuvant for tumorigenesis after *MYC* translocation through inhibition of MYC-mediated apoptosis.

An alternative scenario is that EBV provokes *MYC* translocation. This idea came with the finding that AID, the enzyme responsible for double strand breaks in GC reaction, promotes Ig/MYC translocation (Ramiro et al., 2006). It is also known that EBV infection induces AID activity (Epeldegui et al., 2007). Activating this enzyme could drive B cell maturation independently of GC reaction (Rowe et al., 2014).

In this scenario, GC naïve B cells could be infected and expressed the latency programme III, which drives B cells proliferation (Hochberg et al., 2004; Thorley-Lawson and Gross, 2004). These cells could pass GC reaction and express latency II genes facilitating these EBV-positive B cells enter in memory B cell compartment. Some authors proposed that the ability to prevent apoptosis ensures that the virus-containing cells are protected while passing through the GC, whether or not they have encountered

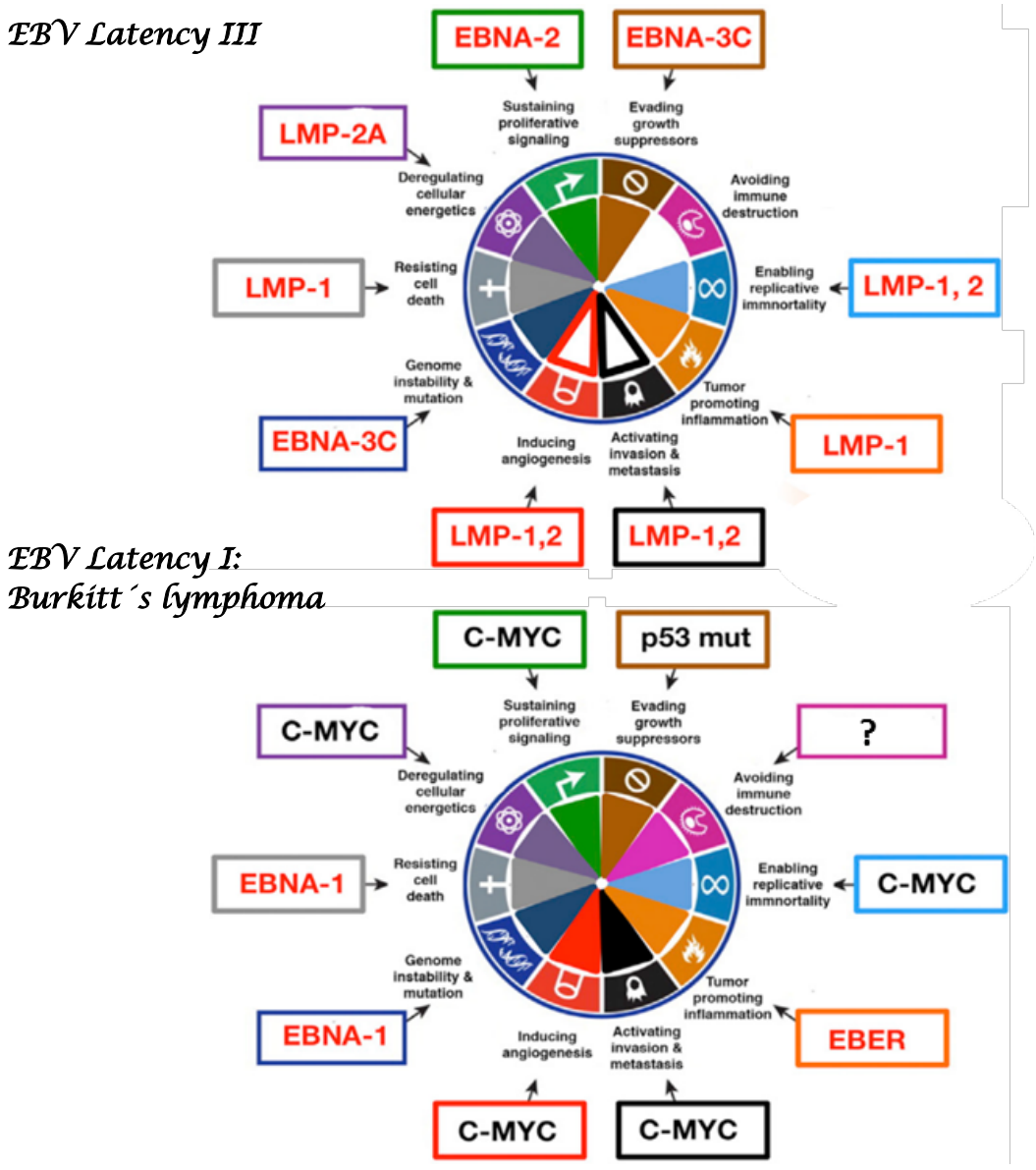
antigen (Henderson et al., 1991; Rowe et al., 2014). Finally, in memory B cell compartment the EBV gene pattern of expression become even more restricted in order to avoid immune surveillance (Gires et al., 1997; Thorley-Lawson et al., 2001).

Other lymphomas have been identified containing a deletion of EBNA2 gene. Cell lines derived from these lymphomas are resistant to apoptosis, which could suggest that loss of EBNA2 confers advantages to these tumours (Kelly et al., 2005). Apparently, EBNA2 expression is required for primary infection but could be gradually lost in order to avoid apoptosis and immune surveillance.

Indeed, small numbers of EBV infected cells, with more restricted patterns of latent gene expression, have been found on GC and in numbers that fit with mathematical models that predict GC involvement in EBV persistence (Rowe et al., 2014).

Finally, if we consider all the pathways involved in tumorigenesis, EBV latent genes are able to influence in most of these pathways **(Figure 1.9)**. However, in BL the pro-tumorigenic activity of these viral latent genes, is replaced by other host-deregulated pathways. Thus, the role of EBV in BL could not be as simple as virus-induced transformation of B lymphocytes, as most of the genes necessary to stablish growth-transforming events in LCLs are absent in BL tumours (Mesri et al., 2014; Rowe et al., 2014).

As mentioned above, there are many theories regarding EBV and *MYC* role in BL. Despite the overwhelming evidence, research and efforts employed to address this issue, until now, the question remains without being completely resolved.



Adapted from Mesri et al., *Cell Host & Microbe. Cell press* (2014)

Figure 1.9. Following the hallmarks of cancer described by Hannahan and Weinberg (2011) we can describe different aspects during tumorigenesis in which all the EBV latent genes are involved. Relating with EBV primary infection or the observations made in B cells infected *in vitro,* EBV latency III programme is displayed affecting all the processes within the cell and inducing tumorigenesis. Whereas in BL pathogenesis mutations in host genes provides different alterations that support the tumour progression in the absence of the expression of EBV latent genes. The suppression of these viral latent genes is necessary to avoid immune surveillance. EBV latent genes are depicted in red while host genes are depicted in black. Genes chosen for hallmarks activation represent one of the available examples and are based on published data collected by the authors. Filled portions within the hallmark pie are used to represent stronger or well-documented hallmark activation. Empty portions represent weaker effects or lack of activation/evidence.

1.4 CR2

CR2 (also termed CD21) is the receptor for EBV and its only way to infect B cells (Lottin-Divoux et al., 2006; Thorarinsdottir et al., 2015). The major EBV glycoprotein gp350/220, mediates the binding to CD21 in the amino-terminal short consensus repeats (SCR) following the endocytosis of the EBV (Nemerow et al., 1985).

CR2 maps on human chromosome 1q32 and encodes a 140 kDa protein (CD21) that possesses an extracellular domain that it is divided in 15 to 16 SCR, a transmembrane domain and a short cytoplasmic tail. CD21 is a membrane glycoprotein that acts as a co-receptor for the BCR and forms complex with other proteins such as CD19 or CD81. CD19/CD21 complex is an important co-receptor that plays a critical role in B cell responses to T cell antigen presentation. CD19 is co-ligated to the BCR through C3d complement fragment, which binds to CD21 and create a bridge between CD19/CD21 complex and BCR **(Figure 1.10)**. Many studies propose that CD19/CD21 complex

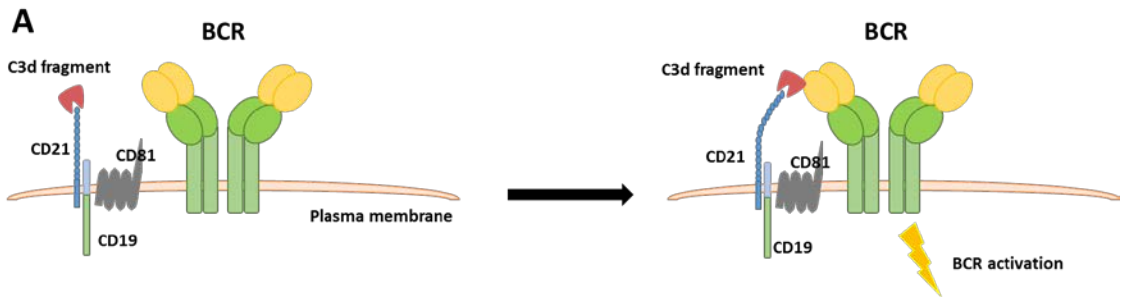


Figure 1.10. Schematic representation of BCR activation through CD21-CD19-CD81 complex. **A**. CD21 recognizes C3d complement activation fragment. These ligands recognize the short consensus repeats of CD21 N-terminal domain. **B**. CD21 forms a bridge between its complex and the BCR. The binding of CD21 to its ligands reduces the threshold of BCR activation.

enhances the signal of BCR *in vitro* and is essential for primary and secondary responses to T cell dependent antigen *in vivo* (Cherukuri et al., 2001).

The importance of CD19/CD21 complex has been proved *in vivo* by the reduced formation of GC and diminished primary antibody responses in mice lacking either CD19 or CD21 (Ahearn et al., 1996; Cherukuri et al., 2001; Engel et al., 1995; Wentink et al., 2015).

In contrast with the human orthologue, the murine *Cr2* gene encodes two alternatively spliced RNA transcripts that generate two distinct glycoproteins: a 145 kDa protein direct homologue of human CD21 and a 190 kDa isoform, produced by the inclusion of an additional five exons encoding six N-terminal SCRs (CR1 or CD35) (Kurtz et al., 1990). Therefore, knockout mice for *Cr2* gene results in mice lacking both CR2 and CR1 proteins (Molina et al., 1990).

CR2 expression is restricted to subpopulations of B cells, some T cells and follicular dendritic cells. Indeed, *CR2* is often used as a marker for GC B cells subpopulations. In human pro- and pre-B cells, the expression of the *CR2* is silenced by methylation of a CpG island in its promotor. Expression in mature B cells is accompanied by the loss of CpG-methylation (Schwab and Illges, 2001). Similarly, B cells downregulate *CR2* upon differentiation into the blast stage (Masilamani et al., 2002).

CR2 promoter includes a characteristic TATA box and a CpG-rich motif (Rayhel et al., 1991). It has also different binding sites for transcription factors regulation such as SP-1 and AP-1 and 2. Additionally, two E-boxes are found in tandem in the proximal region of *CR2* promoter. As we previously mentioned, *CR2* expression is cell-type specific and changes during B cell differentiation. The regulation of *CR2* promoter has been studied to determine the factors that influence the expression of this gene in expressing and non-expressing CR2 cells (Cruickshank et al., 2009; Ulgiati et al., 2002; Zabel and Weis, 2001). It has been shown that the different elements within CR2 proximal promoter (the

region spanning between -315 bp and the transcription start site (TSS)) support the high-level basal transcription of CR2 **(Figure 1.11)**.

Figure 1.11. Schematic representation of CR2 proximal promoter. The binding sites for the main transcription factors that contribute to CR2 promoter regulation are represented.

Objectives

2 OBJECTIVES

MYC is one of the most deregulated oncogenes in cancer. The search for new therapies and new targets against MYC aberrant expression should be an aim in cancer research with especial interest in MYC-driven tumours. Unfortunately, MYC specific inhibition have turned out to be complicated. Therefore, the study of new potential MYC target genes is particularly relevant to develop new drugs and therapies.

Previous results in our lab indicated that CR2 could be a new potential *MYC* target gene. Since CR2 is a receptor of BCR and has a very restricted pattern of expression we focused our experiments in lymphoma cell lines and especially in BL cells in which *CR2* regulation by MYC could be particularly relevant.

In this study we aim:

1. To describe *CR2* as a novel MYC target gene:
    i. Describe *CR2* regulation by MYC in different cell types and conditions
    ii. Assess MYC direct *CR2* regulation
2. Study the relevance of *CR2* regulation by MYC in BL cells physiology.
    i. Regarding EBV infection and BL development
    ii. On BCR signalling downstream pathways

Material and Methods

3 MATERIAL AND METHODS

3.1 Cell culture

Non-adherent cells and adherent cell lines **(Table 1)** were grown in RPMI-1640 or DMEM basal media (Lonza) respectively supplemented with 10% (v/v) fetal bovine serum (Lonza), 150 µg/mL gentamycin (Lab. Normon) and 2 µg/mL ciprofloxacin. In case of stable transfected or transduced cells, growing media was supplemented with the indicated antibiotic concentration.

Table 1. *Cell lines used in this work*

Stable cell line	Origin	Culture media	Selection
Raji	Human B lymphocyte from Burkitt´s lymphoma (ATCC)	RPMI 10% FBS	-
Jurkat	Human T lymphocyte from Acute T cell leukemia	RPMI 10% FBS	-
Ramos	Human B lymphocyte from Burkitt´s lymphoma	RPMI 10% FBS	-
HEK 293T	Human embryonic kidney (ATCC)	DMEM 10% FBS	-
HeLa	Human cervix adenocarcinoma (ATCC)	DMEM 10% FBS	-
K562	Human chronic myelogenous leukemia (CML) (ATCC)	RPMI 10% FBS	-
KMYCER	K562 derived cell line	RPMI 10% FBS	Puromycin 500 ng/ml
Kp27MYCER	K562 derived cell line	RPMI 10% FBS	G418 500 µg/ml Puromycin 500 ng/ml

Before seeding, cells were counted in NucleoCounter® NC-100™ system (Chemometec) and the proliferation rate were estimated with Guava ViaCount Reagent in Guava® PCA (Millipore).

3.1.1 Cell cycle and Apoptosis

Cell cycle was analysed by flow cytometry using Hoechst dye. Briefly, 10^6 cells treated with JQ1 were harvested for each condition, centrifuged 800 x g 5 min and resuspended in previously filtered PBS (3 mM EDTA) in flow cytometry clean tubes. The supernatant was clarified and resuspended in PBS (3 mM EDTA) with 5 µg/mL Hoechst (33342, trihydrochloride,trihydrate (Thermo Fisher Scientific) and incubate 30 min at 37 ^{o}C.

Apoptosis was assayed with Immunostep Annexin V-PE apoptosis detection kit. 10^6 cells were harvested and washed twice with PBS (filtered with 3mM EDTA). Cells were resuspended in Annexin V-binding buffer (10mM Hepes/NaOH (pH 7.4). 2 µL of Annexin V-PE and 7-AAD for 100 µL were added to resuspend cells at concentration 10^6 cells/mL. After 15 min of incubation period (in the dark) cells were resuspended in 250 µL of Annexin V-binding buffer and analyze by flow cytometry.

3.1.2 EBV infection

B95.8 cells were seeded to confluence 0.5-0.9 x 10^6 cells per mL in a flask with 100 mL of RPMI 10% FBS. Let the cells growing for 3 weeks without changing or adding media. After 3 weeks the supernatant were collected, filtered with 0.45 µm filter and storage in 5 mL tubes.

Ramos cells were infected, with lentiviral particles, carrying shRNA against *MYC*, for 48 h and selected with puromycin for other 48 h. Remaining cells were collected and plated

in a T12 and 500 µL of these cells were seeded with 500 µL of the virus supernatant. Cells were harvested at different time points and pelleted for RNA and DNA extraction. RNA and DNA were extracted with Qiagen® kits following manufacturer instructions. 50 ng of DNA were quantified by qPCR analysis. CR2 and LDH-A promoter primers were used as loading control and primers that recognize DNA viral latent genes LMP1 and EBNA1 **(Table 2)** were used to detect EBV infection. RNA were analyzed by RT-qPCR.

Table 2. *Primers for EBV genes*

Primer	Forward	Reverse	Product Size
LMP1	CGCCTAGGTTTTGAGAGCAG	GATGAACACCACCACGATGA	154 bp
EBNA1	GGTTCCAGCCAGAAATTTGA	CTCGCCTGAGGTTGTAAAGG	151 bp

3.1.3 Lentivirus production and cell transduction

HEK293T cells were transfected with PEI as previously described to generate 2nd generation lentiviral particles. Mixture of packaging plasmids and construct of interest was performed in a ratio of 1:3:4 (VSV-G:psPAX2:transfer plasmid). Lentiviral particles-containing supernatants were collected and stored at 4°C in two rounds (48 and 72 h after transfection). Supernatants were clarified at 1500 rpm for 10 min and filtered. Lentivirus concentration: clarified supernatants were mixed with PEG8000 (final concentration of 15%-PEG8000). Mixture was homogenized by inversion and led at 4°C for at least 6 h and a maximum of 7 days. Mixture was centrifuged at 1500xg for 30 min at 4°C, supernatant removed, lentivirus concentrated in serum-free media and stored at -80°C. For lentivirus tittering, HeLa cells were infected with increasing amounts of concentrated lentiviral particles (usually from 0.1 to 10 µL) and selected with puromycin.

Virus tittering, colony forming units (CFU), was calculated as following: number of colonies/µL of virus*103=CFU/mL.

Cells were pelleted and resuspended in the corresponding volume of lentivirus plus 5 µg/mL of polybrene. Different MOIs (multiplicity of infection), were used depending on cell type. When small volumes of lentivirus needed, serum-free media was added until a minimum of 200 µL to allow proper mixture. Mix of cells and lentiviral particles was incubated at 37˚C for 1 h, resuspended every 10 min and plated in half of the volume for the corresponding plate in serum-containing media. After 12-18 h, media was added until reach final volume of the corresponding plate and 48 h after transduction lentivirus-containing media was replaced by fresh media and puromycin selection added (if needed).

Table 3. *Plasmids used in this work*

Plasmids	Construct	Origin
GFPmax		Commercial
pGL3	*Firefly* sp. luciferase reporter gene with no promoter region	Ulgiati et al., 2002
WT	-315bp CR2 promoter in pGL3	Ulgiati et al., 2002
Ebox1Mut	-315bp CR2 promoter with Ebox1 deletion in pGL3	Ulgiati et al., 2002
Ebox2Mut	-315bp CR2 promoter with Ebox2 deletion in pGL3	Ulgiati et al., 2002
4Ebox	*Firefly* sp. Luciferase reporter gene regulated by 4 E-box sequences	T.Berg (Kiessling, et al., 2006)
pRL-null	*Renilla* sp. luciferase reporter gene regulated by the T7 promoter	Commercial (Promega)

pCMV-VSV-G	VSV-G gene encoding enveloped lentiviral protein	Didier Trono (Commercial Addgene)
psPAX2	GAG and POL genes enconding packaging lentiviral proteins	Robert A. Weinberg (Commercial Addgene)
pLKO.1 control	Empty vector	Commercial (Sigma-MISSION)
pLKO.1 shScramble	TR1.5-pLKO-1-puro, Sigma	Roble-Valero, et al., 2017
pLKO.1 shMYC	Short hairpin RNA TRCN0000039640 against human Myc mRNA	Commercial (Sigma-MISSION)
pLKO.1 shMYC	Short hairpin RNA TRCN0000039642 against human Myc mRNA	Commercial (Sigma-MISSION)
pLKO.1 shMYC	Short hairpin RNA TRCN0000039655 against human Myc mRNA	Commercial (Sigma-MISSION)
pLKO.1 shCR2	Short hairpin RNA TRCN0000057113 against human CR2 mRNA	Commercial (Sigma-MISSION)
pLKO.1 shCR2	Short hairpin RNA TRCN0000057114 against human CR2 mRNA	Commercial (Sigma-MISSION)

3.2 RNA extraction and quantification

RNA extraction was carried out using Tri Reagent® (Sigma-Aldrich). Roughly, 0.5-2x10^6 cells were lysed in 0.5 mL of Tri Reagent® for 10 min followed by incubation with 0.1 mL of chloroform for 3 min at RT. Lysates were centrifuged at 13000 rpm for 15 min and nucleic acid containing aqueous phase was transferred to a new 1.5 mL tube. For RNA precipitation, 0.25 mL of isopropanol was added, mixed by inversion, incubated at RT for 10 min and centrifuged for 15 min at 13000 rpm 4˚C. Supernatant was discarded and 0.5 mL of 70% ethanol added. The RNA containing pellet was mixed by vortexing and centrifuged for 5 min at 7500 rpm 4˚C. Supernatant was discarded, RNA air dried and resuspended in RNAse free water. RNA concentration was determined by using a NanoDrop and 200 ng were resolved in an agarose gel to check the integrity of the RNA.

In order to analyze the expression of genes at mRNA level, quantitative real time PCR (RT-qPCR) was assayed. First, reverse transcription (RT) was performed of total extracted RNA from mammalian cell lines. 1 µg of RNA was used for reverse transcription to create complementary DNA (cDNA) of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in a total volume of 20 µl.

The following protocol was set for the reaction:

5 min 25 ºC / 30 min 42 ºC / 5 min 85 ºC.

The cDNA samples were stored at -20ºC until used.

Lately, cDNA was amplified by quantitative PCR using specific primers for the gene of interest.

The primers were designed using the online Primer 3 software tool (http: \\frodo.wi.mit.edu/primer3/) according to PCR standard guidelines: length 18 to 25 bp; GC content 40 to 65 %; no G at the 5' end; no secondary structures; Tm: 50 to 65ºC. PCR conditions were determined depending on the nature and complexity of primers. A PCR mix was prepared with 2X SYBR® Select Master mix (Applied Biosystem), 300 nM forward and reverse primer mix and up to 35 µl of DNAse-free water (used for two 15 µl duplicate reactions) and 2 µL of each sample. PCRs were performed in the CFX Connect™ Real-Time PCR Detection System (Bio-Rad) and MYC and CR2 genes were recognized **(Table 4)**. The qPCR protocol normally used was the following:

For DNA amplification:

95 ºC 5 min / [95 ºC 5 min; 57 ºC 15 s; 72 ºC 15 s] 40 cycles / 95 ºC 1 min

For real time melting curve:

[55 ºC 10 s decreasing by half a degree each cycle] 80 cycles.

Quantitative PCRs were analysed with the CFX ManagerTM software. Threshold cycles (Ct) were determined by default at the beginning of DNA amplification in the exponential phase. The mRNA expression of genes of interest was normalized to mRNA expression of housekeeping genes (RPS14) using the comparative Delta Ct (ΔCt) method:

ΔCt=2 (Ct normalizing gene– Ct gene of interest)

Reactions with water instead of cDNA were used as negative controls to detect possible amplification signals from contaminant DNA or primer dimers.

Table 4. *Primers for RT-qPCR*

Primer	Forward	Reverse	Product Size
RPS14	TATCACCGCCCTACACATCA	GGGGTGACATCCTCAATCC	154 bp
MYC	TCGGATTCTCTGCTCTCCTC	CCTGCCTCTTTTCCACAGAA	151 bp
CR2	CCGACACGACTACCAACCTG	GACAATCCTGGAGCAATGGA	115 bp

3.3 Protein levels analysis by western blot

Roughly, 0.5-2x10^6 cells were pelleted and resuspended in 100 µl of NP40 lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1% NP40 (v/v), 0.1% SDS; protease (Calbiochem) and phosphatase (Sigma-Aldrich) inhibitors added immediately before use). All the steps were performed at 4˚C. Protein samples were sonicated (10 cycles in a Bioruptor® Plus Sonicator device) and finally clarify by centrifugation at 14000 rpm for 20 min at 4˚C. The supernatant was transferred to a new tube and kept frozen until used. Protein quantification was carried out using Bradford Quick Start™ 1X Dye Reagent. Standard linear concentration was measured in a range of 0.125-1.5 mg/mL. Protein sample concentration was estimated with Bradford

colorimetric assay adding 2 µL of each sample in duplicates in a T96 plate with 200 µL of Bradford. The mixture was incubated 10 min in the dark and measured in GLOMAX Multidetection system (Promega).

Samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Blocking was carried out at room temperature using 4% BSA in TBS-T for 1 h. Primary antibodies were used diluted in TBS-T at final concentration referred in **Table 5**. IRDye800/tolRDye680 secondary antibodies were used and signals were recorded with and Odyssey® Infrared Imaging Scanner (LiCor® Biosciences).

Table 5. *Primary antibodies used in this work*

Antibody	Reference and specie	Concentration	Use
c-Myc (N262)	Sc-764 Rabbit polyclonal	1:1000 1%BSA	Wb, IP
c-Myc	9402 Rabbit polyclonal (Cell signaling)	1:3000 5% BSA	Wb, IP
CR2 (C-20)	Sc-7025 Goat polyclonal	1:1000 1%BSA	Wb
β-Actin (I-19)	Sc-1616 Goat polyclonal	1:1000 1%BSA	Wb
β-Actin (C-4)	Sc-47778 Mouse monoclonal	1:3000 1%BSA	Wb
Cyclin A2 (H-432)	Sc-239 Rabbit polyclonal	1:1000 1%BSA	Wb
PARP-1 (H-250)	Sc-7150 Rabbit polyclonal	1:1000 1%BSA	Wb
p-ERK (E-4)	Sc-7383 Mouse mono	1:1000 5%BSA	Wb
ERK (D-2)	Sc-1647 Mouse mono	1:1000 1% BSA	Wb
α-tubulin	Rabbit polyclonal (Nick Cowan)	1:1000 1% BSA	Wb
Annexin V FITC	BD Bioscience Pharmigen	5 µL per test	Flow Cytometry

3.4 Transfection and luciferase assay

Luciferase vectors (see table 3) were transfected into Raji cells using Amaxa Nucleofector™ Technology as described below. 10^6 cells were resuspended in 100 µl Mirus Ingenio® Electroporation solution and mixed with final amount 2 µg DNA plasmids. The mixed solution were electroporated in 2 mm cuvettes (VWR) using Raji Amaxa nucleofector program and resuspended in 1.5 mL RPMI complete media. After 12 h cells were clarified and resuspended in fresh media. Cells were harvested 48 h after transfection.

Alternatively, transfection of HEK293T cells was performed using PEI reagent (Polysciences, Inc). Briefly, PEI and DNA were mixed in free serum DMEM in a ratio 2.5:1 PEI:DNA (µg), vortexed and incubated 10-30 min RT. Culture media was replaced by half the amount of serum-free media and mixture of PEI+DNA was added to cells at 60-70% of confluence. After 12 h of transfection mixture of PEI+DNA was removed and cells were supplemented with complete media. HEK293T cells were also harvested 48 h after transfection.

Dual-Luciferase® Reporter Assay System (Promega) protocol was used following manufactures´s instructions. Briefly, the cells were lysed with Passive lysis buffer 5X diluted in H_2O for 10 min on ice. The samples were frozen at -80 ^oC for at least 15 min, although they could be storage this way for long. The lysed cells were thawed and the supernatant was clarified at 14000 rpm for 30 s. The supernatant was collected in a new 1.5 mL eppendorf. 20 µL of cell lysate was load into a 96-well plate. 100 µl of Luciferase Assay Reagent (LARII) containing the firefly luciferase substrate (luciferin) was added and luminescence was measured within 1 min after addition. After quantifying luminescence, Firefly luciferase reaction was quenched with 75 µL of Stop&Glo ®

Reagent that also contains the Renilla luciferase substrate (coelenterazine) so that initiating the second luciferase reaction. Luminescence from both luciferase reactions was measured in GLOMAX Multidetection system (Promega).

Firefly luminescence values were normalized against Renilla luminescence values used as control of transfection for each sample. Measurements were done in parallel duplicates and values were averaged. Relative light units (R.L.U.) were obtained of experimental values related to empty vector (control) values.

3.5 Chromatin immunoprecipitation

Roughly, 2-3 x 10^7 cells were pelleted in a 50 mL falcon, washed with PBS and centrifuged 1500 rpm 5 min. After washing, the cells were fixed with 10 mL PBS-1% formaldehyde, rotating for 10 min and the fixation was blocked with 125 mM Glycine for 5 min keeping the cells in rotation. Supernatant was clarified at 1500 rpm for 5 min and washed twice with PBS, before storage at -80 ^oC.

Cells were lysed with 1.2 mL lysis Buffer (20 mM Tris HCl pH 8.0; 2 mM EDTA; 0.7% SDS; 1% Triton X-100; 150 mM NaCl; H_2O), adding protease and phosphatase inhibitor immediately before used and led the cells 10 min on ice before sonicate on Bioruptor (as previously described) for 10 cycles (30 s ON, 30 s OFF). Supernatants were clarified at 14000 rpm at 4^oC and collected in a new 1.5 mL eppendorf and storage at -80 ^oC.

DNA fragments were checked diluting 30 µL of lysed sample in 170 µL dilution buffer (0.01% SDS; 1% Triton X-100; 1.2 mM EDTA; 20 mM Tris-HCl pH 8.0; 150 mM NaCl; H_2O) and adding 6 µL RNAse (10 mg/mL SIGMA) and 12 µL NaCL (5M). Lysed sample was descrosslinked shaking the sample overnight at 1500 rpm 65 ^oC in Eppendorf ThermoMixer® C. The DNA was purified with Qiaquick PCR purification kit (Qiagen)

following manufactures´s instructions. Briefly, we added 1 mL PB buffer to each sample and 600 µL of the mix to a column provided and centrifuged 30 s at 12000 rpm. We repeated this step with the remaining volume of each sample. We discarded the supernatant and added 750 µL PE buffer and centrifuged two times to remove all the washing buffer. We changed the column from the collected tube and put them in a new 1.5 mL Eppendorf. We added 50 µL EB buffer, wait for 5 min and centrifuged 1 min at 12000 rpm.

DNA fragments were resolved in an 1.5% agarose gel using 12 µL of purified DNA mixed with 3 µL DNA loading buffer 5X (30% Glycerol; H_2O). In order to match amplicon size, DNA chromatin fragments should be 200 to 500 bp long. We later used this lysate as DNA loading control for qPCR assay.

When the fragments have the proper size for our specific amplicons, we proceed to dilute the sonicated lysate seven times to reduce the amount of SDS. The lysate were diluted in Dilution buffer (protease inhibitor cocktail 1:100, added immediately before use). We also added also 2-3 µg of our antibody to the lysate and incubate the mix rotating at 4^{o}C overnight.

After antibody incubation, we proceed to add Dynabeads® Protein G (Invitrogen) to capture our antibody. We added 30 µl of Dynabeads for each sample. First the Dynabeads were washed with Dilution buffer and blocked for 30 min with Sperm DNA salmon (Invitrogen) (1 µg/ mL for each sample), rotating at 4^{o}C. After blocking, we added the beads to the samples and incubated them for 30 min to 2 h, rotating at 4^{o}C.

Then, Dynabeads were washed in cycles of 5 min of washing with the following buffers that we describe below.

Table 6. *ChIP buffers*

Washes	Volume	Buffer	Composition
1	**1 mL**	**Low Salt**	0.1% SDS;1%Triton X-100;2 mM EDTA;20 mM Tris-HCl pH 8; 150 mM NaCl
1	**1mL**	**High Salt**	0.1% SDS;1%Triton X-100;2 mM EDTA;20 mM Tris-HCl pH 8; 500 mM NaCl
1	**1 mL**	**LiCl**	0.25 M LiCl;1%NP 40 (IGEPAL);1% Sodium Deoxycholate;1 mM EDTA;10 mM Tris-HCl pH 8
2	**1 mL**	**TE**	10 mM Tris HCl pH 7.5;1 mM EDTA
1	**200 µL**	**Elution**	1%SDS; 50 mM Tris-HCl pH 7.5

Samples were incubated with Elution buffer shaking at 65ºC for 30 to 40 min, then they were des-crosslinked with 6 µL RNAsa (10 mg/mL) and 12 µL NaCL 5M and incubated shaking at 65ºC, overnight. Once separated chromatin from proteins we added 2 µM EDTA 0.5 M, 4 µL Tris pH 6.5 1 M and 1 µL Proteinase K (20 mg/ mL, Thermo Fisher Scientific) and incubated shaking for 3 h at 45ºC.

DNA were purified with Qiaquick PCR purification kit (Qiagen), as it has been described above.

As the DNA amount obtained after ChIP is extremely low, we performed qPCR with higher amount of each sample compared with RT-qPCR assay. In this case, 4 µL of each sample were used instead of 2 µL. Primers were designed with primer3 software as mentioned above and primer sequence is detailed in **Table 7**.

Table 7. *Primers for ChIP used in this work*

Primer	Forward	Reverse	Product Size
CR2 +325 bp	ATAAACCGCCTGGTCCTGAT	CCTCATGCAGTGGGTAGGTT	235 bp
CR2 +481 bp	AGGATTCTGCAGGTGCTCAT	TGCTTGGGAACCAGGTCTTA	196 bp
CR2 +141 bp	GGGTTTTCTTGGCTCTCGTC	ATTGCAGTGGTCCCTCAAAG	188 bp

CR2 -35 bp	GTGTGCGCTCAGAACTAGCA	CGACGAGAGCCAAGAAAACC	197 bp
CR2 -198 bp	AGTGTAGTGGGTTGCGTGGT	GCGGGCCCTTAAATAGTGTC	212 bp
CR2 -525 bp	CATGCAGAGAATCTGGGTGA	GTGCCATGCTGTGTTATGCT	246 bp
CR2 +2.1 Kb	GCATTCCTTGGAGAAACAGC	CAGTGGGAGCCCTCAAAATA	153 bp
CR2 +2.2 Kb	GGGTGCGGAAACAATGATAC	CAGTGGGAGCCCTCAAAATA	243 bp
LDH-A	TCCTGACTCAGGCTCATGGC	AGACAACCGACCGGCAGA	103 bp
CR2 Exon9	CAAGAAAGAGGCACCTGGAG	CAGCTTTGCAACGAATCTGA	199 bp
CR2 promoter Luciferase construct	GCTTGTCCCACCCTCACC	GTTCCATCTTCCAGCGGATA	221 bp

Results

4 RESULTS

4.1 MYC downregulation upon JQ1 treatment leads to CR2 downregulation in B and T cells

A previous analysis of gene expression profiling by Affymetrix in human leukaemia K562 cells performed in our laboratory (J.C. Acosta, PhD Dissertation) suggested that CR2 could be a gene induced by MYC. Given the important biological roles of CR2, particularly in BL disease, we wanted to test whether CR2 could be a novel MYC target gene. CR2 is highly expressed in B cells and certain populations T cells. Therefore, we treated Raji cells (BL cell line) and Jurkat cells (acute T cell leukaemia) with JQ1. This compound binds and inhibits BRD4-containing proteins of bromodomain and extraterminal (BET) family. JQ1 is known to potently inhibit MYC transcription in leukaemia and lymphoma cells (Mertz et al., 2011). We tested MYC expression in response to JQ1 and, as expected, JQ1 treatment downregulated *MYC* expression at mRNA levels, determined by RT-qPCR **(Figure 4.1A)** and at protein levels, determined by immunoblot **(Figure 4.1B)** in Raji and Jurkat cells. CR2 expression also decreased at the mRNA and protein levels albeit the decreases was less pronounced than that observed in MYC. On the other hand, *MYC* downregulation is often followed by cell cycle arrest and a decrease in cell proliferation (Bretones et al., 2011). The results showed that treatment of Raji and Jurkat cells, with increasing concentrations of JQ1, stop cell growing in a dose-dependent manner **(Figure 4.1C)**.

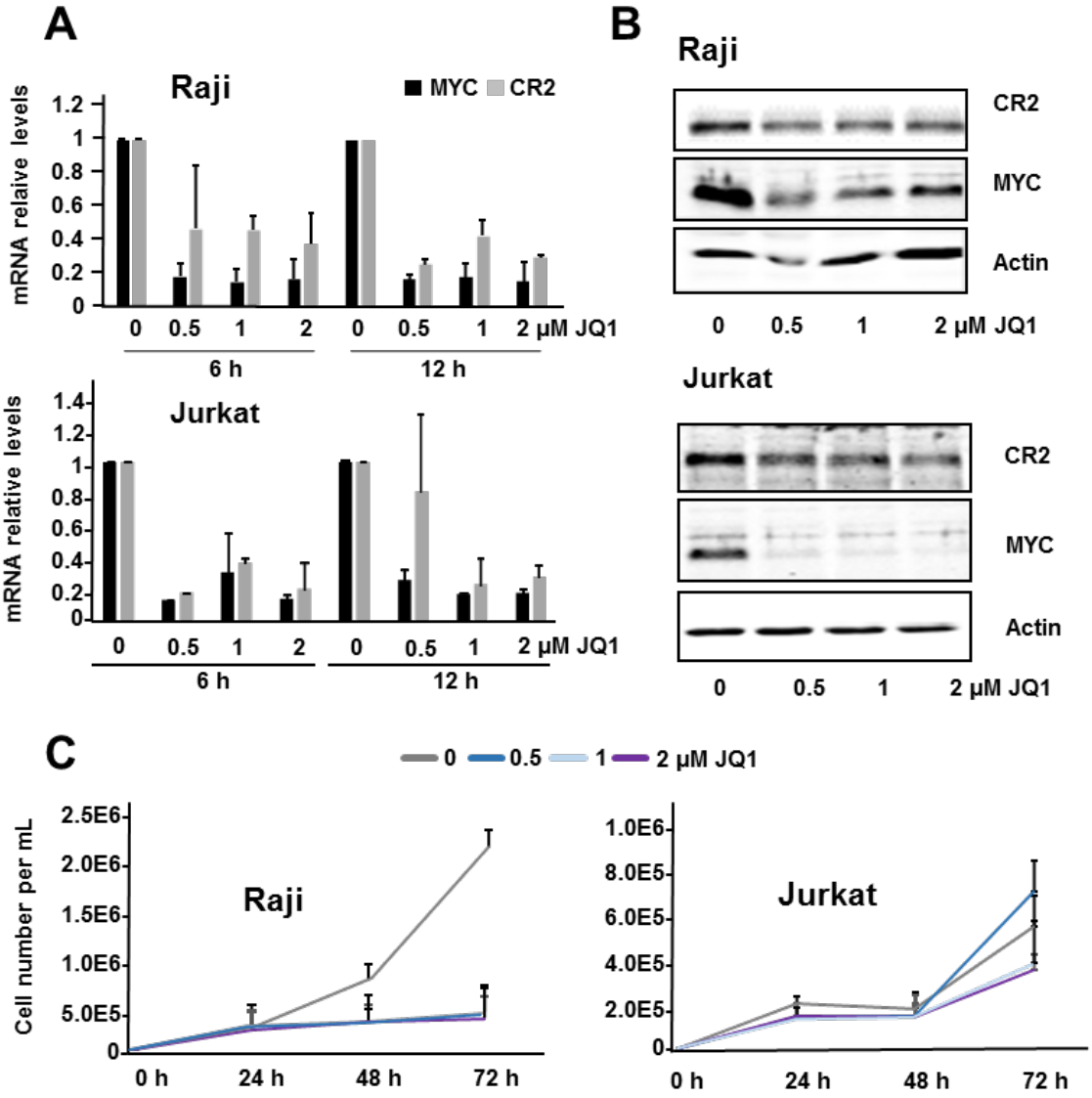


Figure 4.1. Expression of MYC and CR2 mRNA in Raji and Jurkat cells treated with JQ1 at the indicated concentrations for 6 and 12 h. **A**. mRNA levels were determined by RT-qPCR. **B**. Protein levels of MYC and CR2 in Raji and Jurkat cells determined by immunoblot using total lysates. β-Actin levels were determined as protein loading control. **C**. Proliferation arrest of Raji and Jurkat cells treated with the indicated concentrations of JQ1 for 72 h, estimated by cell counting. Data are mean values from two independent experiments ±SD.

It was also noticed an expected arrest in cell cycle in G_0 to G_1 phase **(Figure 4.2A,B)** in both cell lines. Jurkat cells showed a slightly decrease in S phase after 48 h of JQ1 treatment. In contrast, Raji cells showed a sharp decrease in S and G_2 phases even with the lower concentration of JQ1 assayed **(Figure 4.2C)**. The apoptosis analysis showed

an increase of annexin V positive binding both in Raji and Jurkat cells **(Figure 4.3A)**. However, the increase in Raji cells in annexin V binding positive is noticed after 72 h of JQ1 treatment while in Jurkat cells the increase in apoptosis markers is apparent with the lower concentration at short time periods **(Figure 4.3B)**. That implies that JQ1 affected differently both cell lines inducing apoptosis in Jurkat cells. On the contrary, Raji cells suffered a decrease in cell cycle progression and only displayed apoptosis after 72 h of JQ1 treatment.

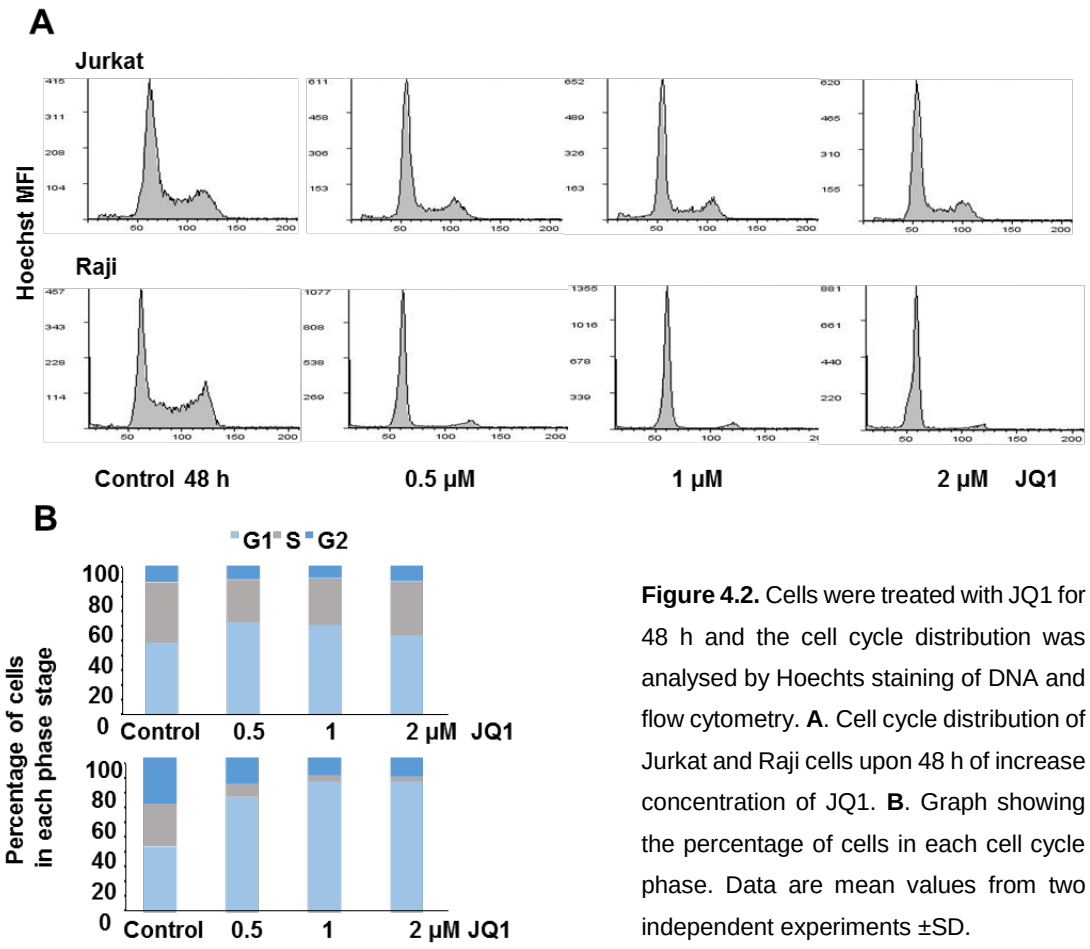


Figure 4.2. Cells were treated with JQ1 for 48 h and the cell cycle distribution was analysed by Hoechts staining of DNA and flow cytometry. **A**. Cell cycle distribution of Jurkat and Raji cells upon 48 h of increase concentration of JQ1. **B**. Graph showing the percentage of cells in each cell cycle phase. Data are mean values from two independent experiments ±SD.

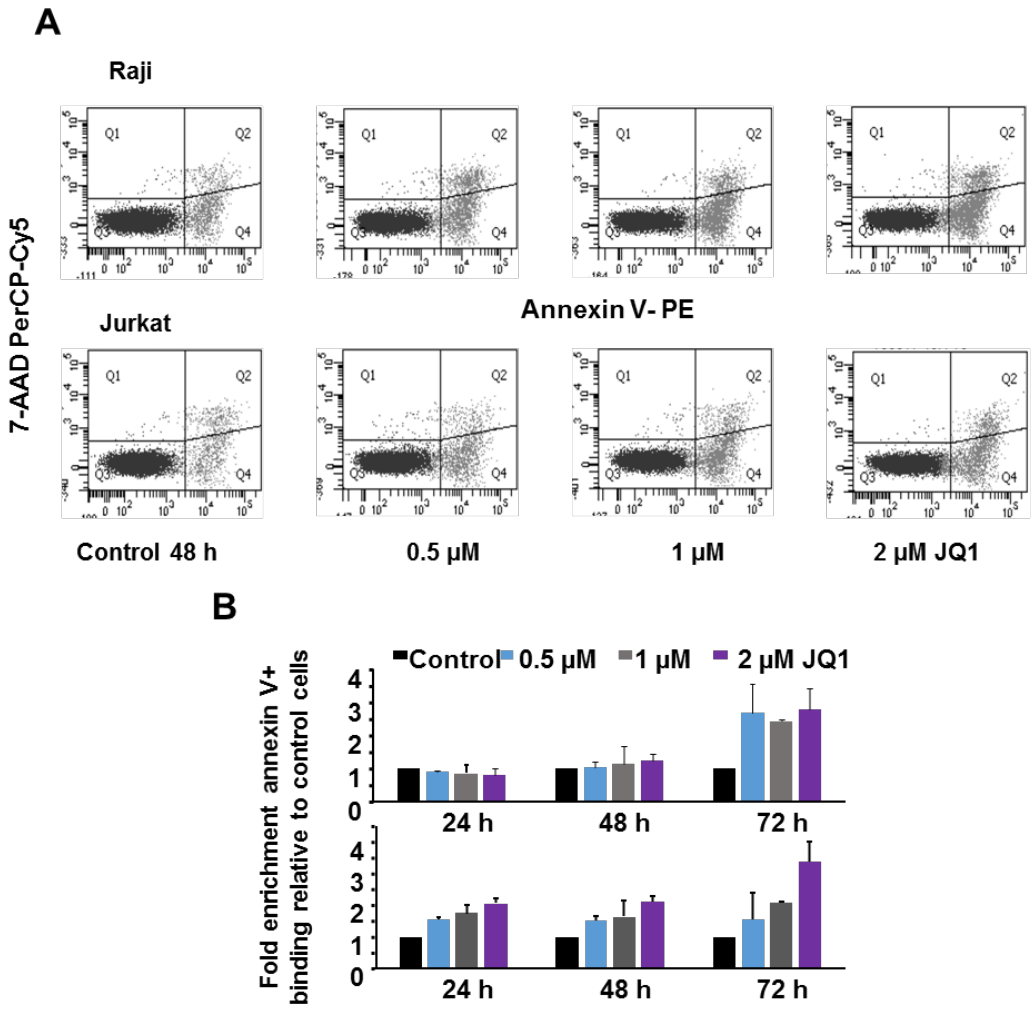


Figure 4.3. Cells were treated with increased concentration of JQ1 for 48 h. Cells were double stained with 7-amino-actinomycin D (7-AAD) and annexin V to distinguish between the necrotic and apoptotic cells. Early apoptotic cells will exclude 7-AAD, while late stage apoptotic cells will stain positively. **A**. The fraction of 7-AAD and annexin V + cells were considered as apoptotic cells by flow cytometry. **B**. Quantification of the data shown in A. The graph shows mean values from two independent experiments ±SD. The results corresponded to the fold increase of apoptotic cells relative to control untreated cells.

Finally, we analysed the protein levels of CR2, MYC, Cyclin A and PARP1 by immunoblot in both cell lines treated with JQ1 for 48 h. PARP1 cleavage was tested in these cells because proteolytic cleavage of PARP1 by caspases is a known hallmark of apoptosis. Cyclin A was used as marker for S phase entry. The results showed that JQ1 treatment resulted in a decrease in Cyclin A and in PARP1 cleavage **(Figure 4.4)**. Raji cells showed

a sharp decrease in Cyclin A while PARP1 protein remain stable. On the other hand, Jurkat cells revealed a smaller Cyclin A decrease protein amount and an important quantity of PARP1 protein in its cleavage form. These findings were in line with the flow cytometry assay of cell cycle profile and the annexin V binding **(Figure 4.2 and 4.3)**.

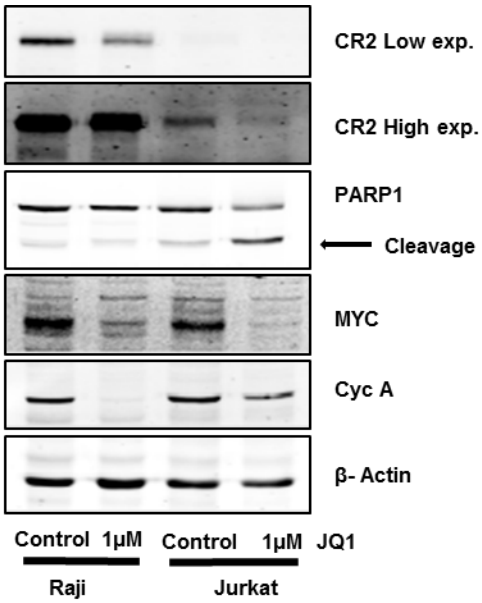


Figure 4.4. Total lysates from Raji and Jurkat cells were extracted 24 h after treatment with JQ1. Immunoblot assay were performed to detect CR2, PARP (entire and cleaved), MYC and CycA. β-Actin were used as loading

In order to know whether *MYC* downregulation upon JQ1 treatment was the major responsible for cell cycle arrest in these cell lines, we used KMYCJ cells, K562 cell line (derived from human myeloid leukaemia) expressing an inducible *MYC* exogenous allele under control of the metallothionein promoter (Acosta et al., 2008). In these cells MYC expression is induced upon treatment with Zn^{2+}. We observed a decrease in proliferation of KMYCJ cells upon JQ1 treatment **(Figure 4.5A)** which is unable to recover *MYC* exogenous expression induced by Zn^{2+}. However, *MYC* overexpression induces CR2 upregulation even in cells arrested **(Figure 4.5B)**. We also confirmed MYC upregulation at protein level **(Figure 4.5C)**. The results also showed that despite the original claim that JQ1 (and derivatives) inhibits the proliferation of lymphoma cells through MYC

downregulation, these cells are arrested by JQ1 even in the presence of high MYC levels.

4.2 MYC silencing in Raji cells downregulates CR2 expression

Although *MYC* downregulation due to JQ1 treatment induces CR2 downregulation, the effect of JQ1 over these cells is not specific on *MYC* (Mertz et al., 2011). Thus, the possibility exists that CR2 downregulation could be due to a direct downregulation of CR2 promoter activity by JQ1 or an indirect effect of this compound through another pathway rather than a result of MYC downregulation. To address this issue we silenced *MYC* expression specifically with short-hairpin mRNAs against *MYC*. We produced lentiviral particles carrying shRNAs against *MYC* and infected Raji cells. Raji cells carrying shMYC were selected with puromycin for further 36 h to eliminate most of the uninfected cells. *MYC* mRNA and protein levels were then analysed in the surviving cells. The expression of MYC was significantly decreased upon lentiviral infection and this decrease paralleled with that of CR2 mRNA and protein levels **(Figure 4.5D)**. The rate of cell proliferation upon lentiviral infection also decreased **(Figure 4.5E)** as expected given the decrease in MYC levels.

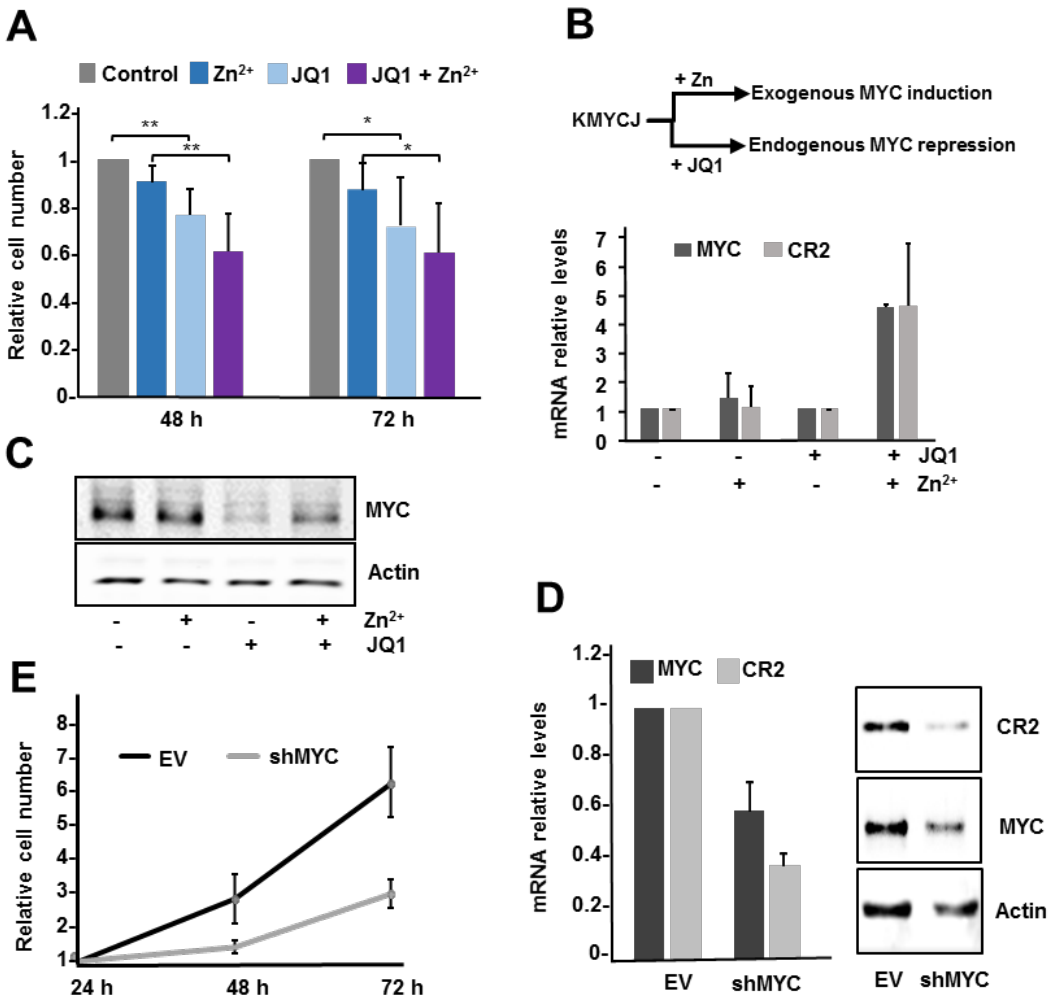


Figure 4.5. A. Cell proliferation of KMYCJ cells treated with 75 µM ZnSO4 and 1 µM JQ1 for 48 and 72 h. Proliferation was measured by cell counting and relativized to that of untreated cells. Data are mean values from four independent experiments ±SD. B. Total RNA was extracted from KMYCJ cells treated for 24 h with 75 µM ZnSO4 and 1 µM JQ1. MYC and CR2 mRNA levels were quantified by RT-qPCR. RPS14 were used for normalization. Data are mean values from two independent experiments ±SD. C. MYC protein levels of Raji cells treated for 24 h with 75 µM ZnSO4 and 1 µM JQ1 determined by immunoblot. β-actin levels were determined as protein loading control. D Raji cells were infected with short-hairpin MYC (shMYC) lentivirus. *MYC* and *CR2* mRNA (left panel) and protein (right panel) expression were assayed by RT-qPCR and immunoblot, respectively. E. Cell proliferation decrease upon MYC repression. The cell counting of Raji cells infected with shMYC or control lentivirus was assayed by cell cytometry. Data are mean values from two independent experiments and normalized to cell counting at the start of the experiment.

4.3 *MYC upregulates CR2 mRNA expression independently of cell proliferation in K562 derived cell line.*

The results so far showed that *MYC* downregulation either upon JQ1 treatment or through specific shRNA downregulation, decreases CR2 expression, suggesting that CR2 could be regulated by *MYC*. However, both assays provoked cell cycle arrest. Thus, it was conceivable that CR2 downregulation in these experimental settings could be a side effect due to cell cycle arrest induced by MYC depletion. To address this issue we treated KMYCJ cells with 10 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) that triggered a dramatic cell cycle arrest in this cell line (Canelles et al., 1997) and with Zn^{2+} to induce *MYC* expression. As expected, TPA induced a proliferative arrest and *MYC* was overexpressed upon Zn^{2+} addition. However, *MYC exogenous* expression was unable to rescue TPA-induced cell proliferation decreased **(Figure 4.6A)**.

Interestingly, we observed CR2 upregulation at mRNA levels that correlates with *MYC* upregulation even when cells remain arrested **(Figure 4.6B)**. As a second approach to study MYC-CR2 interaction in conditions of proliferation arrest, we used another K562 derived cell line, named Kp27MER, that carries a $p27^{KIP}$(CDKN1B, a known cell cycle inhibitor) *allele* under control of metallothionein promoter, as well as a constitutively expressed MYC-ER chimeric protein. This fusion protein becomes activated by 4-hydroxytamoxien (4HT) (Acosta et al., 2008).

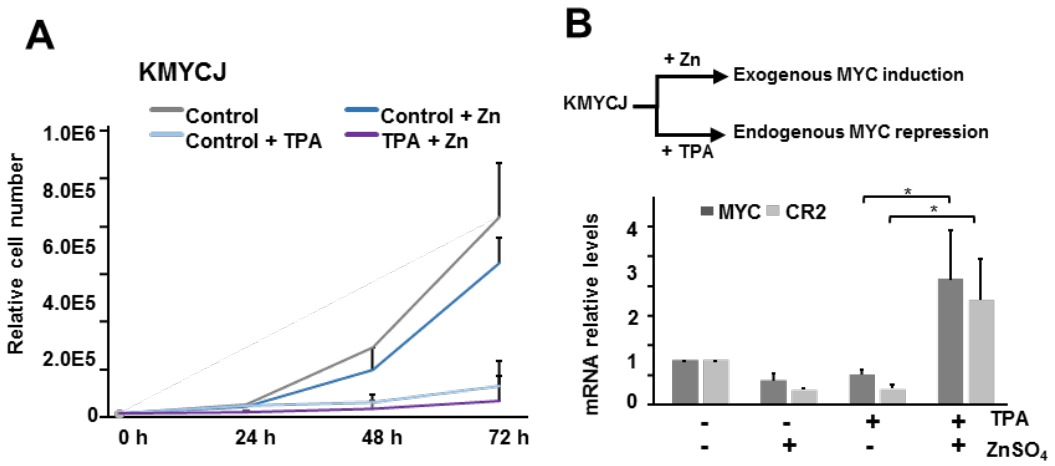


Figure 4.6. A. KMYCJ cells were pre-treated with TPA and 12 h later with Zn^{2+}. Cell counting was determined at different time points. Data are mean values from three independent experiments ± SD. B. KMYCJ cells were treated with 10 nM TPA and 75 µM ZnSO4 as indicated for 48 h and MYC and CR2 total mRNA levels were quantified by RT-qPCR assay. Data are mean values from two independent experiments ± SD.

On the one hand, MYC chimeric version fused with the estrogen receptor (ER) that responds to the ER agonist 4HT is a useful tool. The fusion protein, when expressed in cells, is held in the heat shock complexes in the cytoplasm. When 4HT is added releases MYC-ER and allows nuclear localization and gene regulation (Eilers et al., 1989; Nilsson and Cleveland, 2003). The advantage of this system is that it allows MYC protein activity even in the presence of protein inhibitors, like cycloheximide and thereby preventing a secondary gene regulatory messenger (Cole and McMahon, 1999). MYC-ER protein is degraded very rapidly early after enter the nucleus so MYC target genes have to be analysed within the first hours after activation (Watson et al., 2002).

On the other hand, $p27^{KIP}$ is a CDKs inhibitor and a known cell cycle inhibitor (Bretones et al., 2011). As expected, exogenous expression of $p27^{KIP}$ induce a decrease in cell proliferation after 48h of Zn^{2+} exposure **(Figure 4.7A)**. However, despite this proliferation arrest, we observed an increase in CR2 mRNA expression upon MYC-ER activation with

4HT **(Figure 4.7B)**. As a control for MYC activation we determined the expression of LDHA a MYC *bona fide* target gene, which was up-regulated with 4HT treatment. We conclude that the upregulation of CR2 expression by *MYC* is independent of cell proliferation.

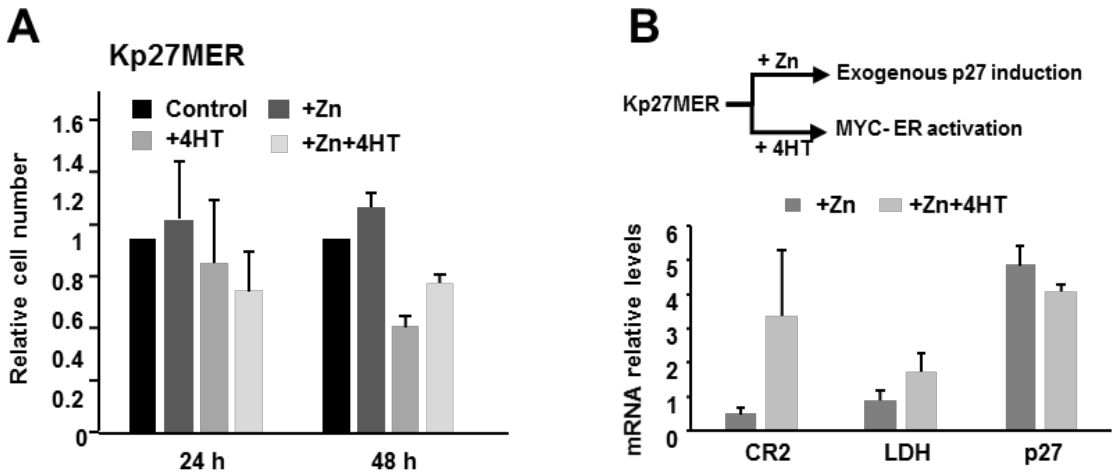


Figure 4.7. A. Kp27MER cells were treated with 200 nM 4HT and 75 µM ZnSO4 as indicated and counted to measure cell proliferation. Data are mean values from three independent experiments ±SD. B. Kp27MER cells were treated as in A and the mRNA levels of CR2, MYC and p27 were measured by RT-qPCR.

4.4 MYC-ER activation upregulates CR2 expression in the absence of protein synthesis.

The former results showed that MYC was able to induced CR2 expression independently from *MYC*-induced effects on proliferation. However, these results do not rule out the possibility that another MYC-target protein not involved in proliferation that could regulate CR2 expression. For example, it was conceivable that another transcription factor induced by MYC was activating CR2 promoter. To test whether CR2 is a direct target of

MYC, we took advantage of a K562 derived cell line expressing MYC-ER named KMER (Acosta et al., 2008).

We treated these cells with the protein synthesis inhibitor cycloheximide to abrogate protein synthesis and we asked for CR2 mRNA expression upon MYC activation in the absence of protein synthesis. It was noted that upon cycloheximide treatment (6 h, 50 µg/ml), MYC protein synthesis was blunted, conversely to the long-lived protein β-Actin **(Figure 4.8A)**. However, the treatment with 4HT activated MYC-ER and induced CR2 mRNA levels **(Figure 4.8B)** even in cycloheximide-treated cells. This result together with those shown above suggest that MYC directly activates CR2 transcription.

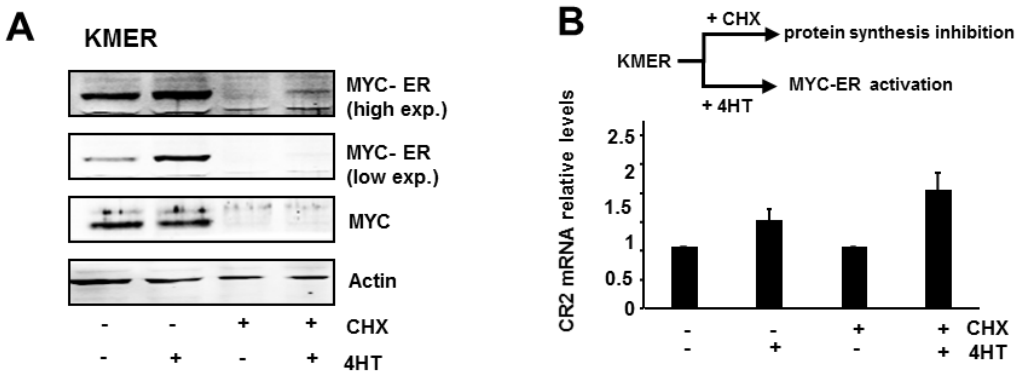


Figure 4.8. A. KMER cells were treated with 200 µM 4HT and 50 µg/ml cycloheximide (CHX) and harvested 6 h after treatment. The levels of MYC (~63 KDa), MYC-ER (~105 KDa) and β-Actin (~43 KDa) were analyzed by immunoblot. β-Actin levels were determined as protein loading control. B. KMER cells were treated as in A and the mRNA of *CR2* were analyzed by RT-qPCR. Data are mean values from three independent experiments ±SD.

4.5 MYC binds directly to CR2 promoter

As predicted in the chromatin immunoprecipitation (ChIP)-seq data generated by the ENCODE Project (UCSC database https://genome-euro.ucsc.edu, Feb. 2019 (GRCh37/hg19)) there are two predicted peaks of MYC and MAX binding in the proximal region of CR2 promoter in K562 cells **(Figure 4.9A)**. In order to confirm MYC binding in BL derived cells we designed different primers along CR2 proximal promoter and in the first intron in Raji cells. Bioinformatic analysis revealed that there are four canonical E-boxes in the region surrounding the CR2 transcription start site. Two of them are located in the promoter near the transcriptional starting site and the other ones in the first intron **(Figure 4.9B)**.

We performed ChIP with anti-MYC antibody in Raji cells and observed an enrichment of MYC binding in the proximal region of CR2 promoter. We used as a negative control the exon 9 of CR2 and a region from the *LDH* promoter region (a *bona fide* MYC target gene) as a positive control for MYC binding **(Figure 4.9C)**. The results indicated that MYC bound to the proximal promoter of *CR2* encompassing two E-boxes clusters in a 20 bp segment mapping 5' close to the transcription start site. The ChIP results are consistent with *MYC* being a direct activator of CR2 transcription.

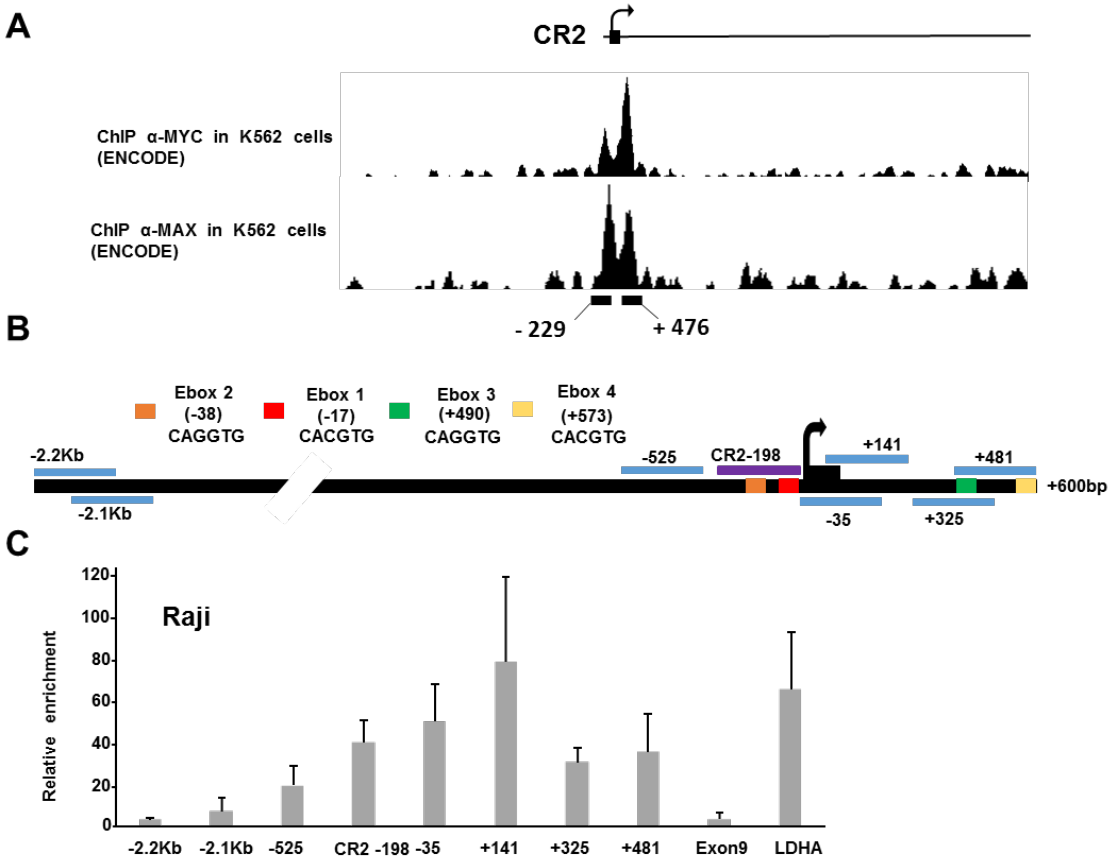


Figure 4.9. A. Schematic representation of *CR2* and MYC-MAX binding sites according with ChIP-seq data from ENCODE project (USC Genome Browser) in K562 cell line. B. Schematic representation of *CR2* proximal promoter and the first exon and intron. The horizontal bars represented the region amplified by different primers used in the ChIP analysis. C. ChIP with anti-MYC antibody on CR2 gene in Raji cells. Exon9 of CR2 gene was used as a negative control and LDHA, a *bona fide* MYC target gene, mapping -85 bp to +19 bp region corresponding to proximal LDHA promoter, was used as positive control. As negative control the chromatin was immunoprecipitated with unspecific IgG. Data are mean values, normalised to IgG, from three independent experiments ±SD.

To confirm this we performed luciferase assays with a reporter harboring 300 bp upstream the *CR2* transcription start site, and containing the two E-boxes. We transfected these construct in K562 and HEK293T cells to test CR2 promoter constructs in non-expressing CR2 cell lines. The results are in accordance with previous results obtained by our collaborators (Cruickshank et al., 2015) **(Figure 4.10A,B)**.

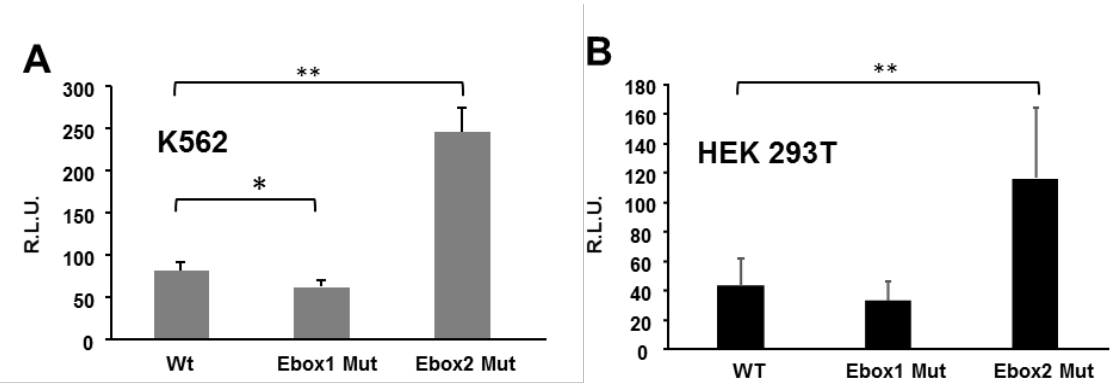


Figure 4.10. CR2 promoter reporters (wild type and carrying mutations in E-Box 1 and E-Box2 as indicated) were transfected into K562 (A) and HEK293T (B) cells. Cells were harvested 48 h after transfection, lysated and the luciferase activity measured. Data are mean values from relative luciferase units (R.L.U.) of three independent experiments ±SD. p-values were calculated by t-Student test. **, $P < 0.05$

We additionally transfected Raji cells with the wild type reporter construct and observed an increase in the luciferase activity compared with the empty vector. Besides, in order to know whether MYC was able to activate CR2 promoter we silenced shMYC concomitantly with luciferase vector transfection and observed that after MYC silencing the luciferase activity dramatically fell **(Figure 4.11A)**. Finally, we performed ChIP assay for MYC binding on these luciferase constructs. HEK293T cells were used because of their high transfection efficiency. The results showed that either E-Box 1 or E-Box 2 deletion significantly decreased MYC binding, as compared with MYC binding observed in the wild type CR2 promoter **(Figure 4.11B)**. Altogether, these results showed that MYC is able to bind CR2 promoter and stimulates its transcription.

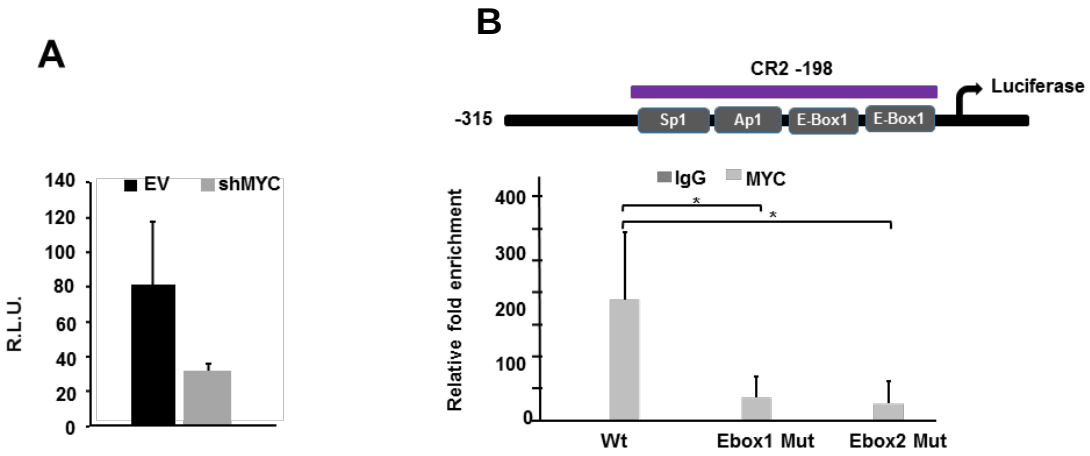


Figura 4.11. **A**. Raji cells were transfected with CR2 promoter luciferase constructs and infected with shMYC lentivirus or control viruses and the CR2 promoter activity was analyzed. The data are mean values from three separate experiments ±SD. **B**. ChIP for MYC was performed on chromatin of HEK293T cells transfected with CR2 promoter luciferase constructs and the mutated E-boxes. Assayed by qPCR, for the amplicon shown schematically above, relative MYC fold enrichment to IgG were represented. Data are mean from two experiments ± SD.

4.6 MYC depletion decreases EBV infection efficiency

So far, our results have established that CR2, the EBV receptor, is a direct target of MYC and that augmented MYC levels increased CR2 protein. Therefore, we next asked whether this increase correlated to a higher infectivity by the EBV.

Raji cells, derived from an EBV-positive BL, express the EBV genes, so they were used as positive control. For the infections we used Ramos cell line, which is a BL cell line but unlike Raji it is EBV-negative. We first depleted MYC in Ramos cells, through shMYC lentiviral transduction and 72 h after lentiviral infection, we incubated the MYC-depleted and control cells with EBV (i.e. B95.8 cells supernatants) for further 48 h. To measure

the level of infection, genomic DNA was prepared from the infected cells and Raji cells, and the EBV DNA from LMP1 and EBNA1 genes was quantified by qPCR.

We first confirmed that Raji cells DNA contained indeed EBV genes and as expected Ramos cells did not **(Figure 4.12A)**. Also, we confirmed that *MYC* (left panel) and CR2 (right panel) expression levels were downregulated at mRNA level after shMYC transduction **(Figure 4.12B)**. Finally, we extracted genomic DNA of Ramos cells exposed to EBV supernatants at 48 h and it was found an increase in the amount of EBV genes over time. Interestingly, cells previously infected with shMYC lentivirus showed less amount of EBV genes in their genome **(Figure 4.12C, D)**. These results indicated that EBV infected less efficiently the cells depleted of MYC.

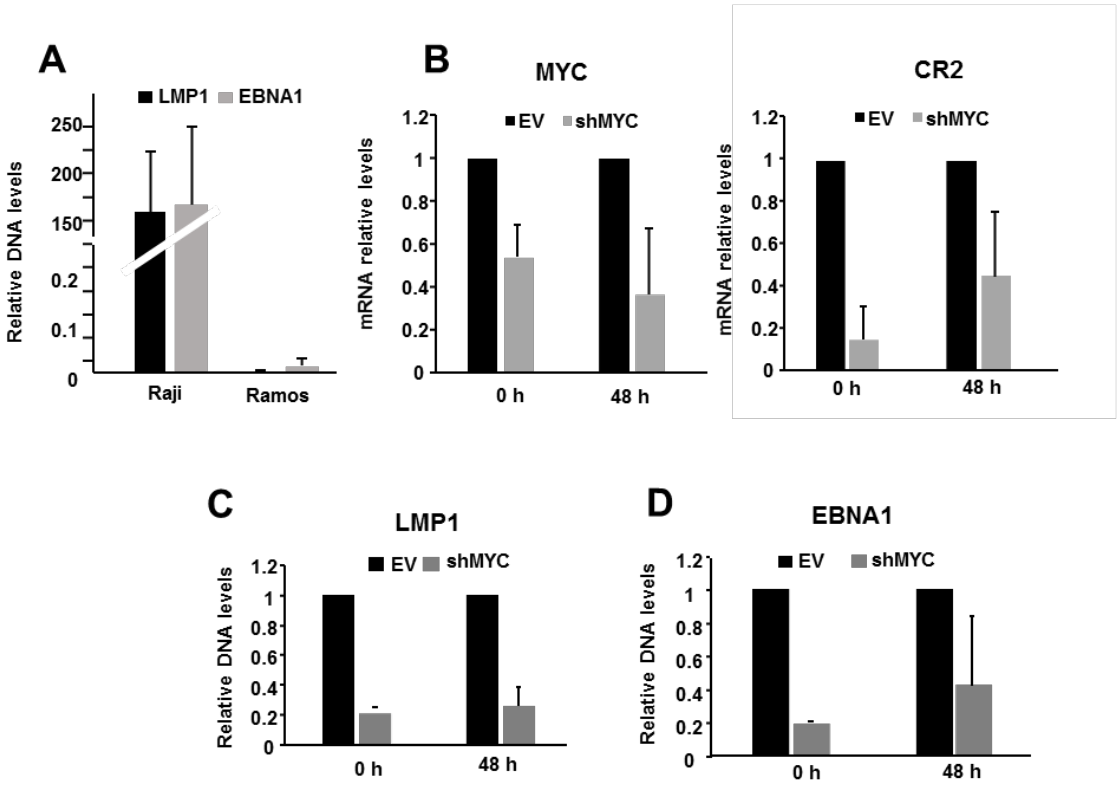
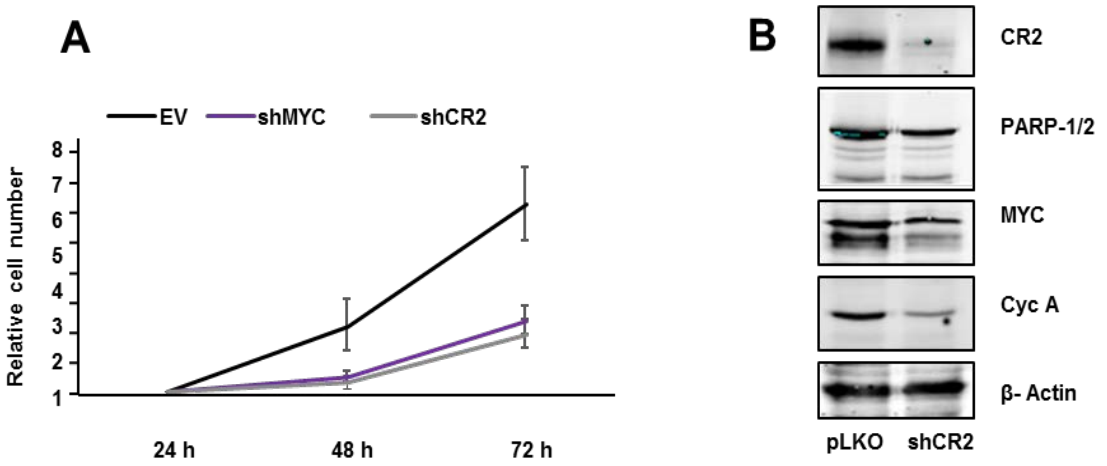


Figure 4.12. A. Raji and Ramos were infected with EBV, total DNA were extracted and relative DNA levels of LMP1 and EBNA1 viral genes were analyzed by PCR. The data was normalized to the DNA levels of LDHA and CR2 (same genomic regions used in ChIP experiments of Figure 4.9). **B**. MYC (left graph) and CR2 (right graph) mRNA levels of Ramos cells infected with shMYC lentiviral particles. **C & D**. Ramos cells were infected with EBV (supernatant from B95.8 stablished cell line) 48 h after infection, total genomic DNA was extracted and quantified by qPCR. Relative total DNA amount of LMP1 and EBNA1 genes from EBV virus were represented. Data are mean values from two independent experiments ±SD.

4.7 CR2 silencing reduces cell proliferation in Raji cells

Given the fact that CR2 is an important co-receptor of BCR we asked the biological relevance of CR2 in our model. If we assume that MYC regulates CR2, this MYC-CR2 pathway could be critical for MYC-induced B cell lymphomas. In order to analyse the CR2 importance on B cells we specifically silence CR2 expression through lentiviral shRNA against CR2. We infected Raji cells for 36 h, selected them with puromycin for further 36 h and measured cell densities for 72 h longer. The results revealed that Raji cells with depleted CR2 suffered a proliferation arrest almost at the same extent as when we silenced MYC expression **(Figure 4.13A)**. Moreover, the absence of cleavage PARP1 and the downregulation of cyclin A indicated that CR2 downregulation also induced cell cycle arrest instead of apoptosis on this cell line **(Figure 4.13B)**, an effect similar to that induced by MYC depletion in these cells.

We also asked which pathway could be CR2 inducing cell cycle arrest in this cell line. Taking to account that CR2 is a BCR co-receptor there are three major pathways described whereby CR2 could be exerting its function: the canonical NF-κB pathway, the MAP kinases pathway or the PI3K-AKT pathway. As mentioned above, in BL cells the NF-κB pathway is shut down, therefore we investigate whether CR2 was affecting the MAP kinase pathway. Preliminary results suggested that CR2 could indirectly contributed to the activation of MAP kinase pathway, as ERK phosphorylation was diminished after CR2 silencing **(Figure 4.13C)**.



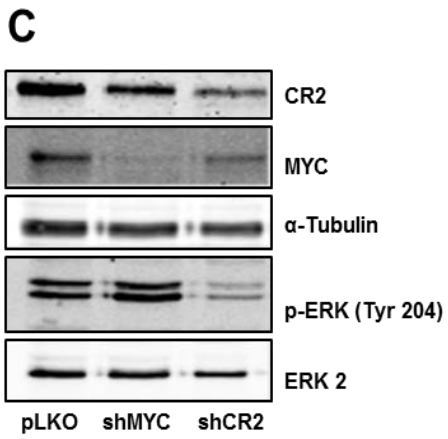


Figure 4.13. Raji cells infected with lentiviral particles against CR2. **A**. Cell proliferation upon CR2 repression. Raji cells infected with shCR2 were counted by flow cytometry. Data are mean values from two independent experiments and normalized to cell counting at the start of the experiment. **B**. Immunoblot of Raji cells infected with lentiviral particles shCR2 for 48 h. Immunoblot confirms CR2 silence. CycA and PARP1 protein levels were assayed. **C.** Immunoblot assay to detect CR2, MYC, p-ERK and ERK 2 (~43 KDa). α-Tubulin were used as loading control (~50 KDa).

Discussion

5 DISCUSSION

5.1 JQ1 treatment downregulates MYC and CR2 expression in lymphoid cells.

The first clue suggesting that CR2 could be a MYC target gene is that in B and T derived cell lines (Raji and Jurkat cell lines), MYC downregulation provoked by JQ1 is followed by CR2 downregulation. JQ1, as other inhibitors of the bromodomain and extraterminal (BET) family of epigenetic readers, has been introduced in clinical assays of B cell malignancies because of their repressive effect over *MYC* (Mertz et al., 2011). It has been postulated that immunoglobulin enhancers are more dependent on bromodomain proteins and subsequently more susceptible to BRD4 inhibition. Thus, MYC expression is remarkably affected in BL cell lines owing to its translocation into the immunoglobulin enhancers (Lopez et al., 2019). However, as JQ1 provokes a decrease in cell proliferation, it is feasible that CR2 downregulation could not be related to MYC levels but to the cell cycle arrest.

Using cells engineered with inducible MYC alleles we observe that CR2 responded to MYC induction even in arrested cells. An interesting result is that JQ1 is able to stop cell proliferation even when MYC expression is induced in K562 cells. These results suggest that the anti-tumoural effect of JQ1 (and likely other BRD4 inhibitors) not only depends on MYC repression. These results are in line with previous studies performed in DLBCL cell lines, in which ectopic expression of MYC was unable to recover cell proliferation arrest induced by JQ1 (Chapuy et al., 2013). Importantly, we have demonstrated that

besides the effects of JQ1 inducing MYC downregulation, both at mRNA and protein levels, it also provokes CR2 downregulation in different lymphoma cell lines.

5.2 MYC directly regulates CR2

MYC downregulation upon JQ1 treatment in Raji and Jurkat cells correlate with CR2 downregulation. In order to check whether CR2 regulation by MYC is independent of cell cycle progression we used two different cellular models both derived from K562 cells. Kp27MER cells overexpress p27, a potent cell cycle inhibitor, upon zinc addition. p27 overexpression induces cell cycle arrest in this cell line and provokes endogenous MYC downregulation. MYC-ER is activated by 4HT but MYC cannot rescue the cell cycle arrest. However, MYC-ER activation is able to induce CR2 even in the absence of cell cycle progression.

Similar results have been observed with KMYCJ cells treated with TPA. These cells carry a Zn^{2+}-inducible MYC allele. In our laboratory, it has been previously described that these cells become arrested upon addition of TPA at low concentration (Canelles et al., 1997). This also induces downregulation of MYC endogenous expression. After adding zinc we induced MYC ectopic expression but this upregulation was not enough to recover TPA-induced cell cycle arrest at the concentrations used in our experiments. Nevertheless, we detected an increase in CR2 mRNA levels upon MYC ectopic expression while the cells remained arrested.

MYC-ER chimeric protein can be used to identify MYC target genes in a context of protein synthesis inhibition. We used KMER cells to investigate CR2 regulation by MYC. If a second protein regulated by MYC was the responsible of CR2 upregulation, inhibition of protein synthesis should avoid this regulation. In contrast, our results showed that CR2

expression is upregulated after 4HT addition in the absence of protein synthesis, suggesting that CR2 is, in fact, a MYC direct target gene.

5.3 MYC binds and upregulates CR2 promoter

MYC binding to CR2 promoter is observed in myeloid K562 cells and published by ENCODE Project (UCSC database https://genome-euro.ucsc.edu,). As there are not BL cell lines analysed in this database we checked whether MYC binds CR2 gene promoter by ChIP. We observe MYC binding sites close to CR2 TSS (in a region containing two E-Boxes) and along the first intron. This is not surprising since MYC often regulates its target genes through binding to their first intron. Reassuringly, MYC binding over CR2 promoter in Raji cells correlates with that published for K562 cells in the ENCODE project.

MYC not only binds CR2 promoter by ChIP but also activates CR2 promoter through luciferase-reporter experiments. We measured CR2 promote activation in cell lines such as K562 and HEK293T cells, observing MYC-mediated activation of the promoter. This activity decreases when MYC is knocked down using lentiviral transduction of shMYC constructs, confirming that MYC stimulates CR2 promoter activity.

Besides, in order to ensure MYC E-box dependent binding we also transfected the mutants in HEK293T cells and performed ChIP of MYC in these cells. The results clearly show that MYC binding in these luciferase constructs is severely impaired in those with the mutated E-box.

In summary: (i) MYC activation using the MYC-ER chimera revealed that MYC activates CR2 transcription in the absence of protein synthesis; (ii) luciferase reporter showed that MYC activates CR2 promoter, and (iii) ChIP experiments demonstrated that MYC binds

CR2 regulatory elements and that binding of MYC to CR2 promoter depends on E-Box elements. All these observations indicate that CR2/CD21 is a *bona fide* MYC target gene.

Moreover, our collaborators have shown that mouse splenic B cells with higher MYC levels also express higher CR2/CD21 levels as assessed by flow cytometry (Virginia Yébenes and Almudena Ramiro, CNIC, Madrid). Taken together these results strongly suggest that CR2 is a new MYC target gene. However, this is not to say that MYC is the only regulator of *CR2*. Other transcription factors are known to recognize and regulate CR2 promoter, as SP1, AP1 and E2A (Cruickshank et al., 2015; Ulgiati et al., 2002). Actually, and therefore MYC levels might not be the only determinant of CR2 levels depending on the cell type or cell differentiation stage. Actually, cancer expression databases reveal that there is not a significant correlation between MYC and CR2 RNA except in B-chronic lymphocytic leukemia (*In silico* Transcriptomics, http://ist.medisapiens.com/login/?next=/). So we hypothesize that CR2 expression is more dependent on MYC during the first stages of lymphoma development but in fully developed BL nodes *CR2* expression does not depend predominantly on MYC.

5.4 EBV infection is less efficient in MYC depleted conditions

We asked whether MYC-dependent CR2 expression had an effect on the efficiency of EBV infection. In order to test this we used a BL cellular model EBV negative to test whether MYC dose and subsequently CR2 levels could be critical to allow the entrance of the virus into B cells. In this context, we infected BL EBV negative cell line (Ramos) with lentiviral particles encoding shMYC to reduce MYC expression and with EBV virus.

Our results revealed that those cells with less MYC were less infected than the corresponding controls.

The canonical theory to explain the link between MYC translocation and EBV infection in BL postulates that EBV genes collaborate or induce MYC translocation and hence B cell malignant transformation. However, our results show that CR2 regulation by MYC could be at least partially responsible for the correlation between BL and EBV infection. MYC upregulation owed to *MYC* translocation would increase the expression of CR2 protein, thus increasing the probability of EBV infection. Both models are depicted in **Figure 5.1**.

According to these results, we hypothesized a “reverse” mechanism by which BL EBV negative cell line could be less efficiently infected upon MYC downregulation. This alternative mechanism is also consistent with the non-transforming EBV genes expressed on BL cells and the absence of MYC translocation in other tumours where EBV infection is prevalent, as nasopharyngeal carcinoma or gastric cancer (Lin et al., 2014; Rowe et al., 1986; Wang et al., 2014).

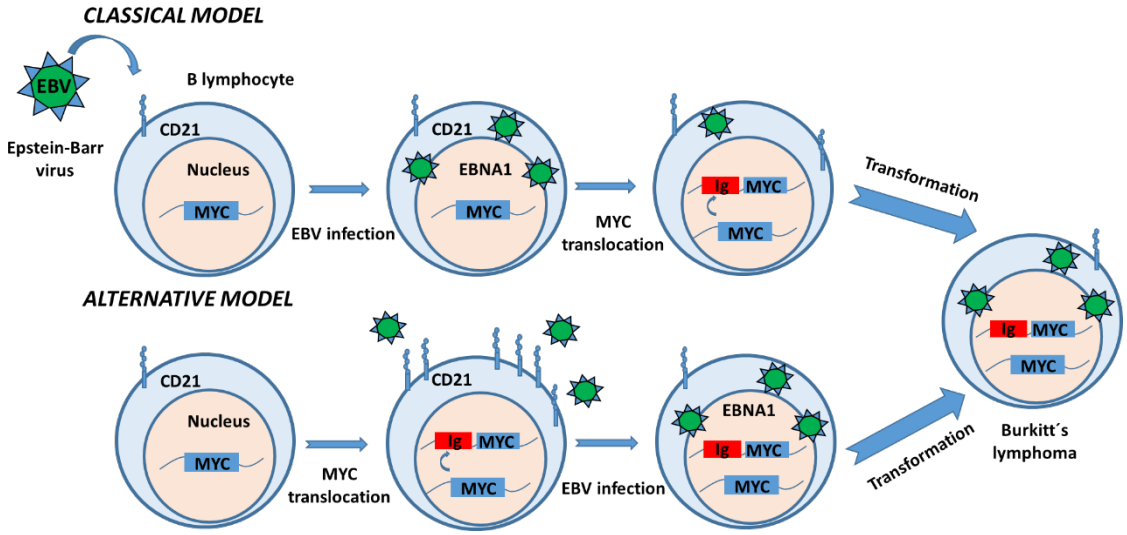


Figure 5.1. Two non-mutually exclusive models depicting the development EBV positive BL cases. In the "classical "model, EBV infection occurs first and promotes MYC translocation. The results presented here allow the proposal of an alternative hypothesis by which *MYC* translocation occurs stochastically in some B cells leading to the overexpression of the EBV receptor, CR2, thus facilitating EBV infection. EBNA1 protein is the only EBV latent protein consistently expressed in BL cells.

In summary, we propose two different scenarios in which EBV infection is related to BL disease. One of them involves EBV infection as a key factor for tumour development. EBV-induced BL model could fit with eBL cases as almost all of them are EBV positive. On the other hand, sBL cases have a minor association with EBV infection. This fact is in line with a second scenario in which MYC translocation could be triggered and enables EBV infection as more viral receptor (CR2) for the virus is available to engage B cells. Both models correspond with the observations gathered until now that propose a GC origin of BL disease and the advantages provided by either MYC translocation or EBV infection for the development of the illness **(Figure 5.2)**.

In any case, MYC and CR2 link in BL provides new insights in the understanding of this disease. Further research should be undertaken to investigate the potential of CR2 regulation by MYC as a new therapeutically approach to BL treatment.

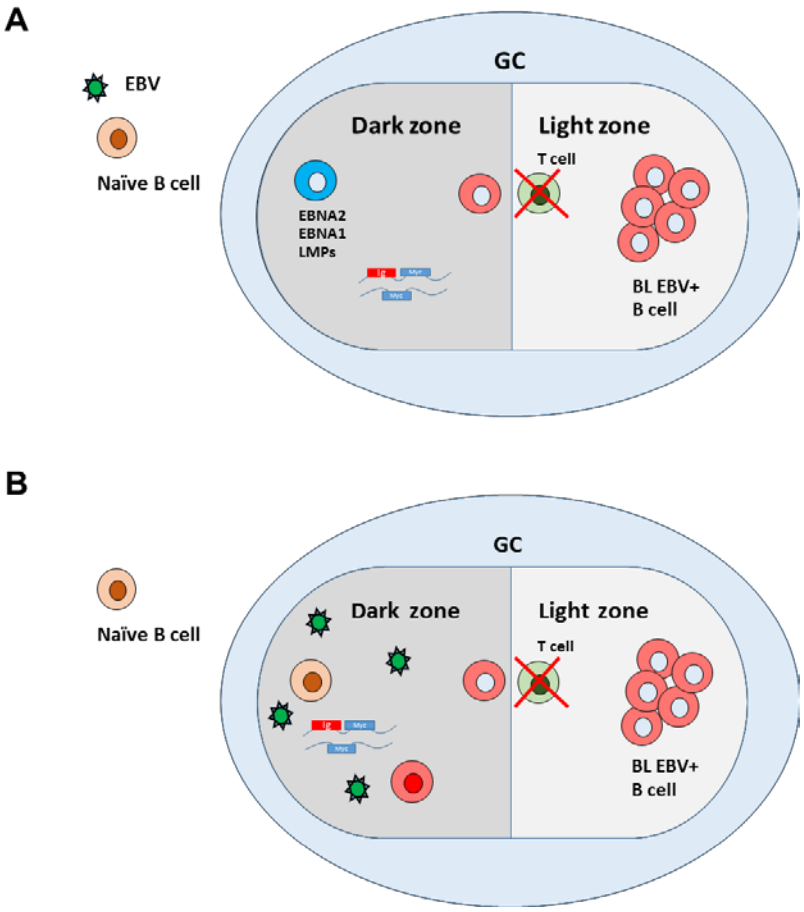


Figure 5.2. Two different models of BL EBV+ **A**. The classical model describes a naïve B cell infected with EBV that begins to express the latency viral genes and their expression provokes MYC translocation. EBV latent genes protect these cells from MYC-induced apoptosis and allow these B cells to pass GC reaction. **B**. A naïve B cell enters into lymphoid tissue and suffers MYC translocation due to a failure in SHM or CSR. This translocation increases the possibility of EBV infection owe to an increase of CR2 receptor availability. These B cells expressed different survival signals and could passed GC reaction avoiding negative selection.

5.5 Implications of MYC-CR2 pathway on B cells

CR2 as a co-receptor of the BCR is essential for B cell differentiation process. For instance, it has been described that CR2 deficiency leads to immunodeficiency problems not only in mouse models but also in humans (Nichols et al., 2015; Pappworth et al., 2009; Thorarinsdottir et al., 2015). Therefore, CR2 function in B cells is crucial for a proper immune response.

We have demonstrated that CR2 silencing on BL cells reduces cell proliferation. It has been described in BL cell lines studies, that BCR signaling enhances downstream pathways that eventually upregulate MYC expression (Benhamou et al., 2016; Sander et al., 2012; Sander and Rajewsky, 2012). MYC upregulation leads to CR2 upregulation, which in turns enhances BCR signaling. Thus, MYC can induce this positive feedback loop maintaining cell proliferation and survival through its target gene, CR2.

Furthermore, CR2 can act through the BCR by three different pathways: the “canonical” NF-κB pathway, the MAP kinase pathway and the PI3K pathway (Vaque et al., 2014). As mentioned before, different studies of BL expression profiles have demonstrated that NF-κB target genes are inactive in BL cells. On the contrary, PI3K pathway has been proven as a key pathway to promote B cells differentiation and survival (Benhamou et al., 2016; Bouillie et al., 1999; Lottin-Divoux et al., 2006) **(Figure 5.3)**.

We used ERK phosphorylated protein as a marker of MAP kinase activation and we observe that after CR2 downregulation ERK phosphorylation is also downregulated. These preliminary results suggest that in CR2 depleted conditions BCR signaling is weaker and could not activate MAP kinase pathway, which eventually affects B cell proliferation.

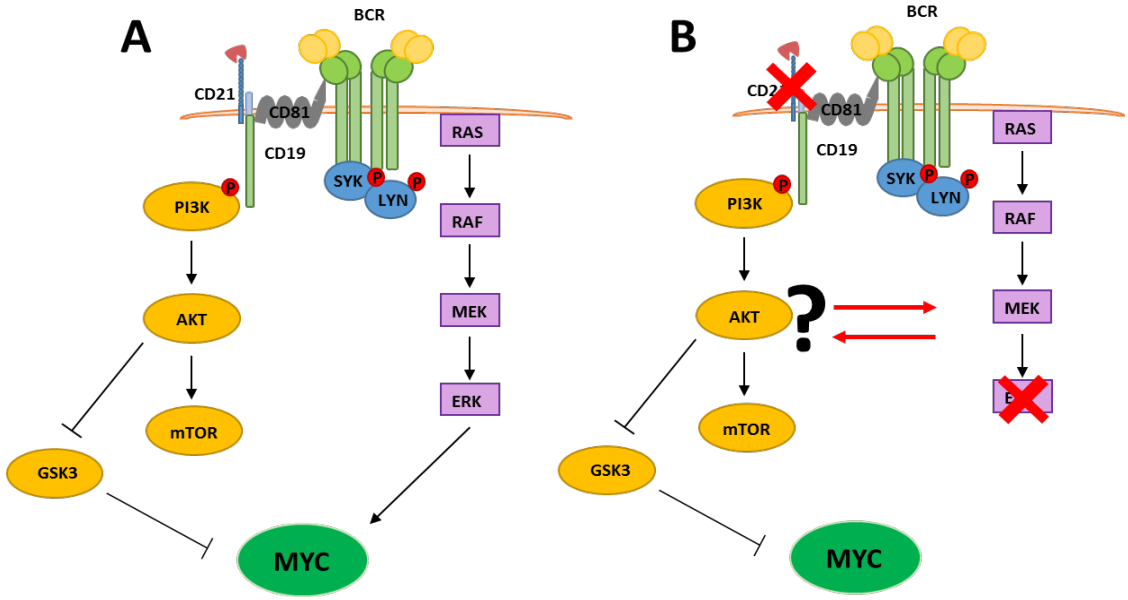


Figure 5.3. Scheme of BCR downstream signaling under CR2 normal and depleted conditions. **A**. After BCR activation spleen tyrosine kinase (SYK) and SRC-family protein tyrosine kinases, such as LYN, activate downstream PI3K-AKT and MAP kinase pathways, which promote cell proliferation. MYC became activated. AKT inhibits GSK3, which phosphorylates MYC at Thr-58 or Ser-62 and marks MYC for its degradation *via* proteasome. MAP kinase pathway activation upregulates MYC expression by different mechanism e.g ERK protein could activate MYC promoter directly. **B.** CR2 depleted conditions, BCR signaling is reduced and ERK and MYC protein levels are downregulated.

It has been described a crosstalk between PI3K and MAP kinase pathway in different tumoural cell lines (Khiem et al., 2008; McCubrey et al., 2007; Rhim et al., 2016). Cell survival and proliferation of tumoural cells often rely on the activity of these pathways. Although, PI3K-AKT pathway is crucial for B cell differentiation and survival the role of MAP kinase pathway in B cells is less known (Khiem et al., 2008). However, MAP kinase pathway is one of the most deregulated pathways in cancer development so it is not surprising that it could be playing an important role in B cell lymphomagenesis.

Since we have not been able to detect PI3K activity upon CR2 downregulation we cannot conclude whether ERK phosphorylation decreased is a direct effect of MAP kinase pathway inactivation upstream or an indirect effect due to BCR reduced signals, through

PI3K pathway. Further research is necessary to unravel the mechanisms by which CR2 downregulation affects BL cells proliferation.

Conclusions

6 CONCLUSIONS

1. MYC silencing in B and T derived cell lines, mediated either by JQ1 or with short hairpin RNA against MYC, induces CR2 downregulation.
2. MYC exogenous expression is able to upregulate CR2 mRNA levels, even when the cells remain arrested. Therefore, CR2 upregulation by MYC is proliferation independent.
3. In the absence of protein synthesis MYC is able to upregulate CR2 mRNA levels. This suggests that CR2 is a MYC direct target gene.
4. In Raji cells MYC binds along CR2 proximal promoter and in the first intron. Furthermore, luciferase assay experiments point out that MYC activates CR2 promoter and upregulates its expression.
5. MYC downregulation followed by CR2 decrease induces less efficient EBV infection in BL cells.
6. Thus, we propose an alternative hypothesis in which MYC levels (due to translocation or other mechanisms) show higher CR2 expression and therefore higher susceptibility to EV infection. This hypothesis is not mutually exclusive with the current assumption.
7. Our hypothesis fits better with sBL cases in which not all the cases are EBV positive, but all of them carry MYC translocation.
8. Our results suggest that CR2-induced proliferative arrest in Raji cells is performed *via* MAP kinase pathway. Further research is required to unravel the mechanism through which CR2 downregulates the activity of this pathway, in BL cells.

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Resumen en castellano

8 RESUMEN EN CASTELLANO

8.1 Introducción

8.1.1 Biología de MYC y su implicación en cáncer

MYC es un factor de transcripción oncogénico de la familia de proteínas hélice-lazo-hélice-cremallera de leucinas o HLH-LZ. MYC está desregulado en aproximadamente la mitad de los tumores humanos (Conacci-Sorrell et al., 2014a; Dang, 2012; Delgado and Leon, 2010a; Vita and Henriksson, 2006). MYC forma heterodímeros con la proteína MAX, también de la familia HLH-LZ, a través del dominio de cremallera de leucinas (LZ). Los dímeros MYC-MAX son la forma activa, uniéndose a secuencias de DNA específicas llamadas secuencias o cajas E (E-boxes, secuencia canónica CACGTG) en las regiones reguladoras de los genes. Se ha estimado en 1.000 a 15.000 el número de sitios de unión a MYC en los diferentes modelos estudiados, con uno o más sitios de unión a MYC en el 15% de los genes humanos, estimándose en unos 1.000 los genes regulados por MYC sea positiva o negativamente, pues casi la mitad de ellos son reprimidos por MYC (revisado en (Meyer and Penn, 2008).

Debido al amplio número de genes que regula, que a su vez están implicados en prácticamente todas las funciones esenciales dentro de la célula, MYC se encuentra desregulado en al menos la mitad de los tumores humanos (Dang, 2012; Schaub et al., 2018; Walz et al., 2014). Su alteración es especialmente relevante en enfermedades hematológicas, tales como leucemias o linfomas, en las que, además, la desregulación de MYC suele ser indicativo de un peor pronóstico de la enfermedad (María G. Cortiguera, 2015).

8.1.2 MYC en linfomas y centro germinal

MYC se encuentra frecuentemente alterado en la mayoría de linfomas y leucemias humanas. La desregulación de MYC en estas enfermedades está asociado frecuentemente a un peor pronóstico de la enfermedad y a una mayor mortalidad (Cai et al., 2015; Nguyen et al., 2017).

Al contrario que en otros tipos tumorales, MYC se encuentra frecuentemente traslocado en linfomas y leucemias, generalmente en regiones cromosómicas codificantes para distintos componentes del sistema inmune, tales como el TCR o el BCR y en los genes que codifican para las inmunoglobulinas. La consecuencia de esta translocación es una sobreexpresión de MYC fuera del control endógeno de su propio promotor (Delgado and Leon, 2010b; María G. Cortiguera, 2015).

La mayor parte de los linfomas humanos tienen origen en células B de centro germinal. Los centros germinales son estructuras dinámicas que se forman en el interior de los nódulos linfáticos o el bazo. Estas estructuras se generan a partir de la activación de linfocitos B foliculares para su encuentro con los linfocitos T dependientes de antígeno. Durante una respuesta inmune normal los centros germinales se forman para permitir la maduración de linfocitos B, su proliferación y diferenciación, con el objeto de generar células B de memoria o células plasmáticas (Allen et al., 2007a; Allen et al., 2007b; Victora and Nussenzweig, 2012).

Las células B naïve que entran dentro del centro germinal expresan BCL6, un gen muy importante en la regulación del centro germinal. Una vez dentro del centro germinal estas células B sufren un proceso denominado hipermutación somática y una expansión clonal, incrementando de este modo la población de linfocitos B con alta afinidad. Cuando estos linfocitos han dejado de proliferar en la zona oscura del centro germinal pasan a la zona clara donde se encuentran con las células T foliculares. En este paso

la expresión de BCL6 baja y la expresión de MYC aumenta ya que desaparece el efecto represor de BCL6 sobre el promotor de MYC. En la zona clara del centro germinal los linfocitos B sufren el cambio de clase de las inmunoglobulinas. Los linfocitos que hayan sido seleccionados podrán diferenciarse a linfocitos B de memoria o a células plasmáticas secretoras de anticuerpos. La diferenciación a célula plasmática se produce cuando aumenta la expresión de BLIMP1 que a su vez reprime la expresión de MYC (Dominguez-Sola et al., 2012; Klein et al., 2003; Victora et al., 2012).

Muchos autores sostienen que el papel de MYC en centro germinal, principalmente en la zona clara, es determinante para dirigir la diferenciación de los linfocitos B hacia células de memoria, en lugar de células plasmáticas y además evitar su eliminación por selección negativa y muerte por apoptosis (Dominguez-Sola et al., 2012).

En resumen, los centros germinales son estructuras de alta inestabilidad cromosómica, en el que se dan alteraciones de los linfocitos B necesarios para una correcta respuesta inmune. Aunque es un proceso estrictamente regulado, errores en cualquiera de las vías celulares implicadas en la maduración de linfocitos, da lugar a distintos tipos de linfomas. La mayoría de los linfomas humanos conocidos proceden de células B con origen en el centro germinal. Muy frecuentemente la translocación de genes importantes para la homeostasis del centro germinal tales como *BCL6*, *BCL2* o *MYC* está presente en este tipo de tumores. Estos linfomas se caracterizan por ser especialmente agresivos y tener un peor pronóstico.

8.1.3 Linfoma de Burkitt

El linfoma de Burkitt (BL) es el más común de los linfomas no-Hodgkin en niños y adolescentes, aunque también supone aproximadamente un 30% de los casos en adultos mayores de 60 años, lo que en números absolutos excede los casos en niños.

Una de sus características principales es la translocación del oncogen *MYC* en el locus de las inmunoglobulinas (Ott et al., 2013; Said et al., 2014).

Existen dos variantes clínicas del BL. La primera es el BL endémico propio de la región ecuatorial de África, el cual se caracteriza por estar asociado con el virus del Epstein Barr (EBV) en prácticamente todos los casos (Magrath, 2012; Ott et al., 2013; Yustein and Dang, 2007). Esta variante afecta principalmente a niños y en concreto a niños varones. Por último, existe el BL esporádico, que afecta principalmente a pacientes inmunosuprimidos de Europa y los Estados Unidos. Su incidencia es de 3 millones de personas al año, afectando más a varones. Aproximadamente el 30% de estos casos están asociados igualmente al EBV (Said et al., 2014).

8.1.4 EBV

El EBV pertenece a la familia de los herpesvirus y sus dianas principales son las células epiteliales y los linfocitos B. Además del BL, está asociado a otros tipos de neoplasias, tales como, el carcinoma gástrico o el carcinoma nasofaríngeo. La infección primaria es silenciosa, pero puede dar lugar a enfermedades infecciosas tales como mononucleosis infecciosa y ciertos tipos de enfermedades linfoproliferativas. La unión del virus se produce a través de la interacción de la glicoproteína viral gp350 y un receptor del complemento (CR2/CD21) del linfocito huésped (Yustein and Dang, 2007).

El EBV entra en sus células diana por endocitosis. Una vez dentro comienza a expresar los genes virales encargados principalmente de mantener las células proliferando, evitar la apoptosis y evadir el sistema inmune del huésped. La expresión de los genes virales depende del estado de latencia del virus. En el caso de estado de latencia III, característico de enfermedades linfoproliferativas tras un transplante o linfomas asociados con virus como el SIDA, se expresan los genes nucleares (*EBNA1*, -2, -3A, -

3B, -3C y –LP) y las proteínas latentes de membrana (*LMP1* y 2). El estadio de latencia II corresponde a linfomas de Hodking y los genes virales que se expresan preferentemente son el *EBNA1* y los LMPs. Por último, el estado de latencia I corresponde al linfoma de Burkitt e incluye la expresión de los genes *EBNA1* y los RNAs pequeños, principalmente (Fish et al., 2017; Roughan and Thorley-Lawson, 2009; Thorley-Lawson et al., 2013)

8.1.5 CR2/CD21

El CR2 (también llamado CD21) es una proteína de 145 kDa codificada dentro de un cluster de genes que regulan la activación del complemento y que en humanos está localizado en el cromosoma 1q32. Se expresa principalmente en células B, en ciertos subtipos de células T y en células dendríticas, aunque también se ha descrito en basófilos, células epiteliales, mastocitos y queratinocitos (Zabel and Weis, 2001). El CR2 forma un complejo con las proteínas CD19 y CD81, que sirven de correceptores al receptor de células B (BCR), en la superficie de células B. Deficiencias en CR2 se han asociado a una mayor actividad del BCR manteniendo activa la vía de PI3K en leucemia linfocítica crónica de células B (B-CLL), contribuyendo de esta manera a un peor pronóstico de la enfermedad (Nichols et al., 2015).

Por otro lado, CR2 es un marcador de diferenciación de células B, encontrándose niveles altos o moderados en linfocitos B inmaduros y maduros y siendo prácticamente indetectable en precursores de células B y células plasmáticas (Cruickshank et al., 2009)

8.1.6 Linfoma de Burkitt, MYC e infección por EBV

MYC se encuentra traslocado en prácticamente todos los casos de linfoma de Burkitt. Sin embargo, la relación con la infección por EBV no es tan clara. En el caso del linfoma de Burkitt endémico está presente en casi todos los casos, mientras que en los casos de linfoma de Burkitt en países desarrollados sólo ocurre en un 20-30 % de los casos. Muchos autores sostienen que ambos eventos deben estar relacionados. La hipótesis más aceptada afirma que la infección por EBV debe desencadenar cambios en los linfocitos B que favorezcan la translocación de MYC y, por tanto, su sobreexpresión (Rowe et al., 2014; Said et al., 2014). También se ha propuesto que algunas proteínas virales como EBNA1 protegen a la célula de la apoptosis causada por los altos niveles del MYC traslocado.

Sin embargo, hay datos que no apoyan esta hipótesis. Por ejemplo, los dos genes más implicados en la transformación por EBV, es decir, EBNA2 y la mayoría de genes virales, no se expresan en linfoma de Burkitt. En segundo lugar, la gran mayoría de los linfomas de Burkitt con traslocación de MYC que ocurren fuera de África y Nueva Guinea son EBV-negativos. En tercer lugar, sólo una fracción de sujetos infectados con EBV y que han sufrido mononucleosis infecciosa desarrollan linfoma de Burkitt.

Por lo tanto, y a pesar de que existe una gran cantidad de evidencias que relacionan ambos sucesos, sigue sin estar clara cuál es la relación de MYC y el EBV en el origen y desarrollo del linfoma de Burkitt.

8.2 Objetivos de este proyecto

Resultados previos en nuestro laboratorio indican que CR2 puede ser una possible nueva diana de MYC. Puesto que CR2 es un co-receptor del BCR y se expresa en un número limitado de líneas celulares, decidimos restringir nuestro estudio a líneas celulares derivadas de linfomas, especialmente Linfoma de Burkitt, ya que la traslocación de MYC en este linfoma es especialmente relevante.

Los objetivos de este estudio son los siguientes:

1. Caracterizar CR2 como una nueva diana de MYC:
   i. De forma independiente a la proliferación celular o la síntesis de proteínas.
   ii. Describir su regulación directa.
2. Estudiar la importancia de la vía MYC-CR2:
   iii. Con la infección por EBV y el desarrollo de BL.
   iv. Su implicación en la regulación del BCR.

8.3 Resultados y Discusión

8.3.1 El tratamiento con JQ1 baja la expresión de MYC y CR2 en líneas celulares de linfoma B y T.

En nuestro laboratorio ya hemos demostrado que el tratamiento de células Raji (células B humanas derivadas de Burkitt, con MYC translocado) y K562 (leucemia mieloide crónica) con JQ1 regula negativamente la expresión de MYC. Tras el silenciamiento de MYC debido al tratamiento con JQ1 observamos igualmente una bajada en los niveles de CR2 a nivel de RNA mensajero (mRNA) y proteína. Además, se procedió a realizar ensayos de proliferación, ciclo celular y muerte celular por apoptosis.

El JQ1 es un inhibidor de proteínas con bromodominios y aunque se ha demostrado que reduce eficazmente la expresión de MYC en células B, no es específico de MYC. Por tanto, se silenciaron los niveles de MYC de forma más específica infectando con partículas lentivirales que contenían shMYC. Se observó que la bajada en la expresión de CR2 se correlacionaba con una bajada en los niveles de MYC tanto a nivel de mRNA como de proteína.

Como era esperable los ensayos de proliferación revelaron una bajada proliferativa tras el descenso en los niveles de expresión de MYC. Luego la bajada en la expresión de CR2, podría ser debida a un efecto indirecto de la parada proliferativa provocada por la bajada en los niveles de MYC.

Además, tratamos la línea celular derivada de K562 (KMYCJ), que son células derivadas de la línea celular K562 con un alelo de MYC inducible con zinc, con JQ1 para observar si la bajada proliferativa inducida por este compuesto era capaz de recuperarse al

sobreexpresar MYC. Observamos que las células tratadas con JQ1 y con Zinc no eran capaces de recuperar la parada proliferativa inducida por el JQ1.

Estos resultados indican la bajada proliferativa en células de linfoma inducida por el JQ1 no se debe únicamente a la bajada de MYC, puesto que su sobreexpresión no es capaz de recuperar el crecimiento normal de estas células. Este efecto ya se ha observado previamente en células de linfoma difuso de células B grandes (DLBCL) (Chapuy et al., 2013).

8.3.2 La regulación de CR2 por MYC es independiente de la parada proliferativa

Puesto que los resultados anteriores mostraban que el tratamiento con JQ1 provocaba una bajada proliferativa acompañada de una bajada en los niveles de MYC y CR2, quisimos descartar que la bajada de CR2 se debiera a un efecto indirecto de la parada proliferativa.

Realizamos dos experimentos con dos tipos celulares diferentes disponibles en nuestro laboratorio. Primero utilizamos las KMYCJ, mencionadas en el apartado anterior y las pre-tratamos con TPA para inducir una parada proliferativa. Las células sufrieron una bajada en el crecimiento celular tal como esperábamos y esa bajada proliferativa no pudo recuperarse con la sobreexpresión de MYC inducida al añadir zinc. Sin embargo, los análisis de qPCR indican una subida del mRNA de *CR2* tras la inducción de *MYC*, incluso aunque las células permanecen en parada proliferativa.

Por otro lado, también utilizamos la línea celular Kp27MER que posee un alelo inducible de p27, un inhibidor del ciclo celular. Al mismo tiempo también expresa constitutivamente la proteína quimérica MYC-ER activable con 4-Hidroxitamoxifeno. Al sobreexpresar p27 en estas células se produce una para de ciclo inducida por p27 que

no se recupera tras la activación de la proteína quimérica MYC-ER. Sin embargo, al igual que en el experimento anterior los niveles de mRNA de CR2 aumentaron tras la activación de la proteína MYC-ER, aunque las células mantienen la parada de ciclo.

Por lo tanto, podemos concluir que la regulación de CR2 por MYC no es dependiente del estado proliferativo de las células.

8.3.3 CR2 es una diana directa de MYC

Aunque los experimentos anteriores mostraban que al bajar la expresión *MYC* bajaba también el CR2, esto aún no demuestra que CR2 sea una diana directa, ya que MYC puede estar induciendo otro factor de transcripción que a su vez activa el promotor de CR2. Para comprobar que la inducción de CR2 es un efecto directo de MYC recurrimos a línea KMER. Esta línea deriva igualmente de la línea celular K562 y contiene la proteína quimérica MYC-ER. Tratamos estas células con 4HT (para activar MYC-ER) y con cicloheximida, un inhibidor de síntesis de proteínas. Los resultados indicaban que tras la inhibición de la síntesis de proteínas desaparecía el MYC endógeno. Por otro lado, cuando tratamos las células con cicloheximida y 4HT se activaba la proteína quimérica y los resultados de qPCR indicaban que la activación de la proteína MYC-ER aumenta la expresión de CR2 a nivel de mRNA, incluso en ausencia de síntesis de proteínas. Estos datos sugieren que MYC induce directamente la expresión de CR2.

8.3.4 MYC se une al promotor de CR2

Para determinar la unión directa de MYC al promotor de CR2, obtuvimos de nuestros colaboradores (Daniela Ulgiati. University of Western Australia) diferentes construcciones del promotor de CR2 en vectores luciferasa. El promotor del CR2 contiene dos cajas E, en la región 5’ proximal al inicio de transcripción. Además del

promotor normal, teníamos dos construcciones más con ambas cajas E mutadas. Transfectamos estas construcciones en las líneas celulares K562 y HEK293T para comprobar la activación del promotor de CR2. Observamos un incremento en la actividad luciferasa en todas las construcciones comparadas con el vector vacío. Además, transfectamos las construcciones en nuestro modelo celular, las células Raji y observamos que la actividad del promotor disminuía tras el silenciamiento de MYC.

Puesto que en la región proximal del promotor del CR2 también existen secuencias para la unión de otros factores de transcripción quisimos verificar que MYC el responsable de la activación del promotor. Usamos las diferentes construcciones luciferasa en la línea celular HEK293T. Tras ser transfectadas procedimos a realizar un ChIP de MYC. Los resultados mostraron la unión de MYC al promotor original y observamos una reducción de esta unión en las construcciones con las cajas E mutadas.

Por otro lado, los datos de ChIP en la base de datos ENCODE indica que MYC se une al promotor de CR2 en la línea celular K562. Pero no hay datos en ninguna línea celular derivada de linfoma de Burkitt. El proyecto ENCODE y otros estudios han demostrado que la afinidad de la unión de factores de transcripción a un locus del genoma puede variar significativamente de una línea celular a otra. Dada su posible importancia en linfomagénesis, estudiamos la unión de MYC al promotor de CR2 en Raji. Realizamos por tanto un ChIP de MYC en células Raji, observando que existe un enriquecimiento de MYC en la región proximal del promotor de CR2, confirmando así los resultados observados en la base de datos ENCODE para otras líneas celulares como K562.

Tomados en su conjunto, todos estos datos confirman que CR2 es una diana directa de MYC.

8.3.5 Validación funcional de la transactivación de CR2 por MYC

Todos los resultados anteriores parecen indicar que existe una regulación directa de CR2 por MYC, por tanto, se decidió estudiar los efectos biológicos de la reducción de niveles de CR2. Para ello se encargaron shRNAs de CR2 para realizar una validación funcional del silenciamiento de CR2 en células de linfoma de Burkitt.

8.3.5.1 Efecto de la ruta MYC-CR2 en la infección por virus Epstein-Barr

Por último, decidimos infectar una línea celular de linfoma de Burkitt Epstein-Barr negativa (Ramos) (Freesen and Hausen; 1976) con virus de Epstein-Barr. Las células productoras de virus fueron donadas por nuestros colaboradores (José R. Regueiro González-Barros. Universidad Complutense de Madrid). Estos virus fueron obtenidos creciendo la línea celular B95.8, que es una línea derivada de linfocitos B productora del virus del Epstein-Barr.

El objetivo del experimento fue infectar células con diferentes niveles de expresión de MYC y, por tanto, de CR2, para inferir si los niveles del receptor se correlacionan con la eficiencia de la infección. Para medir la eficiencia de la infección se diseñaron primers que reconocerían diferentes genes del virus. Se comprobaron los niveles de expresión de MYC y CR2 por RT-qPCR y los niveles de infección midiendo la presencia de genes del virus de Epstein-Barr en el DNA de las células por PCR.

Los resultados sugieren que las células expuestas al sobrenadante con virus del Epstein Barr son capaces de integrar el DNA viral tal como se observa en los resultados de qPCR de DNA genómico en las células Ramos. También se observó que existe una

correlación entre los bajos niveles de MYC y CR2, con una reducida eficiencia de la infección por EBV.

8.3.5.2 Efecto del silenciamiento de CR2 en la línea celular Raji

Se procedió a infectar la línea celular Raji con partículas lentivirales que contenían un shRNA contra CR2. Los ensayos de proliferación indican que tras el silenciamiento de CR2 hay una bajada en el crecimiento celular. Se confirmó el silenciamiento de CR2 por inmunoblot y se miraron otros marcadores de viabilidad celular y proliferación. La ausencia de rotura de PARP1 indicaba que las células no estaban muriendo por apoptosis, aunque la reducción en los niveles de ciclina A confirmaba lo observado en los ensayos de proliferación, indicando que la bajada de CR2 induce parada proliferativa, aunque no muerte celular, al menos por apoptosis.

Puesto que el silenciamiento de CR2 provocaba una parada proliferativa bastante significativa en esta línea celular nos preguntamos qué vía celular podría estar alterando el silenciamiento de CR2 para provocar este fenotipo. Puesto que el CR2 es un co-receptor del BCR, cabe suponer que las vías alteradas fueran vías dependientes de la actividad del BCR. Existen 3 vías principales activadas por el BCR: la vía de PI3K-AKT-mTOR, la vía de las MAP quinasas y la vía canónica de NF-κB.

8.3.5.3 Efecto sobre las vías reguladas por el complejo de proteínas al que pertenece CR2

Se procedió a infectar la línea celular Raji con partículas lentivirales que llevaban shMYC y shCR2 y se analizó por inmunoblot distintos marcadores de cada una de las vías. Se

comprobó la fosforilación de IκB, AKT y ERK, para determinar cambios en la actividad de alguna de las 3 vías. Tan sólo han podido observarse cambios en la fosforilación de ERK, aunque quedaría por determinar si la bajada en la fosforilación de ERK es un efecto directo del silenciamiento de CR2 o podría deberse a una regulación de ERK por una vía diferente a la vía directa de las MAP kinasas. Para ello, se está tratando de determinar si existen cambios en la fosforilación de AKT, lo que podría indicar también una alteración de la vía PI3K-AKT.

8.4 Conclusiones

1. La regulación negativa de MYC en líneas derivadas de células B y T, mediada tanto por JQ1 como por shRNAs, provocan una bajada proporcional en los niveles de CR2.

2. La sobreexpresión de MYC en líneas celulares derivadas de K562 indujo la expresión de mRNA de CR2 en células en arresto proliferativo. Por tanto, la inducción de CR2 por MYC es independiente del estado proliferativo de las células.

3. En condiciones de ausencia de síntesis de proteínas, MYC es capaz de inducir la expresión de mRNA de CR2.

4. El ChIP realizado en las Raji sugiere que MYC se une a lo largo del promotor del CR2. MYC activa el promotor de CR2 de manera dependiente de la presencia de cajas E. Por tanto, MYC regula directamente el promotor de CR2, activando su transcripción.

5. El silenciamiento de CR2 con shRNA provoca una bajada proliferativa y la inactivación de ERK2 sugiriendo que la inactivación de la vía de las MAP kinasas podría ser la vía a través de la cual la bajada de CR2 provoca una parada proliferativa.

6. La bajada en los niveles de expresión de MYC y, por tanto, del receptor del virus, provoca una bajada en la eficiencia de la infección de EBV en células B humanas.

7. La translocación de MYC podría ser el mecanismo a través del cual aumentaran los niveles del receptor del EBV y, por tanto, favoreciera la infección de los linfocitos B y, en última instancia, inducir la transformación de estas células.

8. Esta hipótesis estaría más en consonancia con el subtipo de linfoma de Burkitt esporádico, en el que la asociación del virus con esta enfermedad ocurre en un 20-30% de los casos.