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Role of ZEB factors in B cell activation and malignant progression

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Role of ZEB factors

in B cell activation and malignant progression

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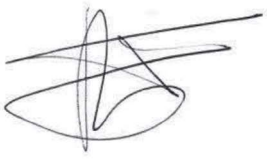
Dr. Antonio Postigo
Director



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A la meva mare i al meu pare,

Gemma i Vicent.

Though nothing can bring back the hour of splendor in the grass, of glory in the flower.
We will grieve not, rather find strength in what remains behind.

William Wordsworth.

PREFACE

The experimental study presented in this doctoral thesis was performed at the Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS) in Barcelona in the laboratory of Transcriptional Regulation of Gene Expression under the supervision of Dr. Antonio Postigo (PI at IDIBAPS' Hematology-Oncology Department) and Dr. Gaël Roué (former senior scientist at IDIBAPS Hemato-Oncology Division). The work was supported by an FPI (Formación Personal Investigador) scholarship from the Spanish Government (Ministerio de Ciencia e Innovación) associated with the project SAF2014-52874-R to Dr. Antonio Postigo.

ABSTRACT

ZEB1 and ZEB2 are two transcription factors best known for their role driving a dedifferentiation process, commonly referred to as epithelial to mesenchymal transition (EMT). This process is carried out either in multiple physiological and pathological conditions, such as normal development and tumor progression. More recently, their role in T cell development and T cell leukemias have been studied, but their role in B-cell activation and B cell malignant progression remains still poorly understood.

In this PhD dissertation, it was found that both ZEB1 and ZEB2 factors are expressed during the differentiation of the B cell lineage and specifically in the Germinal Center (GC) B cells. ZEB1 is required for GC formation and to prepare a correct T-dependent response in front of a specific antigen. Due to different chromosomal alterations and mutations, the GC B cells can undergo malignant transformation and give rise to different subtypes of B cell lymphoma, being diffuse large B cell lymphoma (DLBCL) the most common. It was found that ZEB1 and ZEB2 are involved in the progression of DLBCL as they regulate the DLBCL proliferation and cell metabolism, being ZEB1 a marker of poorer prognosis and associated to higher proliferation rate of tumoral cells and ZEB2 having an inversed pattern. These reversed expression patterns of ZEB1 and ZEB2 were also found in multiple myeloma (MM), where ZEB2 acts as an anti-tumoral marker and is associated with a pre-malignant stage, the monoclonal gammopathy of undetermined significance (MGUS). ZEB1 acts as a pro-tumoral gene, associated with the malignization of the disease and is associated with different hallmarks: poorer treatment response, cell migration, and in the bone formation.

The results set ZEB1 and ZEB2 as potent prognosis markers in lymphomas and highlight that their potential as therapeutic targets needs to be assessed in the future.

RESUM

ZEB1 i ZEB2 són dos factors de transcripció descrits pel seu paper en dur a terme un procés de desdiferenciació, conegut com la transició epiteli-mesènquima. Aquest procés es duu a terme tant en condicions fisiològiques com patològiques, com pot ser durant el desenvolupament o en la progressió tumoral i metàstasi. Recentment s'ha descrit el seu paper en el desenvolupament de cèl·lules T i en leucèmies de cèl·lula T, però el seu rol durant el desenvolupament i activament de les cèl·lules B i en la progressió de tumors hematològics de cèl·lula B encara no és del tot conegut.

En aquesta tesis, s'ha trobat que els dos factors, ZEB1 i ZEB2, s'expressen durant la diferenciació del llinatge de cèl·lules B i específicament en les cèl·lules B del Centre Germinal. Per la formació de centres germinals i per donar a lloc una bona resposta humoral enfront un antigen específic és necessària la intervenció de ZEB1. En limfomes difusos de cèl·lula gran, ZEB1 i ZEB2 estan implicats en la progressió de la malaltia, ja que en regulen la seva proliferació i metabolisme cel·lular. En aquest cas, ZEB1 està associat a un pitjor prognàtic i ZEB2 té una funció inversa, associant-se a un millor pronòstic. Aquest patró també es pot observar en el cas de mieloma múltiple, en què ZEB2 està associat a un estadi premaligne de la malaltia i ZEB1 actua com un gen protumoral, associat a diferents marcadors com podria ser una pitjor resposta al tractament, migració cel·lular i formació d'osteoclasts.

Aquests resultats situen ZEB1 i ZEB2 com marcadors de prognosi en limfomes, sent considerat ZEB1 com un gen promotor de la tumorigènesi i marcador de mal pronòstic i ZEB2 sent un marcador de bon pronòstic. També suggereixen ZEB1 com una potencial diana terapèutica en limfomes.

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2-DG	2-deoxy-d-glucose
AML	acute myeloid leukemia
B-ALL	B cell precursor acute lymphoblastic leukemia
BCR	B cell receptor
BM	bone marrow
CFA	complete Freund's adjuvant
CM	central memory T cell
DLBCL	diffuse large B cell lymphoma
EM	effector memory T cell
EMT	epithelial to mesenchymal transition
FBS	fetal bovine serum
GC	germinal center
Ig	immunoglobulin
Ip	intraperitoneally
KLH	keyhole limpet hemocyanin
MCL	mantle cell lymphoma
MGUS	monoclonal gammopathy of undetermined significance
MM	multiple myeloma

MZ	marginal zone B cell
NHL	non-Hodgkin lymphoma
OxPhos	oxidative phosphorylation
PBS	phosphate-buffered saline
sRBC	sheep Red Blood cells
T-ALL	t-cell acute lymphoblastic leukemia
TCA	tricarboxylic cycle acid
Tfh	T follicular helper
TMA	tissue microarray
WHO	world health organization

INTRODUCTION

INTRODUCTION

1. B CELL DEVELOPMENT

The immune system's role is to recognize and protect from pathogens through two immunological responses, with significant crosstalk between them. First, the innate immune response will provide the first line of defense, creating an inflammatory response by macrophages, dendritic cells, neutrophils, eosinophils, basophils, and natural killer cells (Bonilla and Oettgen, 2010).

Secondly, the adaptive immune will be involved, orchestrated by lymphocytes, with a precise specificity against pathogens. It consists of B cells and T cells that will generate effector and memory lymphocytes, capable of activating pathogen-specific effector pathways and producing an immunologic memory that will be able to create a faster and robust response in case of an encounter with the same pathogen in the future (Bonilla and Oettgen, 2010; Kavathas et al., 2019).

1.1. B cell lineage differentiation

B cells derive from hematopoietic stem cells in primary lymphoid organs (fetal liver and bone marrow) and during their development, they will migrate to secondary lymphoid organs (spleen and lymph nodes) (Figure 1). In the bone marrow, the hematopoietic stem cell differentiates into a common lymphocyte progenitor that will express surface immunoglobulins (Ig) to become pro- and pre-B cells (Kurosaki et al., 2009).

During this stage, B lymphocytes undergo DNA antigen receptor gene rearrangements in the variable regions of the heavy chain (V(D)J recombination) and along this development have different cell signaling pathways and transcription factors activated (e.g., IKZF1, SPI1, TCF3, IRF4, IRF8, IKZF3) (Matthias and Rolink, 2005; Nutt and Kee, 2007). Once they become immature or naive B cells, they migrate to the spleen via the bloodstream to become transitional B cells (Figure 1).

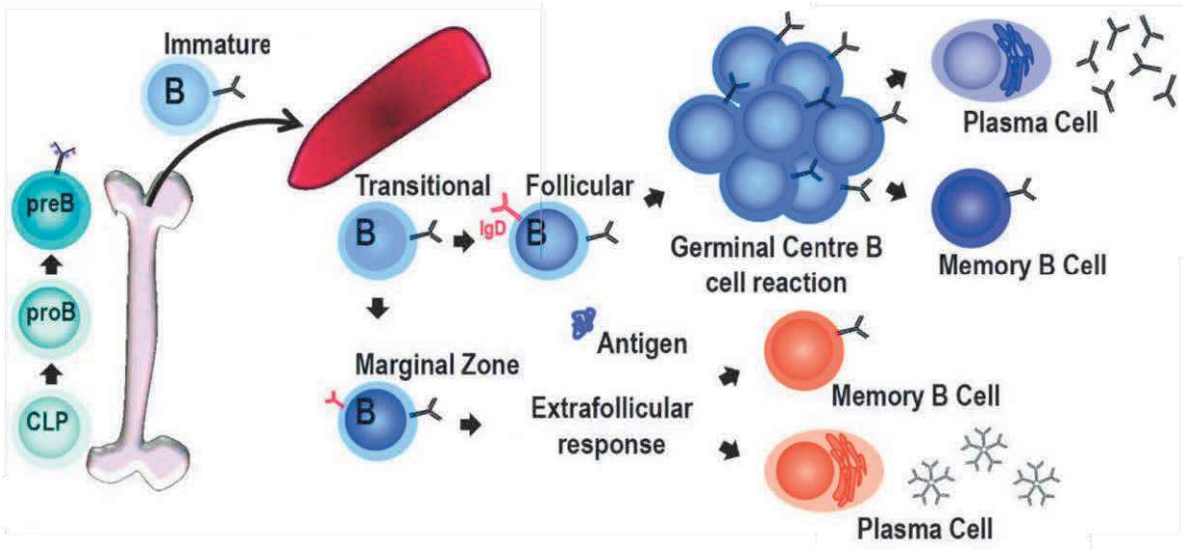

Figure 1. Scheme of B cell development and differentiation after the encounter with an antigen. B cell development from the common lymphoid progenitor (CLP) until differentiated memory B cells and plasma cells. Figure adapted with permission from Yam-Puc et al., 2018.

The spleen is divided into three main regions; the red pulp, where the blood is filtered and the macrophages reside; the white pulp, where the T and B lymphocytes compartments are; and the marginal zone (MZ), surrounding the white pulp (Bhattacharya, 2018). Within the white pulp, there is a structure named germinal center (GC), where the B cells with an affinity for a specific antigen are selected and high-affinity B cell receptors (BCRs) are formed. Finally, these cells will differentiate into plasma or memory B cells (Bhattacharya, 2018; Yam-Puc et al., 2018).

1.2. Germinal center dynamics

Morphologically, GCs can be divided into two zones that are visually recognizable by histology (Figure 2). The light zone contains different cell populations: macrophages, dendritic cells, T cells, and B cells and the dark zone, which is comprised of almost all B cells (Mesin et al., 2016).

These structures are formed in response to a T-dependent response and will give rise to high-affinity memory B cells and plasma cells producing specific antibodies. In a T-dependent response, T cells support B cells during the antibody response by recognizing the antigen on the B cell surface. Then T cells will become activated and,

in turn, can trigger the B cell activation (Parker, 1993; Zotos and Tarlinton, 2012). This response can be experimentally induced in mice by the administration of different antigens: keyhole limpet hemocyanin (KLH) and sheep red blood cells (sRBC) (Lebrec et al., 2011) and the results of this dissertation study this response.

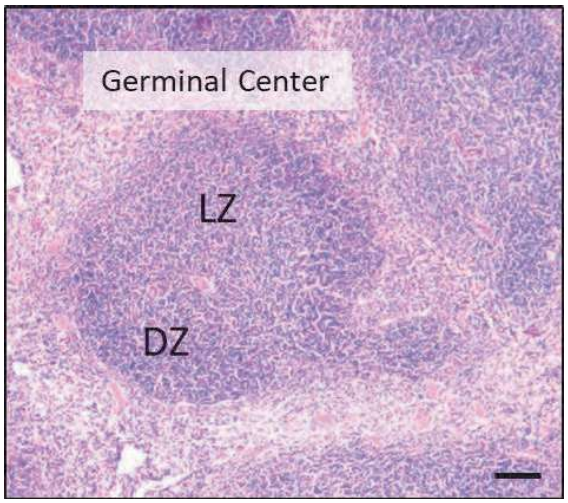

Figure 2. Histological structure of the Germinal Center. Hematoxylin and Eosin (H&E) staining of the murine spleen showing a GC with the two differentiated zones: dark (DZ) and light (LZ) zones. Scale bar represents 100 µm.

Next, the transitional B cells in the spleen complete their maturation and differentiate into Follicular B cells or Marginal Zone B cells, depending on microenvironment factors and the BCR signaling (Cariappa et al., 2001; Yam-Puc et al., 2018) (Figure 1).

Subsequently, MZ B cells will migrate to the marginal zone area of the spleen and will be the first to be in contact with the antigens circulating in the blood. These cells will be mainly performing a T-independent response, and once they are activated will migrate to the red pulp and differentiate to plasma cells without depending on T cells or onto the formation of the GC (Pillai et al., 2005). Although their primary function is to act in the T-independent response, MZ B cells have also been described to interact directly with T cells and directly differentiate into plasma cells (Attanavanich and Kearney, 2004).

Later, Follicular B cells will interact with the T helper follicular (Tfh) cells and result in the full activation and clonal expansion of the B cells that will migrate into the center of the follicle and rapidly proliferate to form an early GC (De Silva and Klein,

2015). Tfh cells originate from CD4+ naïve cells that have encountered a dendritic cell. If CXCR5 is expressed, Tfh cells will migrate to the follicle and undergo differentiation where they will meet and help the B cells, with whom they have a symbiotic relationship, and get activated (Crotty, 2014).

Once the GC is mature, two zones that will correspond to two types of GC B cells can be distinguished (dark and light) (Figure 2). The dark zone is formed by densely pack B cells, referred to as centroblasts, which are highly proliferative B cells and will undergo immunoglobulin somatic hypermutations. The light zone consists of another type of B cells, referred to as centrocytes, but also of Tfh cells and follicular dendritic cells. Centrocytes will be selected by their affinity for the specific antigen and be activated (Basso and Dalla-Favera, 2015). Finally, centrocytes either will reentry the dark zone to undertake more rounds of immunoglobulin somatic hypermutations or will differentiate into memory B cells or plasma cells, as GC B cells have the ability to migrate between both, dark and light zones (Figure 3) (De Silva and Klein, 2015).

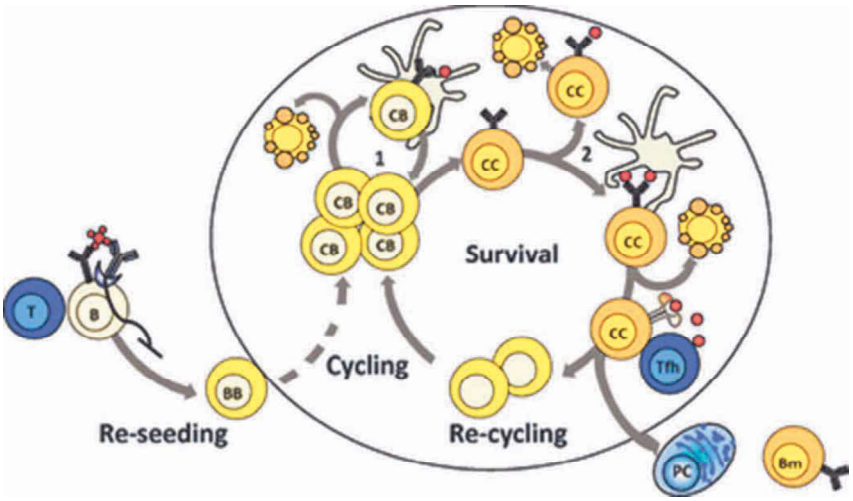

Figure 3. Scheme of GC dynamics between centroblasts and centrocytes. Centroblasts (CB) might exit the cell cycle to become centrocytes (CC), then the centrocytes might re-enter the cell cycle (re-cycling) or differentiate into plasma B cells or memory B cells. Figure adapted with permission from Vinuesa et al., 2010.

Plasma cells are terminal differentiated non-proliferative B cells able to secrete high-affinity antibodies that will neutralize the invading pathogen within a short time response. If there is an antigen-recall response, memory B cells can reenter the GC and

undergo rapid expansion and differentiation into plasma cells (De Silva and Klein, 2015).

Another cell of the microenvironment that plays a pivotal role in the GC formation is a specialized stromal cell, the follicular dendritic cell. These cells will capture the antigen, soon after the immunization, and will present it to the B cell (Vinuesa et al., 2010).

1.3. Transcriptional regulation of Germinal Center formation and function

This process is orchestrated by a highly coordinated network of transcriptional factors and epigenetic modifications (Figure 4), which will activate or repress signaling pathways to regulate B cell maturation.

One of the best-characterized factors is the transcriptional factor B cell lymphoma 6 (BCL6), a master regulator of the GC formation that controls the fate of GC B cells and Tfh cells. It directly represses the expression of TP53, ATR, p21 inducing a rapid proliferation and tolerance to the DNA damage (essential for the somatic hypermutations that occur in the GC). BCL6 also promotes the migration of B cells into the follicle and the B/Tfh interaction. Finally, the expression of BCL6 represses the plasma cell differentiation by repressing PR/SET domain containing 1 (PRDM1/Blimp-1) (Basso and Dalla-Favera, 2012). PRDM1, along with X-box binding protein 1 (XBP1), is essential for the formation of antibody-secreting cells (plasma cells) (Recaldin and Fear, 2016).

MYC is essential for almost all proliferating cells; however, its expression is almost localized in the centrocytes to facilitate the reentry into the dark zone (Recaldin and Fear, 2016). Centroblasts and centrocytes can be classified by the signature surface proteins, with centroblasts having $CXCR4^{hi}$, $CD83^{lo,}$ and $CD86^{lo}$ and centrocytes being $CXCR4^{lo}$, $CD83^{hi}$ and $CD86^{hi}$. According to this model, a decreased expression of CXCR4 is coordinated with reduced expression of proliferation-associated genes and these

changes will help the B cells migrate from the dark to the light zone (Bannard et al., 2013).

Another important master regulator is Paired Box 5 (PAX5), which is expressed through the B cell maturation and required for the commitment to the lymphoid lineage, in the beginning, activating genes critical for the B cell identity (CD19, CD21) finally to repress XBP1 (Recaldin and Fear, 2016).

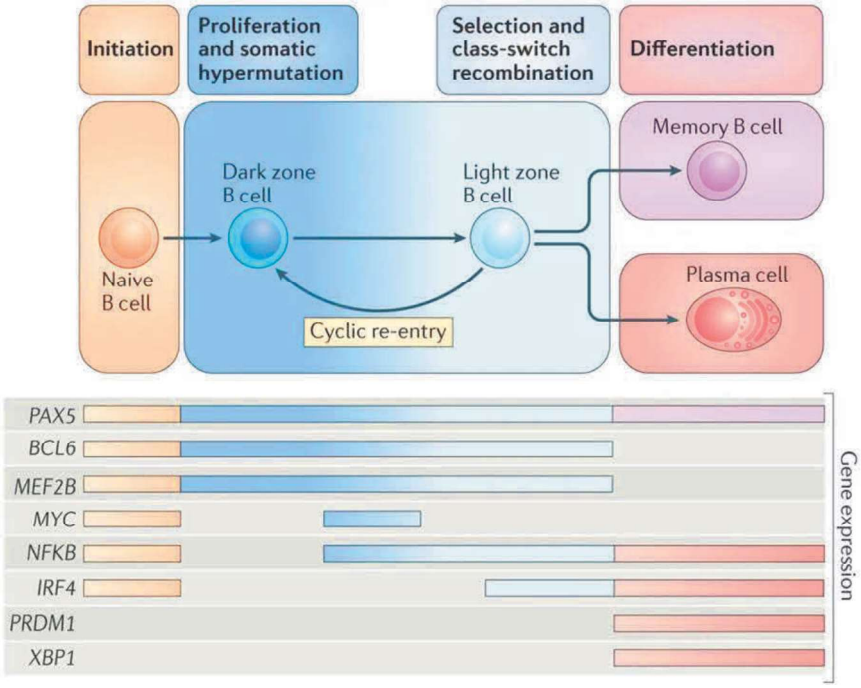

Figure 4. Expression pattern during the initiation and formation of GC. During the B cell differentiation, there is a differential expression of master regulators of the GC. Figure adapted with permission from Basso and Dalla-Favera, 2015.

In addition to being regulated at the transcriptional level, B cells undergo a series of epigenetic changes, essentials for the maturation of the cells. Naïve or immature B cells have chromatin with a compacted conformation, and during a T-dependent response, it will decompact, promoting interactions with different transcription factors (Azagra et al., 2020). Changes in DNA methylation will also be promoting the B cell commitment as the methylation occurs during the stem cells stage, but it is lost upon differentiation (Zhang and Good-Jacobson, 2019).

2. HEMATOLOGIC B CELL MALIGNANCIES

B cell neoplasms arise from the malignant transformation of B cells at different stages of their differentiation and maturation. The characteristics of each malignancy will rely on the properties of the cell of origin and the molecular mechanisms underlying the disease progression. The World Health Organization classifies more than 40 types of B cell lymphomas and leukemias depending on their stage of normal B cell differentiation from where they originate (Table 1) (Swerdlow et al., 2016).

Chronic lymphocytic leukemia
Monoclonal B-cell lymphocytosis
B-cell prolymphocytic leukemia
Splenic marginal zone lymphoma
Hairy cell leukemia
Splenic B-cell lymphoma/leukemia
Splenic diffuse red pulp small B-cell lymphoma
Hairy cell leukemia-variant
Lymphoplasmacytic lymphoma
Waldenström macroglobulinemia
Monoclonal gammopathy of undetermined significance (MGUS), IgM
μ heavy-chain disease
γ heavy-chain disease
α heavy-chain disease
Monoclonal gammopathy of undetermined significance (MGUS), IgG/A
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Monoclonal immunoglobulin deposition diseases
MALT lymphoma
Nodal marginal zone lymphoma
Pediatric nodal marginal zone lymphoma
Follicular lymphoma
In situ follicular neoplasia
Duodenal-type follicular lymphoma
Pediatric-type follicular lymphoma
Large B-cell lymphoma with IRF4 rearrangement
Primary cutaneous follicle center lymphoma
Mantle cell lymphoma
In situ mantle cell neoplasia
Diffuse large B-cell lymphoma (DLBCL)
Germinal center B-cell type
Activated B-cell type
T-cell/histiocyte-rich large B-cell lymphoma
Primary DLBCL of the central nervous system
Primary cutaneous DLBCL, leg type
EBV+ DLBCL, NOS
EBV+ mucocutaneous ulcer
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK+ large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
HHV8+DLBCL, NOS
Burkitt lymphoma
Burkitt-like lymphoma with 11q aberration
High-grade B-cell lymphoma, with MYC and BCL2 and or BCL6 rearrangements
High-grade B-cell lymphoma, NOS
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

Table 1. World Health Organization (WHO) classification of mature B cell neoplasms. Table adapted from Swerdlow et al., 2016.

In this dissertation, we will be focused on Diffuse Large B Cell Lymphoma (DLBCL) and Multiple Myeloma (MM), as they are two of the most common B cell malignancies. A better understanding of the pathogenesis will help to have a more reliable prognosis of the disease, as well as to improve current treatment and therapeutic strategies as both are still incurable and have high unmet medical needs.

2.1. Diffuse large B cell lymphoma

Diffuse large B cell lymphoma (DLBCL) is the most common non-Hodgkin lymphomas (NHL) in adults and is characterized by the presence of large neoplastic cells with a diffuse pattern of growth. NHL is the most common hematologic malignancies with an incidence of 115,118 new cases in Europe and 48,096 deaths in 2018, of which 1,245 new cases and 459 deaths took place in Catalonia alone (Asociación Española Contra el Cáncer, 2018; Ferlay et al., 2018).

The age of diagnosis is between 65-75 years, and it usually arises *de novo,* but it can also progress from chronic lymphocytic leukemia or follicular lymphoma (Swerdlow et al., 2016). The staging of DLBCL, according to the Ann Arbor system, establishes four stages (Table 2), which correlate with an increasingly poorer prognosis. Different parameters (age, blood levels of lactate dehydrogenase, extranodal involvement, and the stage) are also used as prognosis predictors (Tilly et al., 2015).

Stage	
I	Involvement of a single lymphatic region (I) or localized involvement of single extralymphatic organ or site (IE).
II	Involvement of two or more lymphatic regions on the same side of the diaphragm (II) or localized involvement of a single extralymphatic organ or site and one or more lymphatic regions on the same side of the diaphragm (IIE).
III	Involvement of lymphatic regions on both sides of the diaphragm.
IV	Diffuse or disseminated involvement of one or more extralymphatic organs with or without lymphatic involvement

Table 2. Ann Arbor staging classification. Table from Tilly et al., 2015.

2.1.1. Classifications of DLBCL

Several classifications of DLBCL have been established. The two most commonly used are the cell of origin and consensus cluster classification. The cell of origin of DLBCL corresponds to the clonal expansion of the B cells in the GC. Depending on the staging of differentiation studied by different parameters (immunophenotyping, gene expression, and morphological features) there is a classical classification (cell-of-origin classification) of the subtypes of DLBCL: GCB cell-like (GCB), activated B cell-like (ABC), and unclassified (Figure 5) established by the WHO and revised in 2016 (Swerdlow et al., 2016). This classification has been recently updated by Schmitz et al. where they classified DLBCL patients in four genetic subtypes, named MCD (based on *MYD88* and *CD79B* mutations), BN2 (based on *BCL6* and *NOTCH2* mutations), N1 (based on *NOTCH1* mutations) and EZB (based on *EZH2* and *BCL2* mutations) (Schmitz et al., 2018).

In turn, the consensus clustering classification, based on the gene expression profiles and metabolic fingerprints, classifies the DLBCL in three subsets: BCR, OxPhos, and HR (Monti et al., 2005). The B cell receptor/proliferation cluster (BCR-DLBCL) is characterized by an increased expression of the BCR components and a higher glycolytic flux, switching the cell metabolism into anaerobic glycolysis, characteristic of the tumoral cells. The oxidative phosphorylation cluster (OxPhos-DLBCL) is associated with an increased expression of mitochondrial components that will lead to an elevated mitochondrial activity, ATP production, and fatty acid oxidation. This subset does not have functional BCR signaling; therefore, it is not sensitive to the BCR inhibitors (Caro et al., 2012; Norberg et al., 2017). Finally, the host response cluster (HR-DLBCL) is marked by a T-cell-rich inflammatory immune cell infiltrate and an associated inflammatory response (Monti et al., 2005).

2.1.2. DLBCL lymphomagenesis

The molecular pathogenesis of this malignancy is crucial for better identification of new therapies. DLBCL lymphomagenesis includes chromosomal alterations and deregulations of transcriptional and epigenetic regulatory networks in normal B cells.

DLBCL shows a high genomic instability that leads to chromosomal alterations, and in Figure 5 are exemplified some of the most common dysregulated pathways and oncogenic alterations (Pasqualucci, 2013). *BCL6* deregulation is a critical pathogenic mechanism of DLBCL lymphomagenesis, as chromosomal aberrations are present in 35% of patients. Another frequent alteration is related to the histone modification genes (*CREBBP* and *MLL2*) (Pasqualucci and Dalla-Favera, 2015). There are also mutations and deletions associated with specific groups of the cell of origin classification. In GCB-DLBCL, somatic mutations of *BCL2* account for 34% of cases, heterozygous somatic mutations of *EZH2* have been reported in 22% of the patients and MYC translocations occur in 6-15% of the patients (Colomo et al., 2017). The genomic landscape of ABC-DLBCL is associated with the activation of the BCR signaling pathway by mutations in *CD79B/A* and *CARD11* as well as alterations in the TLR pathway (*MYD88* mutations) (Pasqualucci, 2013; Pasqualucci and Dalla-Favera, 2015).

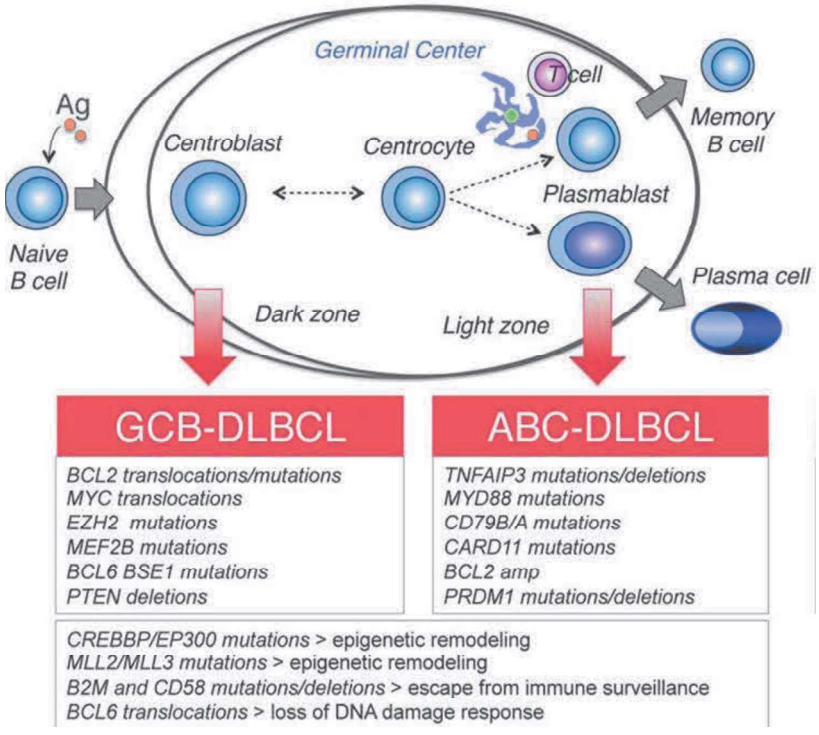

Figure 5. Genomic alterations in DLBCL subtypes. Scheme of the most common mutations, translocations, or deletions associated with GCB or ABC DLBCL subtypes. Figure adapted with permission from Pasqualucci, 2013.

2.1.3. Current treatment regimes in DLBCL

Around 60% of DLBCL patients will have a good response and prolonged survival, whereas the rest will relapse (Alizadeh et al., 2000). Regardless of the classical classification, there is a remarkable heterogeneity within each subtype that will have a direct impact on the treatment and prognosis (Pasqualucci and Dalla-Favera, 2015).

The first well-established treatment was a combination of different chemotherapeutic agents named CHOP consisted of cyclophosphamide, doxorubicin, vincristine, and prednisone (Fisher et al., 1993). This treatment was then supplemented with immunotherapy, the anti-CD20 monoclonal antibody named rituximab. The R-CHOP became the standard therapy, achieving durable remission up to 60% of the cases (Roschewski et al., 2014). Unfortunately, the specific group that shows resistance to the R-CHOP or relapses after remission will have a particularly poor outcome, and the efforts to improve R-CHOP have generally failed to show clinical benefits (Liu and Barta, 2019).

This resistance suggests that there is a differential response to R-CHOP according to different DLBCL subtypes (Roschewski et al., 2014). For this reason, the understanding of different DLBCL subtypes has led to a molecularly targeted precision and personalized combinations that prevent and treat relapsed patients. Some of the most important agents are a proteasome inhibitor (bortezomib), a BTK inhibitor (ibrutinib), a P13K inhibitor (idelalisib), a SYK inhibitor (R406) or a CXCR4 inhibitor among others that are in different clinical phases for different B cell lymphomas (Camicia et al., 2015; Gonzalez-Santamarta et al., 2020; Recasens-Zorzo et al., 2019).

2.2. Multiple Myeloma

Multiple Myeloma (MM) is a B cell malignancy characterized by the malignant transformation and expansion of clonal malignant plasma cells in the bone marrow. The differentiated plasma cells are the cell of origin of this malignancy, and when becoming tumoral, they will proliferate and produce monoclonal immunoglobulin protein, causing one of the hallmarks of MM, the bone osteolysis (Hideshima and

Anderson, 2002). MM is the second most common hematologic malignancy with an incidence of 48,297 new cases in Europe and 30,860 deaths in 2018, of which 518 new cases and 315 deaths took place in Catalonia alone (Asociación Española Contra el Cáncer, 2018; Ferlay et al., 2018).

The age of diagnosis of MM is between 65-70 years and its classified into three stages for its prognosis depending on the bone lesions, hemoglobin level, monoclonal protein level, creatinine level, and CRAB features (hypercalcemia, renal failure, anemia, and bone lesions) (Rajkumar et al., 2014).

2.2.1. Classification of MM

During the progression of MM, it is possible to distinguish different stages of clonal evolution (Figure 6). The asymptomatic pre-malignant stage is the monoclonal gammopathy of undetermined significance (MGUS). It is characterized by an accumulation of monoclonal immunoglobulin (M-protein) and the absence of clinical signs (Blade et al., 2008; de Larrea et al., 2018; Landgren et al., 2009).

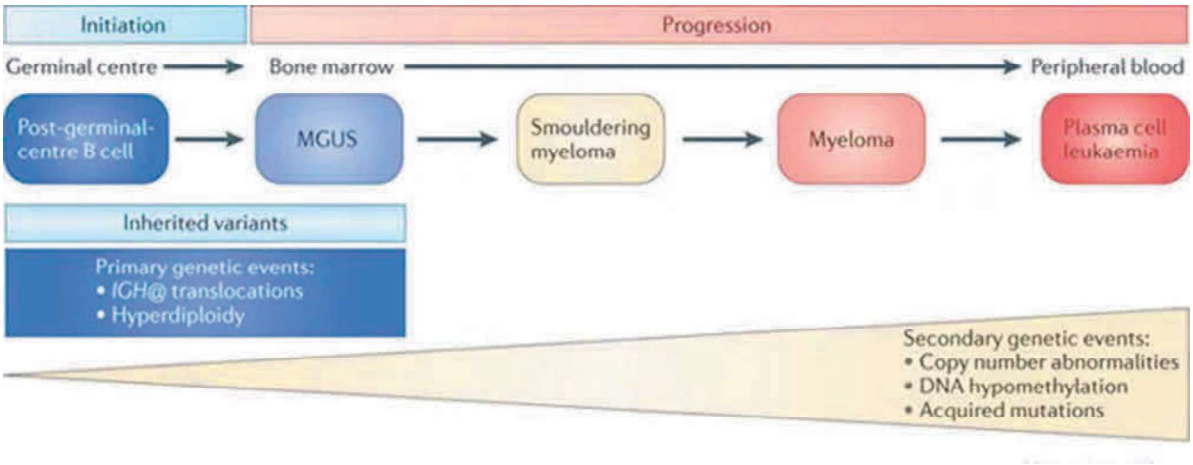

Figure 6. Initiation and progression of MM. Progression of the malignancies from a pre-malignant stage (MGUS) to multiple myeloma, and their associated genetic events. Figure adapted with permission from Morgan et al., 2012.

The MGUS can evolve to an asymptomatic MM (smoldering MM) and finally to symptomatic MM with a progression rate of 1% per year (Morgan et al., 2012). MGUS almost always precedes MM, but only 10% of patients with MM have a history of

preexisting MGUS. It might be due to MGUS being mostly asymptomatic and being detected only by accident. The evolution from MGUS to MM requires multiple genomic events, although the malignant transformation is still unclear (Rajkumar and Kumar, 2016).

2.2.2. Myelomagenesis

Although several mutations and alterations will result in cytogenetically different plasma malignancies, MM is considered a unique disease (Rajkumar and Kumar, 2016). The genetic alterations, associated with the myelomagenesis, rely on chromosomal translocations and hyperdiploidity, affecting the immunoglobulin transcriptional regulatory regions (50% of cases). Some of the most common dysregulations due to IgH translocations are Cyclin D aberrant expression or **the IgH-WHSC1** translocations. Other secondary mutations dysregulate different signaling pathways (NK-κB, MAPK) (Barwick et al., 2019; Manier et al., 2017).

An essential early event in the progression of MM is the homing or migration of the malignant plasma cells to the BM niche. The CXCL12/CXCR4 axis plays a pivotal role in it. The posterior migration to other niches through the blood and its dissemination is promoted by hypoxia through the activation of the epithelial-to-mesenchymal (EMT) process (Azab et al., 2012).

One of the most critical factors determining initiation and progression in the myelomagenesis is the role of the bone marrow microenvironment. The network formed between the MM cells and the microenvironment cells in the bone marrow (hematopoietic precursors, osteoblasts, osteoclasts, dendritic cells, and endothelial cells) will result in a release of cytokines and growth factors that will help the MM cells to proliferate, migrate and resist chemotherapy agents (Balakumaran et al., 2010).

2.2.3. Current treatment regimes in MM

The initial treatment of MM depends on the eligibility of the patient (depending on the age) for an autologous stem cell transplant. Some of the most frequent agents used are thalidomide, dexamethasone, lenalidomide, bortezomib, and melphalan (for

patients who are not candidates to the transplant). These agents have been used in two or three-drug combinations, to increase the overall survival and the progression free-survival, except in elderly patients (Rajkumar and Kumar, 2016). Recently, new targeted therapies involving daratumumab, an anti-CD38 antibody, are showing an increased efficiency when combined with other drugs in newly diagnosed MM cases, as well as in other B cell neoplasms (Abdallah and Kumar, 2019; Vidal-Crespo et al., 2020).

Although current therapies can extend the patients' median survival to 5 to 7 years, MM remains largely incurable (Rajkumar and Kumar, 2016; Richardson et al., 2010).

3. ROLE OF THE TRANSCRIPTION FACTORS ZEB1 AND ZEB2

3.1. Structure and mechanism of action of ZEB factors

The Zinc finger E-Box binding (ZEB) family of transcription factors is constituted by two factors: ZEB1 (also known as TCF8 or δEF1, among others) and ZEB2 (also known as SIP1, among others) (Dongre and Weinberg, 2019). They were first identified in a common ortholog in *Drosophila* known as *zfh-1,* expressed in the mesoderm of early embryos (Lai et al., 1991).

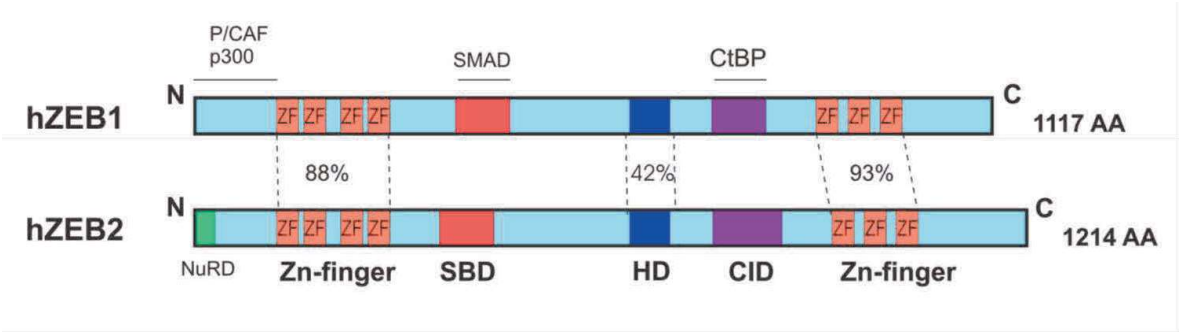

Figure 7. Schematic representation of *ZEB1* and *ZEB2* structure. Both transcription factors possess two zinc-finger domains, a homeodomain (HD), and SMAD (SBD) and CtBP (CID) binding domains. Figure adapted with permission from Goossens and Haigh, 2012.

Both genes have very similar genomic structure encoding for N-terminal and C-terminal zinc finger DNA binding domains (Figure 7). These domains bind to regulatory regions of target genes containing E-box like sequences (CANNT), with CACCTG and CAGGTA as those for which the ZEB factors have the highest affinity binding (Ikeda and Kawakami, 1995; Sekido et al., 1994). More centrally located, *ZEB1* and *ZEB2* have binding sites for corepressors, as the CtBP interaction domain (CID) or coactivators (p-300 or P/CAF) (Goossens and Haigh, 2012) (Figure 7). Whether ZEB1 or ZEB2 are acting as activators or repressors is more likely to be cell-type specific and dependent on posttranslational modifications (Lehmann et al., 2016).

There is a high degree of similarity between ZEB1 and ZEB2 in their zinc-finger domains and they bind to similar target genes. Although they commonly have similar

patterns, their roles in the activation or repression of these genes do not need to be similar. For example, in the TGF-β signaling, ZEB1 and ZEB2 have been described to be regulators of the pathway with opposing roles. While ZEB1 synergizes with SMAD to have an activation role, ZEB2 acts as a repressor by repressing SMAD functions (Soen et al., 2018).

The ZEB factors can be modulated at different levels by epigenetic modifications (histone modifications and DNA methylations), alternative splicing, miRNA regulation (miR-200 and miR-34), transcriptional control (signaling pathways as Wnt, Shh, Hippo, TFGβ or other EMT factors), protein stabilization, and subcellular localization (Dongre and Weinberg, 2019; Skrypek et al., 2017).

3.2. Expression and roles of ZEB1 and ZEB2 in embryonic development and cancer

The ZEB factors are best well-known for their role as drivers of the epithelial-to-mesenchymal transition (EMT). The EMT is a reversible and plastic process that induces the transition from an epithelial-like cell to a more motile mesenchymal phenotype by repressing E-cadherin, among other genes (Vandewalle et al., 2009). Other well-known EMT-transcription factors that play a pivotal role in the program are those that belong to the SNAIL (SNAI1, SNAI2, SNAI3) and TWIST (TWIST1, TWIST2) families, among others. The EMT factors have acquired more importance as new roles in the regulation of different cell processes have been described (Stemmler et al., 2019).

In normal development and embryogenesis, the EMT plays a crucial role in the gastrulation and neural crest formation and, later on, in the cartilage, bone and muscle formation, and the development of hematopoietic cells (van Grunsven et al., 2000; Vandewalle et al., 2009). *Zeb1*$^{-/-}$ deficient mice die before birth and exhibit multiple skeletal defects and a severe T cell deficiency in the thymus (Takagi et al., 1998). *Zeb2*$^{-/-}$ deficient mice also die before birth due to malformations in the neural crest (Van de Putte et al., 2003). Mutations in both *ZEB1* and *ZEB2* are associated with syndromic malformations, and a mutation or deletion in one allele of ZEB2 causes

Mowat-Wilson syndrome, which is characterized for intellectual disability, delayed development and intestinal disorders (Dastot-Le Moal et al., 2007).

One of the most studied roles of the EMT transcription factors and, in consequence, of ZEB1 and ZEB2 is in cancer. They are described to be involved in some of the cancer hallmarks defined by Hanahan and Weinberg (Hanahan and Weinberg, 2011). As described before, the loss of E-cadherin is one of the significant traits of EMT and is also an essential event in tumor metastasis and invasiveness in carcinomas (Caramel et al., 2018; Vandewalle et al., 2009). ZEB factors are involved in the acquisition and maintenance of cancer stem cells in pancreatic cancer (Wellner et al., 2009). ZEB1 and ZEB2 are also linked to increased angiogenesis in the tumor, as both factors are overexpressed in the endothelial cells of the tumor microenvironment (Fu et al., 2020; Liu et al., 2016). They are related to the regulation of the cell cycle and proliferation in a tissue-dependent manner. In a colorectal model, ZEB1 blocks cellular senescence via activation of Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1) (De Barrios et al., 2017). Finally, ZEB factors confer resistance to chemotherapy and radiotherapy in different carcinomas, like breast or pancreatic cancer (Meidhof et al., 2015; Zhang et al., 2018).

3.2.1. ZEB1 and ZEB2 in the hematopoietic lineage

The hematopoietic progenitors arise from the hemogenic endothelium and involve changes in the phenotype of the endothelial cell, including the EMT (Yokomizo et al., 2011). From expression arrays, it is shown that both factors are expressed at different levels in the hematopoietic lineage (Figure 8) (Goossens and Haigh, 2012). In the hematopoietic stem cell compartment, both factors are mutually and equally expressed, and once the lineage commitment initiates, there is an oscillation that will drive cell fate decisions. In the lymphoid lineage, ZEB1 is more expressed in the common lymphoid progenitor, the early T cell differentiation, and late B cell differentiation, and ZEB2 is highly expressed in the maturation of Natural Killer cells. In the myeloid lineage, on the one hand, ZEB2 is more expressed in the common myeloid progenitor and will be important in the monocyte differentiation, as on the other hand, ZEB1 expression increases in the erythrocyte differentiation.

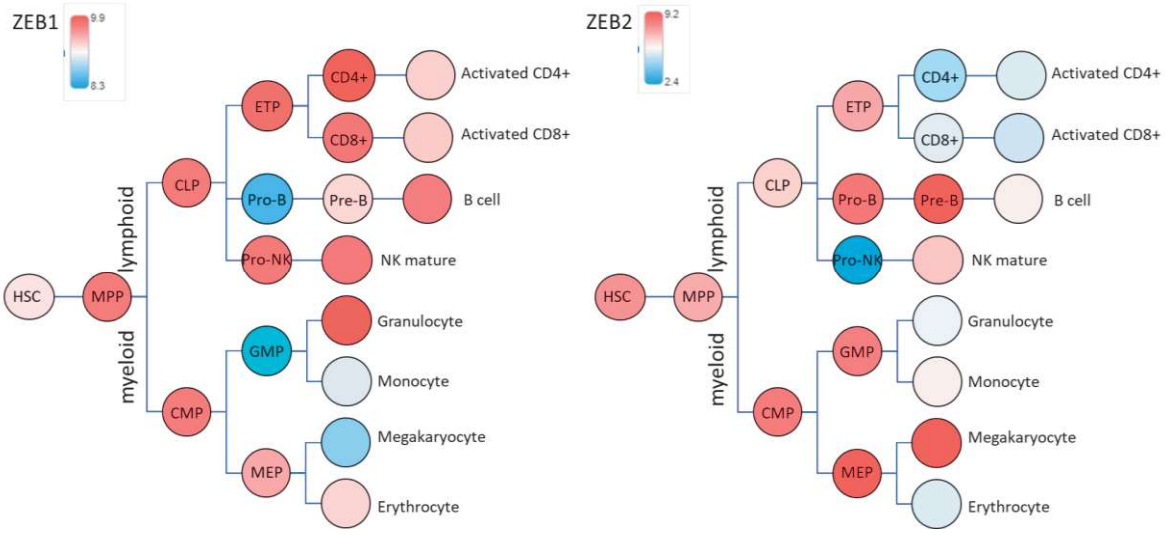

Figure 8. Scheme of *ZEB1* and *ZEB2* expression in the hematopoietic lineage. CLP (common lymphoid progenitor), CMP (common myeloid progenitor), ETP (early T-cell Progenitor), GMP (granulocyte monocyte progenitor), HSC (hematopoietic stem cell), MEP (megakaryocytic erythroid progenitor) and MPP (multipotent progenitor). Data obtained from Bagger et al., 2016.

Different studies in mice have shown that ZEB1 has an essential role in T cell development. *ZEB1* directly represses α4-integrin (*ITGA4*), a gene highly expressed in hematopoietic stem cells, and restricted to lymphocytes and myeloid subpopulations upon differentiation (Goossens and Haigh, 2012; Scott and Omilusik, 2019). A mouse model, harboring a truncated C-terminal deletion of *Zeb1*, shows atrophy in the thymus and a blocked T cell development in an early stage, as *ZEB1* binds directly to *CD4* (Hidaka et al., 2008). ZEB1 is also repressing other genes involved in the T cell differentiation: *IL2* and *GATA3* and binds to other hematopoietic genes as *IGH* (Goossens and Haigh, 2012).

Another mouse model with a C-terminal truncation, named *Cellophane,* presents B cell defects and impaired proliferation in the spleen (Arnold et al., 2012). Other studies using the *Cellophane* mouse present *Zeb1* as essential for the transition from $CD4^{-}CD8^{-}$ to $CD4^{+}CD8^{+}$ cells, differentiation of Natural Killer T cells, and a key regulator of T cell receptor (TCR) signaling (Zhang et al., 2020). ZEB1 is also directly binding to BCL6, which is both important in the GC reaction and the lymphomagenesis, as already described (Papadopoulou et al., 2010).

A specific ZEB2 loss in the hematopoietic lineage results in the accumulation of hematopoietic stem cells, which suggests a role of the factor in the mobilization and homing of these cells. It also affects most lineages producing differentiation defects and a loss of mature B cells in blood due to a block transition from pre-B cells to pro-B cells. *Zeb2* also acts in the T cell development, regulating genes that are necessary for the formation of the effector and memory T cells (Goossens et al., 2011; Li et al., 2017b; Scott and Omilusik, 2019).

Taking together both expression patterns in the hematopoietic lineage, the opposing patterns between the expression and or binding of ZEB1 and ZEB2 to target genes might be relevant in cell fate decision points (Soen et al., 2018).

3.2.2. ZEB1 and ZEB2 in hematologic malignancies

Taking together the importance of ZEB1 and ZEB2 in different carcinomas and their expression in hematopoietic lineages, it is expected that they will have crucial roles in different hematologic malignancies. However, their complete role is not fully understood, and they can act both as tumor suppressors or oncogenes, depending on the tumor subtype (Table 3).

	ONCOGENE	TUMOR SUPPRESSOR
ZEB1	MCL DLBCL AML	T-ALL
ZEB2	T-ALL AML	B-ALL

Table 3. *ZEB1* and *ZEB2* as oncogenes or tumor suppressors genes. Table adapted from Soen et al., 2018.

In T-cell acute lymphoblastic leukemia (T-ALL), *Zeb2* overexpression *in vivo* is associated with increased leukemia stem cell properties and spontaneous development of T-ALL (De Coninck et al., 2019; Goossens et al., 2019). However, ZEB1 is a mediator of the oncogene *LMO2* and *in vivo* loss-of-function mice develop T-ALL

spontaneously (Sun et al., 2010). T-ALL cell lines also showed low levels of ZEB1 that resulted in inhibited proliferation (Nakahata et al., 2010).

In B cell malignancies, these factors are described to have an essential role in some subtypes. In Mantle Cell Lymphoma (MCL), a downregulation of ZEB1 shows a decreased tumor growth *in vivo* and an increased sensitivity to chemotherapy in cell lines (Sanchez-Tillo et al., 2014). In DLBCL, ZEB1 is associated with poorer survival of the patients (Lemma et al., 2013) and altered expression of ZEB1 has been seen in pathogen-associated DLBCL (Huang et al., 2014), but a potential role of ZEB1 in the pathogenesis of DLBCL or the mechanism involved on it has not been studied yet.

In B cell precursor acute lymphoblastic leukemia (B-ALL), a loss of *ZEB2* DNA binding capacity is involved in the initiation and tumor progression (Roberts et al., 2014). In MM, no association with ZEB1 nor ZEB2 has yet been described, but a subset of tumoral cells within the tumor has been described to acquire an EMT-like phenotype to migrate to the BM niche (Muz et al., 2014). MM with extramedullary disease and a poorer prognosis show an upregulation of other EMT transcription factors (SNAI1, SNAI2, and TWIST1) regulated by CXCR4 (Roccaro et al., 2015). *ZEB2* genomic locus rearrangements are associated with aggressive B cell lymphomas and translocation involving *ZEB2* and *BCL11B* loci are identified in early T-ALL (Goossens and Haigh, 2012).

In acute myeloid leukemia (AML), ZEB1 is a direct target of MLL-AF9 and a knockdown of *Zeb1* decreased the invasiveness *in vitro* and *in vivo* (Stavropoulou et al., 2016). ZEB2 expression was also crucial for the growth of AML (Li et al., 2017a; Meyer, 2017).

RATIONALE AND OBJECTIVES

RATIONALE AND OBJECTIVES

The overall objective of this dissertation is to investigate the role of ZEB1 and ZEB2 in the normal B cell differentiation and activation processes, and in the context of two of the most common B cell malignancies, DLBCL and MM.

The results of this thesis aimed to address the following specific objectives:

1. Expression and role of the ZEB factors in the GC dynamics and its activation.
2. Expression and role of the ZEB factors in the progression of DLBCL.
3. Expression and role of the ZEB factors in the progression and dissemination of MM.

MATERIALS AND METHODS

MATERIALS AND METHODS

Established cell lines.

MM cell lines (MM1.S and RPMI2886) were kindly provided from Dr. Ghobrial (Dana-Farber Cancer Institute, Boston, USA) and Dr. Fernández-Larrea (Dept. of Hematology, Hospital Clínic, Barcelona, Spain) respectively. The NHL cell lines (DOHH2, WSU-DLCL2, WSU-FSCCL, Karpas-422, SC-1, OCI-Ly8, U2932, Farage and Pfeiffer) and the human embryonic kidney 293T cells were obtained from the American Tissue Culture Collection cell line depository (ATCC, Manassas, USA).

MM and DLBCL cell lines were grown in Roswell Park Memorial Institute (RPMI-1640) medium (Lonza, Basel, Switzerland) supplemented with either 10% or 20% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, USA) and 1% penicillin/streptomycin (Lonza). The 293T cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) medium (Lonza) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Lonza).

MM and DLBCL human samples.

MM human samples were obtained from Dr. Fernández-Larrea (Hospital Clínic). RNA was processed as described below and retrotranscribed to cDNA. Procurement and use of human samples had the approval of the ethical research committee of the Hospital Clínic of Barcelona under reference HCB 2014/0820 and were obtained in line with the Helsinki Declaration and with the prior consent from patients.

A series of formalin-fixed and paraffin-embedded slides of human DLBCLs were obtained from Dr. Fernández-Aceñero (Dept. of Pathology, Hospital Gregorio Marañon, Madrid, Spain) and Dr. Colomo (Dept. of Pathology, Hospital del Mar, Barcelona, Spain) and were included into Tissue MicroArrays (TMA). The procurement and use of human samples had the approval of the ethical research committee of the Hospital Clínic of Barcelona under reference HCB 2014/0820 and were obtained in line with the Helsinki Declaration and with the prior consent from patients.

Plasmids and lentiviral particles.

Inducible vectors of interference (TRIPZ) and overexpression (pLUT) from Dr. Ivanov (West Virginia University Cancer Institute, Morgantown, USA) were used. For the production of lentiviral particles, we used the envelope (pMDG2) and packaging (pSPAX2) vectors along with the lentiviral vectors containing short hairpins targeting *ZEB1* (TTTACAACTGTTTGTAGCG), *ZEB2* (TTGAACTTGCGATTACCTG) or a scrambled non-targeting hairpin sequence as control.

293T cells were used to produce lentiviral particles expressing the vector of interest. The cells were transfected with the plasmids mentioned above (envelope, packaging, and vector of interest). Viral supernatants were collected at 48h and 72h post-transfection and concentrated by centrifugation at 100,000g for 2h. Virus titration to ensure an optimal infection rate was determined using 293T cells.

Viral supernatants were used to infect the different DLBCL and MM cell lines by centrifugation at 600g for 2h at 32ºC. For the creation of stable cell lines, cells were selected by treating with puromycin (Thermo Fisher, Waltham, USA), and for the induction of the shRNA, cells were treated with 1 mg/ml of Doxycycline (Thermo Fisher).

RNA extraction and Quantitative Real-Time PCR.

Total RNA was extracted with TRIzol Reagent (Thermo Fisher) and retrotranscribed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). mRNA levels were quantified using quantitative real-time PCR (qRT-PCR) with the GoTaq® qPCR Master Sybr Green Mix (Promega, Madison, USA). Primers used are listed in Table 4. Relative expression was analyzed using the Opticon Monitor software (Bio-Rad, Hercules, USA) with the ΔΔCT method and normalized with a housekeeping gene (*GAPDH, B-ACTIN,* and or *GUSB*).

Target gene	Forward (5' to 3')	Reverse (5' to 3')
B-ACTIN	CCCAGCACAATGAAGATCAA	ACATCTGCTGGAAGGTGGAC
BBC3	GACCTCAACGCACAGTACGAG	AGGAGTCCCATGATGAGATTGT
BIRC5	GCCCAGTGTTTCTTCTGCTT	CCGGACGAATGCTTTTTATG
CXCR4	ATAGTCCCCTGAGCCCATTT	AGCAGGTAGCAAAGTGACGC
DKK1	GATATCCCAGAAGAACCACACTGACT	GGACCAGAGTGTCTTGCACAA
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
GFM1	GTGTTGGATGGTGCAGTCCT	TCGAACAATCTGACCAAAGTCTC
GLUT1	CTTTGTGGCCTTCTTTGAAGT	CCACACAGTTGCTCCACAT
GUSB	CTCATTTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTTA
MKI67	CGTCCCAGTGGAAGAGTTGT	CGACCCCGCTCCTTTTGATA
NEK2	CCAGCCCTGTATTGAGTG	ACTTCCGTTCCTTTAGCA
PDK1	ACCAGGACAGCCAATACAAG	CCTCGGTCACTCATCTTCAC
TUFM	CATCAATGCGGCTCATGTGG	CATGCTCCACCCCAATCTGT
YARS1	CTCCACCTTTTCCCGTCTCA	GACATGGGACAAAGTAAGCC
ZEB1	AGCAGTGAAAGAGAAGGGAATGC	GGTCCTCTTCAGGTGCCTCAG
ZEB2	GAAAAGCAGTTCCCTTCTGC	GCCTTGAGTGCTCGATAAGG

Table 4. Primers used for Quantitative Real-Time PCR.

Cell migration assay.

Prior to the assay, MM cell lines were incubated with a starvation medium consisting of RPMI-1640 with 1 % FBS for 12h. 0.5 x 10^6 cells were labeled with 1 μM CFSE (5(6)-Carboxyfluorescein diacetate N-succinimidyl ester, Sigma-Aldrich) and

seeded in the upper chamber of a transwell in an 8 µm pore insert, placed over a 12-well-plate (Corning, Corning, USA). 30 nM rhCXCL12 (ImmunoTools, Friesoythe, Alemania) was added to the lower chamber and incubated for 4 hours. The results were read, and the fluorescence quantified with a GloMax®-Multi Detection System (Promega) microplate reader.

Proliferation and viability assay.

DLBCL and MM were seeded onto 96-well-plates and incubated at the indicated time points with or without different drug treatments at the indicated doses. After the indicated time points, 5 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) diluted in PBS was added and incubated for 3-4h. The formazan precipitated was diluted with DMSO (Dimethyl sulfoxide, Thermo Fisher), and the colorimetric assay was detected at 560 nm absorbance using a 750 mm absorbance as reference wavelength with the GloMax®-Multi Detection System (Promega) microplate reader. Each measurement was made in triplicate, and values were represented with respect to cells with the scrambled shRNA, as reference.

To assess the proliferation of DLBCL, an EdU uptake assay was performed using the Click-it EdU kit (Thermo Fisher). On the indicated time point, cells were incubated with 10 µM of EdU for 2h. Cells were washed and incubated with a Fixable Viability Dye (Thermo Fisher) and incubated for 30 min at 2-8ºC, protected from light. Then, cells were fixed and permeabilized and incubated with the Click-it reaction cocktail for 30 min at room temperature, protected from light. Once the cells were washed, the analysis was assessed in a BD FACSCanto2 (BD Biosciences, San Jose, USA), and the data were analyzed using FlowJo (FlowJo, Ashland, USA).

Immunohistochemistry and immunofluorescence.

Mouse tissue samples were formalin-fixed overnight, and paraffin-embedded (FFPE) and sections of 4 µm were cut. Other samples were OCT-embedded (Optical cutting temperature compound, Electron Microscope Sciences, Hatfield, USA), frozen and stored at -80ºC. 7 µm cryosections were cut with Leica Cryostat (Leica, Wetzlar, Germany).

Formalin-fixed and paraffin-embedded slides were deparaffinized with xylenes and rehydrated using standard protocols before being incubated with 10 mM Sodium Citrate (pH6) for antigen retrieval. Frozen slides were fixed with 1% paraformaldehyde and permeabilized with PBS 1% NP-40 and PBS 0.25% X-Triton.

For DAB (3,3-Diaminobenzidine) immunostainings, all slides were incubated with 0.3% H_2O_2 to block endogenous signaling and a blocking solution to minimize unspecific IgG binding (5% goat/donkey normal serum depending on the secondary antibody, 4% BSA and 0.5% Tween-20 in PBS) followed by overnight incubation with the primary antibody and a 1-hour incubation with an HRP-conjugated antibody. The staining was developed using a DAB peroxidase substrate kit (Vector Laboratories, Burlingame, USA), counterstained with hematoxylin (Sigma-Aldrich) and mounted in Dibutylphthalate Polystyrene Xylene solution (DPX, Sigma-Aldrich). Hematoxylin and eosin (H&E) staining were performed right after rehydration by hematoxylin and eosin incubations.

For immunofluorescence assays, slides were incubated with 0.1% $NaBH_4$ PBS to block endogenous signaling, instead of H_2O_2. Detection of the primary antibody was performed with immunofluorescent-conjugated secondary antibodies and DAPI ProLong Antifade (Thermo Fisher) was used to counterstain the nucleus.

All slides were analyzed in an Olympus BX41 (Olympus, Tokyo, Japan) and with ImageJ software (National Institute of Health, Bethesda, USA). Antibodies used in this dissertation are listed in Table 5. The intensity and proportion of positive cells in the DLBCL TMA slides were assessed by external pathologists.

Flow cytometry analysis and cell sorting (FACS).

Mouse spleens were collected at the indicated time points. Splenocytes were obtained by mechanical disaggregation of the tissue and filtered through a 70 µm cell strainer. Erythrocytes were removed using Red Blood Lysis Buffer (Sigma-Aldrich). A single-cell suspension was incubated in a blocking solution and stained with a combination of different fluorophore-labeled antibodies (listed in Table 5). Expression of cell surface proteins was assessed in either a BD FACSFortessa analyzer or a BD

FACSCanto3 (BD Biosciences, San Jose, USA) and the data were analyzed using FlowJo (FlowJo, Ashland, USA). Cell counting was done with Perfect Count Beads (Cytognos, Salamanca, Spain).

Antibodies.

Primary Antibodies	Source	Clone
Flow cytometry analysis		
B220-PB	BD Bioscience	RA3-6B2
CD21-PECy7	eBioscience	8D9
CD23-PE	BD Bioscience	B3B4
CD25-PE	BD Bioscience	PC61
CD3-A488	BioLegend	17A2
CD3-PECy7	Tonbo Bioscience	145-2C11
CD44-FITC	BD Bioscience	IM7
CD4-PB	BioLegend	GK1.5
CD62L-APC	eBioscience	MEL-14
CD8-PE	BD Bioscience	53-6.7
CD95-APC	BD Bioscience	Jo2
CXCR5-APC	BioLegend	L138D7
GL7-A488	Thermo Fischer	GL7
MHCII-FITC	Miltenyi	M5/114.15.2
PD1-PE	Tonbo Bioscience	J43.1
Immunohistochemistry / Immunofluorescence		
BCL6	SantaCruz	N-3
CD45R-PE	eBioscience	RA3-6B2
CD68	SantaCruz	KP1
S100A4	SantaCruz	PA5-18601
ZEB1	Sigma-Aldrich	HPA027524
ZEB1	Sigma-Aldrich	AMAB90510
ZEB2	Sigma-Aldrich	HPA003456

Table 5. List of antibodies used in different experiments.

Generation of conditional *Zeb1* knockout mice.

The conditional *Zeb1* flox mouse model was generated in collaboration with the Transgenic Services Facility, Centro de Biologia Molecular Severo Ochoa (CBMSO, Madrid, Spain).

Briefly, two sgRNAs were designed to elicit double-strand breaks (DSBs) flanking exon 6 in the *Zeb1* gene, along with two ssDNA oligos containing the corresponding LoxP site and a restriction enzyme (Table 6, Figure 9).

sgRNA 5'	TTACAGACACCTCTAACACAAGG
sgRNA 3'	AGTACCAGCAAACCCTTTCTTGG
ssDNA 5'	AGCTAAGTCCCTTCAAGTGCCTGGTCACTGAGGAAAGCTGGGGTTACAGACACC TCTAACGCTAGCATAACTTCGTATAGCATACATTATACGAAGTTATACAAGGCT TCCTCCCCAAAAGGGAGCCGTACAGACATGAAAATATTTATCAATCAAAGGC
ssDNA 3'	AACCAAAGGTTAACCTAACTCCTAACAAAGGAGTTGGCACACGAAGTACCAGCA ACCCTGAATTCATAACTTCGTATAATGTATGCTATACGAAGTTATTTCTTGGCT TATGGTGAATGGGAACATGGTTGTTTAATAGTGATCATAAGCAAAGAAGA

Table 6. DNA and RNA sequences for the generation of the *Zeb1* flox mouse model.

A mixture of *in vitro* transcribed RNA (100 ng/µl for *Cas9* and 50 ng/µl for each sgRNAs) and ssDNAs (100ng/µl for each) was injected into the cytoplasm of B6CBAF2 zygotes, using standard procedures (Behringer et al., 2014). Zygotes that survived the injections were transferred into the oviducts of pseudopregnant foster mothers for development to term. The progeny was then crossed with wild type C57BL6/Jcrl mice and the presence of LoxP sequences correctly inserted was analyzed by DNA sequencing.

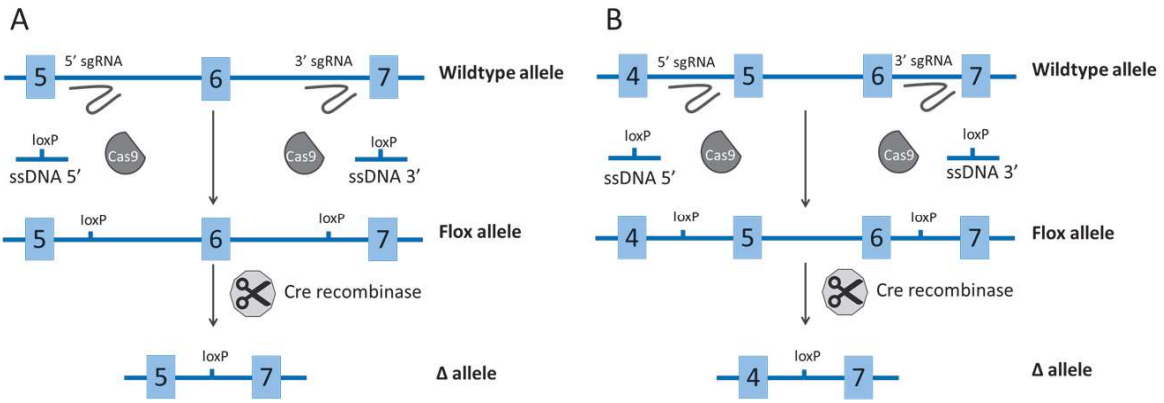

Figure 9. Scheme of *Zeb1* and *Zeb2* mouse model generation. **(A)** The strategy used for the generation of the *Zeb1* flox mouse model and **(B)** the *Zeb2* flox mouse model.

Generation of conditional *Zeb2* knockout mice.

The conditional *Zeb2* flox mouse model was generated in collaboration with the Mouse Mutant Core Facility at the Insitute for Research in Biomedicine (IRB, Barcelona, Spain) according to a method described elsewhere (Yang et al., 2013).

sgRNA 5'	TGAAACACACAAAAAAAGGAAATTTA
sgRNA 3'	TTGAAATGACTGAGTAGGGCTAATTCA
ssDNA 5'	ATAACTTCGTATAGCATACATTATACGAAGTTATGGATCCGCTAGC
ssDNA 3'	CCAGGCTCTAGAGAGCTCATAACTTCGTATAGCATACATTATACGAAGTTAT

Table 7. DNA and RNA sequences for the generation of the *Zeb2* flox mouse model.

Briefly, 2 sgRNA were designed to flank the exon 5 and 6 of the *Zeb2* gene along the ssDNA oligos, containing the LoxP site and a restriction enzyme (BamHI and XbaI) (Table 7, Figure 9).

A mixture of the sgRNA (1,25 ng/µl for each) ssDNA (5 ng/µl for each) and Cas9 protein (30 ng/µl) was injected in the pronucleus of the zygotes and transferred into foster mothers for development to term. Offspring were genotyped by PCR analysis of DNA obtained from tail biopsies using primers flanking the LoxP insertion sites and

positives were identified by the presence of the correct band size corresponding to the size of the inserted sequence. To ensure that the LoxP sites were inserted correctly, we carried out a restriction fragment-length polymorphism assay (RFLP), nested PCR, and DNA sequencing on the fragment.

Transgenic mice for the GC B cell-specific deletion of Zeb1 or Zeb2.

Conditional *Zeb1* floxed mice (loxP-flanked Zeb1 allele, *Zeb1* $^{fl/fl}$) were crossed with the transgenic *Cγ1* cre strain to obtain *Zeb1* $^{fl/fl}$; Cγ1$^{cre/wt}$ (hereafter referred to as *Zeb1*$^{\Delta GC}$). Conditional Zeb2 floxed mice (loxP-flanked Zeb2 allele, *Zeb2* $^{fl/fl}$) were crossed with *Cγ1* cre strain to obtain *Zeb2* $^{fl/fl}$; Cγ1$^{cre/wt}$ (hereafter referred to as *Zeb1*$^{\Delta GC}$). As control group, we used *Zeb1* $^{fl/fl}$; *Cγ1*$^{wt/wt}$ (*Zeb1*^{WT}) and *Zeb2* $^{fl/fl}$; *Cγ1*$^{wt/wt}$ littermates (*Zeb2*^{WT}) (from now on referred as control mice). The *Cγ1* cre mice model were obtained from Dr. Melnick (Cornell University, New York, USA) (Béguelin et al., 2016).

The use of mice in this dissertation followed the guidelines established by the Animal Experimental Committee of the University of Barcelona School of Medicine (Barcelona, Spain) and was approved under the protocol numbers 321/18 and 251/19.

Assessment of GC formation and immunizations in mice.

8-10 week old mice of the four genotypes (*Zeb1*^{WT}, *Zeb1*$^{\Delta GC}$, *Zeb2*^{WT} *and Zeb2*$^{\Delta GC}$) were immunized intraperitoneally (ip) with 100 µg 2,4,6-trinitrophenyl (TNP)-keyhole limpet hemocyanin (KLH) conjugate (TNP_{31}-KLH, Biosearch Technologies, Novato, USA) in Complete Freund's Adjuvant (CFA, Sigma-Aldrich) at day 0 and boosted at day 14 with 100 µg TNP_{31}-KLH diluted in PBS. Serum samples were collected from nonlethal mandibular bleedings at day 0 (before the immunization), 7, 14, and 21 days following the immunizations. Mice were euthanized on day 21 to obtain the spleen and lymph nodes for further analysis. When specified, 8-10-week-old mice were immunized intraperitoneally with 500 µl of 2% sheep Red Blood Cells (sRBC, Innovative Research, Novi, USA) in PBS on day 0 and boosted at day 10. Mice were euthanized at day 20 to collect the spleen and lymph nodes for further analysis.

Basal experiments with no immunizations were also performed, and mice were euthanized at 8-10 weeks of age to collect serum, spleen, and lymph nodes for further analysis.

Determination of antibody production.

The detection of antibody production was assessed by enzyme-linked immunosorbent assay (ELISA). For the detection of TNP-specific antibodies, high binding plates (Corning) were coated with 4 μg/ml TNP-BSA and for the detection of total IgG and IgM with 3 μg/mL anti-mouse IgG (Sigma, M2650) or 3 μg/mL anti-mouse IgM (Jackson Immunoresearch, West Grove, USA, 115-006-075). Serum samples were diluted in 2% BSA-PBS and incubated with HRP conjugated anti-mouse IgG (Sigma, A3673) or biotin-conjugated anti-mouse IgM (Jackson Immunoresearch) and streptavidin-peroxidase (Roche, Basel, Switzerland). The reaction was developed with TMB (Abcam, Cambridge, UK), stopped with a Stop Solution (1N HCl and 0.6N H_2SO_4), and read at 405 nm with a GloMax®-Multi Detection System (Promega) microplate reader.

Gene expression array data and survival plots.

Association between *ZEB1* and *ZEB2* in different B cell populations was examined in different published arrays: GSE26408 (Green et al., 2011) and GSE38696 (Victora et al., 2012).

Correlation between the expression of ZEB1 and ZEB2 and overall survival in DLBCL patients was examined in two array databases, GSE10846 and GSE34171 (Lenz et al., 2008). Association between *ZEB1* and *ZEB2* in DLBCL array, classified upon their metabolic phenotype by Caro et al., was examined in a published array: GSE10846 (Lenz et al., 2008).

Association between ZEB1 and ZEB2 in different MM arrays was examined in different published arrays: GSE9782 (Mulligan et al., 2007), GSE4204 (Driscoll et al., 2010), GSE24080 (Mitchell et al., 2016), GSE5900 (Zhan et al., 2007), GSE27838 (Garg

et al., 2012), GSE71935 (Helsmoortel et al., 2016), and GSE7307 (From the Human Body index by Roth and co-workers).

Statistical analysis.

GraphPad Prism v. 8 (GraphPad Software Inc., San Diego, USA) was used to perform all quantitative analysis. A non-parametric Mann-Whitney test was used to assess the statistical significance between groups classified as non-significant for $p>0.05$ or significant for values of $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). In the histogram plots, the bars represented the mean with the error bar (standard error of the mean). For the Kaplan Meyer survival plots, a Cox proportional hazard regression was used.

RESULTS

RESULTS

1. ROLE OF ZEB1 AND ZEB2 IN B CELL DIFFERENTIATION AND ACTIVATION

1.1. Characterization of ZEB1 and ZEB2 expression in B cells

In the hematopoietic lineage, ZEB1 and ZEB2 factors have been seen to be frequently, but not always, mutually exclusive (Soen et al., 2018). Both factors are expressed in the human tonsils and the spleens of mice. Its expression is located in the GC B cells and also in some cells from the microenvironment (dendritic cells, histiocytes, and endothelial cells) (Figure 10).

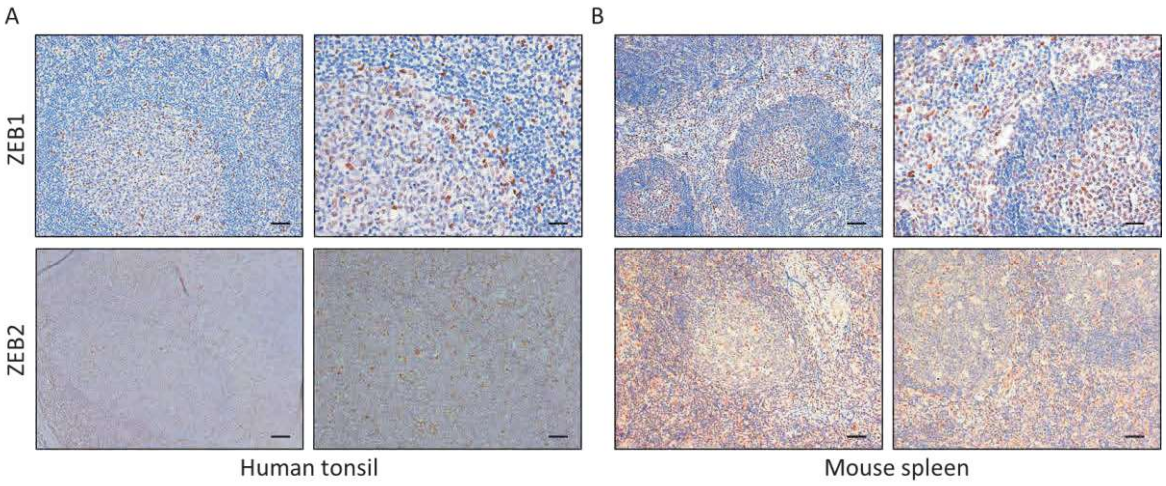

Figure 10. ZEB1 and ZEB2 expression in human tonsils and mouse spleens. Representative images of immunostainings with ZEB1 or ZEB2 in **(A)** human tonsils and **(B)** mouse spleen. Scale bars represent 100 μm and 50 μm.

Analysis of a published array (GSE26408, n=4) showed a reversed pattern of the mRNA expression of both factors along with the B cell differentiation and confirmed the expression of both, ZEB1 and ZEB2, in the GC B cells, with a higher expression of ZEB1 (Figure 11.A). We also analyzed a published array of centroblasts and centrocytes of the mice spleen (GSE38696, n=4) and observed a significant switch

between ZEB1 and ZEB2, being ZEB1 higher in the centroblasts and ZEB2 higher in the centrocytes (Figure 11.B).

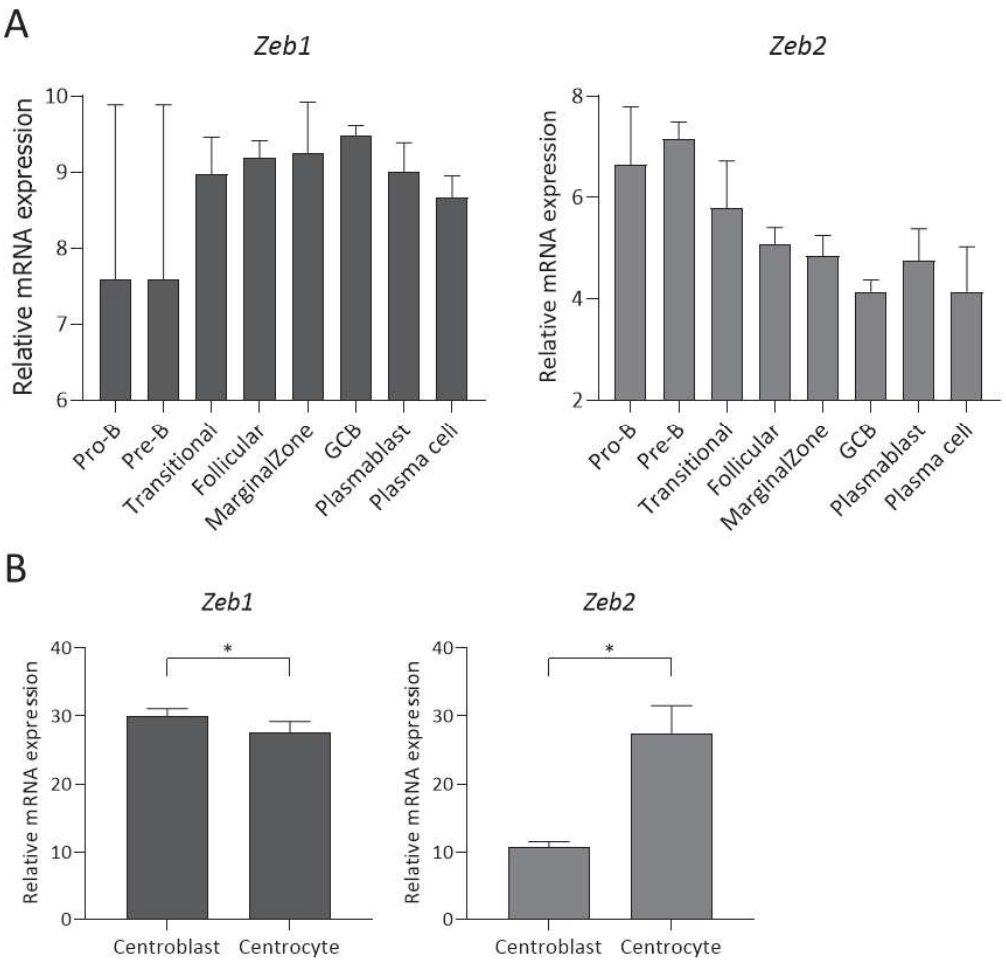

Figure 11. Zeb1 and Zeb2 expression in the B cell lineages. **(A)** *Zeb1* and *Zeb2* expression of B cells of different stages of development purified from the bone marrow and spleen of mice (GSE26408) **(B)** *Zeb1* and *Zeb2* expression of centroblasts and centrocytes from LN from mice (GSE38696).

1.2. Deletion of *Zeb1* and *Zeb2* in GC cells *in vivo*

To characterize the specific role of the ZEB factors in the activation and differentiation of B cells, we generated conditional knockout mice for *Zeb1* and *Zeb2*, described in Material and Methods.

In the *Zeb1* flox model, the exon 6 codifies for a large proportion of the central ZEB1 protein and upon cre-mediated deletion, resulted in a truncated form of the protein due to a premature stop of translation. In the *Zeb2* flox model, upon the cre deletion, it resulted in a truncated form of the protein that lacks the exon 5 and 6.

These mice bearing the floxed gene (*Zeb1* or *Zeb2)* in both alleles were crossed with transgenic cre mice targeting *Cγ1* promoter, which relies on the property of the GC B cells to undergo Ig class switch recombination obtaining an efficiency of more than the 85% in the GC B cells (Casola et al., 2006). The mouse models ($Zeb1^{\Delta GC}$ and $Zeb2^{\Delta GC}$) had a knockout expression of *Zeb1* or *Zeb2,* when the *Ighg1* gene is expressed in the GC, compared with its control.

At the histological level, we observed differences in the architecture of the spleen in the $Zeb1^{\Delta GC}$ and $Zeb2^{\Delta GC}$ mice. There were fewer GC in both models, being more evident in the $Zeb1^{\Delta GC}$. Follicles in the $Zeb2^{\Delta GC}$ mice have a less organized structure, lacking a clear distinction between the light and dark zones (Figure 12). Therefore, we decided to check the dynamics in the different B cell populations.

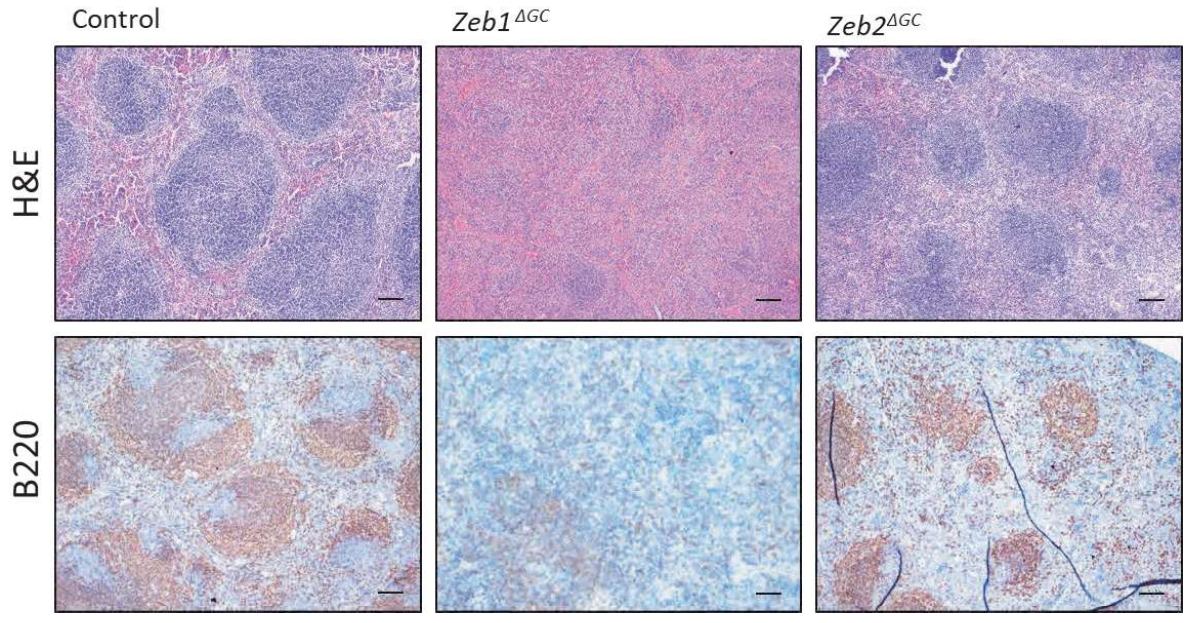

Figure 12. Immunostaining of spleen of the mice. Representative images of spleens with hematoxylin and eosin (H&E) staining and B220 staining. Scale bar represents 100 μm.

1.3. Characterization of B cells in *Zeb1*$^{\Delta GC}$ and *Zeb2*$^{\Delta GC}$ mice

We examined the different B cell populations in the spleen of non-immunized mice and compared it to mice that developed a T-dependent response by a protocol of immunizations with TNP_{31}-KLH, described in Materials and Methods.

As expected, the immunization was accompanied by a significant increase in the size of the spleen and the number of total splenocytes and of B (B220+) cells (Figure 13. B and E-F), but no differences were observed among the different groups. The immunization was also accompanied by an increase in the spleen weight, except in the *Zeb2*$^{\Delta GC}$, which did not increase upon immunization and had a significantly smaller spleen, compared with its control counterparts (Figure 13.C).

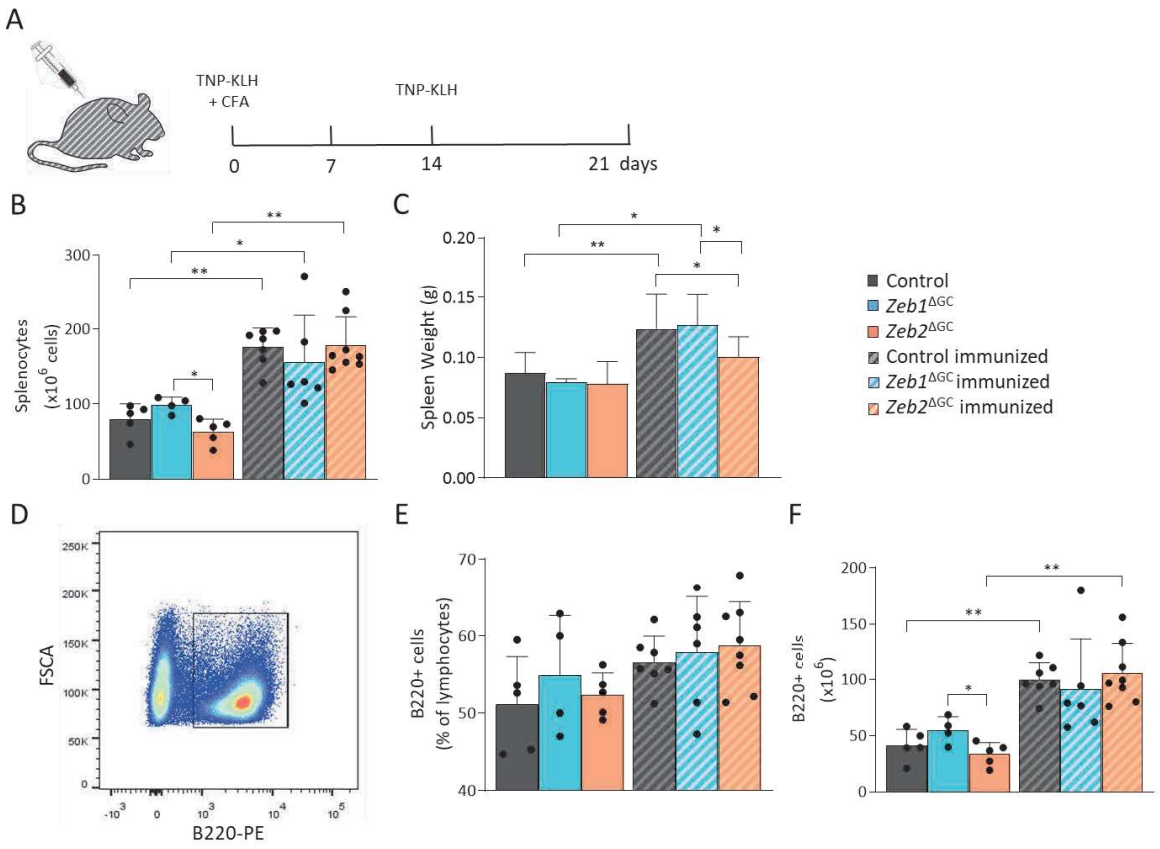

Figure 13. *Zeb1* and *Zeb2* in the spleen and B cells. **(A)** Scheme of the protocol used for immunizations. 8-10 weeks-old mice were injected i.p. with TNP-KLH on day 0, boosted on day 14, and were euthanized on day 21. Non-immunized mice were 8-10 weeks old. **(B)** FACS analysis of the total number of splenocytes. **(C)** Spleen weight of non-immunized and

immunized mice **(D)** Representative FACS plot of the B cell gating. **(E)** FACS analysis of the proportion and **(F)** the total number of the B cells.

We then checked the different B cell subpopulations of the spleen (Figure 14). Upon immunization, there were significant increases in the Follicular B cells ($CD21^{+}CD23^{-}$) and GC B cells ($CD95^{+}GL7^{+}$) of the control mice but not in the MZ B cells ($CD21^{+}CD23^{+}$). In the *Zeb1*$^{\Delta GC}$, no significant differences, except for a slightly increased number of MZ, were observed comparing the non-immunized and immunized mice and in the *Zeb2*$^{\Delta GC}$ results were similar to the control activation.

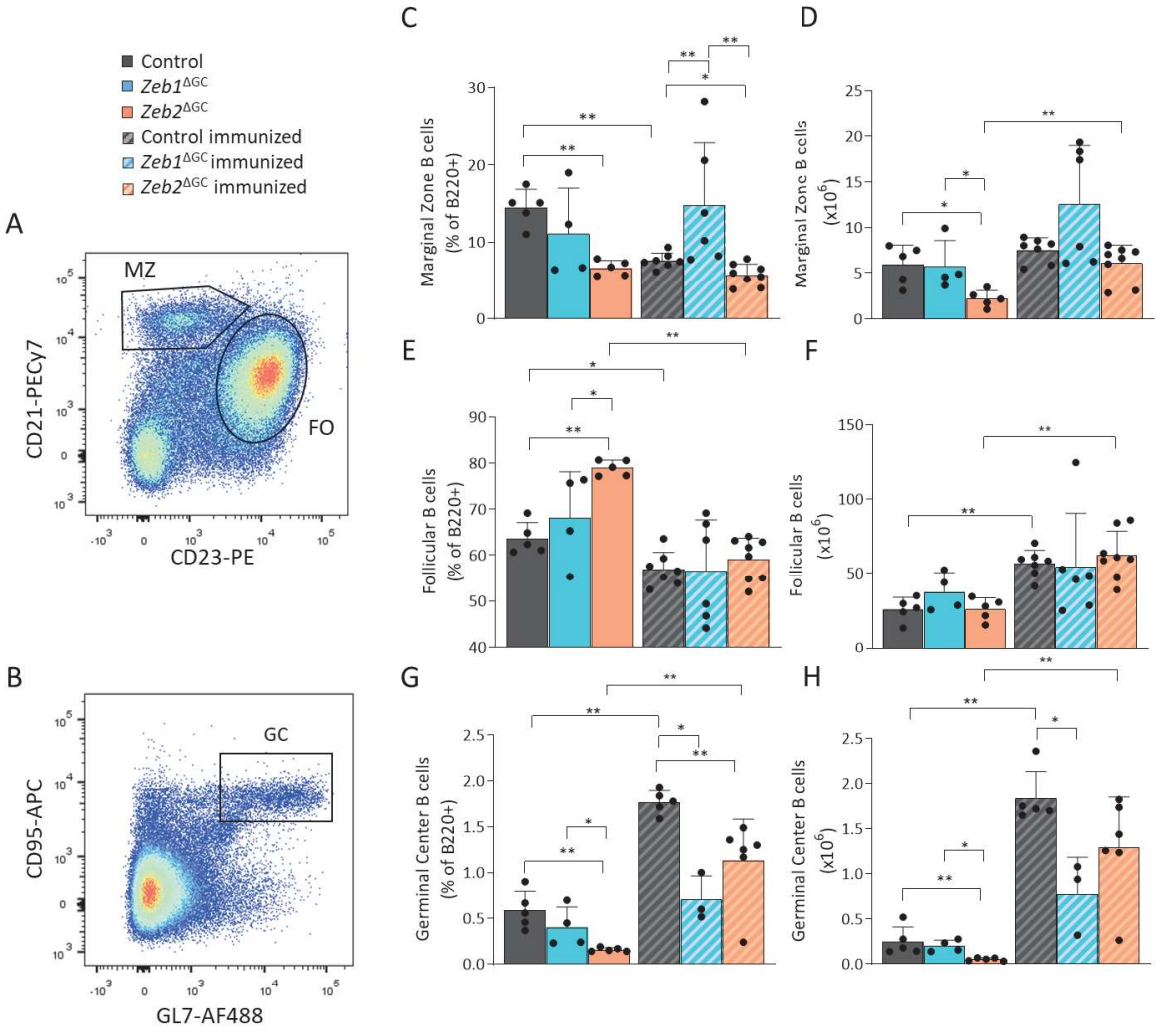

Figure 14. *Zeb1* and *Zeb2* in the B cell subpopulations. **(A)** Representative FACS plot of the MZ and Follicular (FO) cell gating. **(B)** Representative FACS plot of the GC B cell gating. **(C)** FACS analysis of the proportion and **(D)** the total number of the MZ cells. **(E)** FACS analysis of

the proportion and **(F)** the total number of the Follicular cells. **(G)** FACS analysis of the proportion of and **(H)** the total number of the GC B cells.

In non-immunized conditions, *Zeb1*$^{\Delta GC}$ showed similar results to the control mice, but the *Zeb2*$^{\Delta GC}$ mice showed a decreased number of MZ and GC B cells. In the immunized group, there was a trend to an increased MZ and a significant decrease in the GC B cells in the *Zeb1*$^{\Delta GC}$. The *Zeb2*$^{\Delta GC}$ mice showed similar results to the control, and the decreased GC in the non-immunized conditions was reversed as we can see that there is a tendency to have a lower number of GC B cells, but these differences were not statistically significant.

One of the master genes regulators of GC is BCL6, a transcriptional repressor essential for the initiation of the GC reaction, the migration of GC precursors into the follicle, and the differentiation into memory B cells and plasma cells (Basso and Dalla-Favera, 2012; De Silva and Klein, 2015). We checked its expression in the spleens of non-immunized mice and observed decreased levels of BCL6 expression in both *Zeb1*$^{\Delta GC}$ and *Zeb2*$^{\Delta GC}$ compared with their control counterparts (Figure 15).

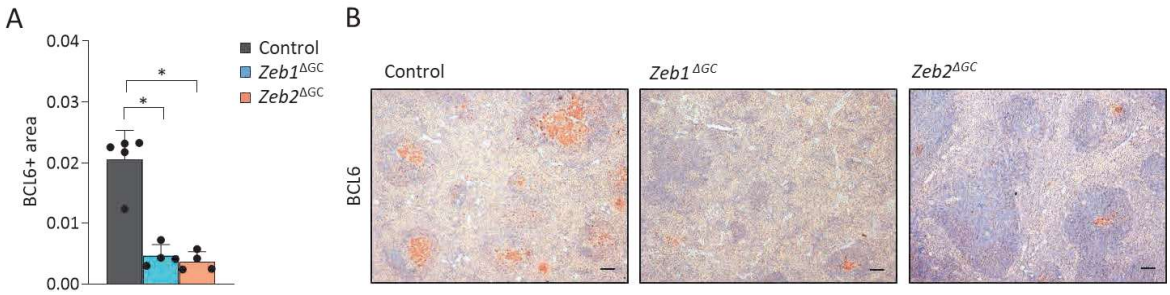

Figure 15. BCL6 expression in spleen. **(A)** Percentage of BCL6+ area in the spleen **(B)** Representative images of immunostaining of BCL6. Scale bar represents 200 μm.

1.4. Characterization of T cells in *Zeb1*$^{\Delta GC}$ and *Zeb2*$^{\Delta GC}$ mice

We then studied whether Zeb1 or Zeb2 knockout expression in the GC B cells affects the absolute and relative number of T cell subpopulations in the spleen (Figure 16). A significant decrease in the number of cells was observed in both CD4+ and CD8+ T cells in the non-immunized Zeb2$^{\Delta GC}$ compared with its control. This effect was

reverted upon immunization, as we observed an increase in the T cells of the Zeb2$^{\Delta GC}$ upon immunization. However, no other significant differences were observed among the groups or upon immunization. In order to see if there was an activation of these cells, we decided to look at the different subsets of CD4+ and CD8+ T cells: Naïve (CD62L+CD44-), Effector Memory (EM, CD62L-CD44+) and Central Memory (CM, CD62L+CD44+) (Figure 17.A).

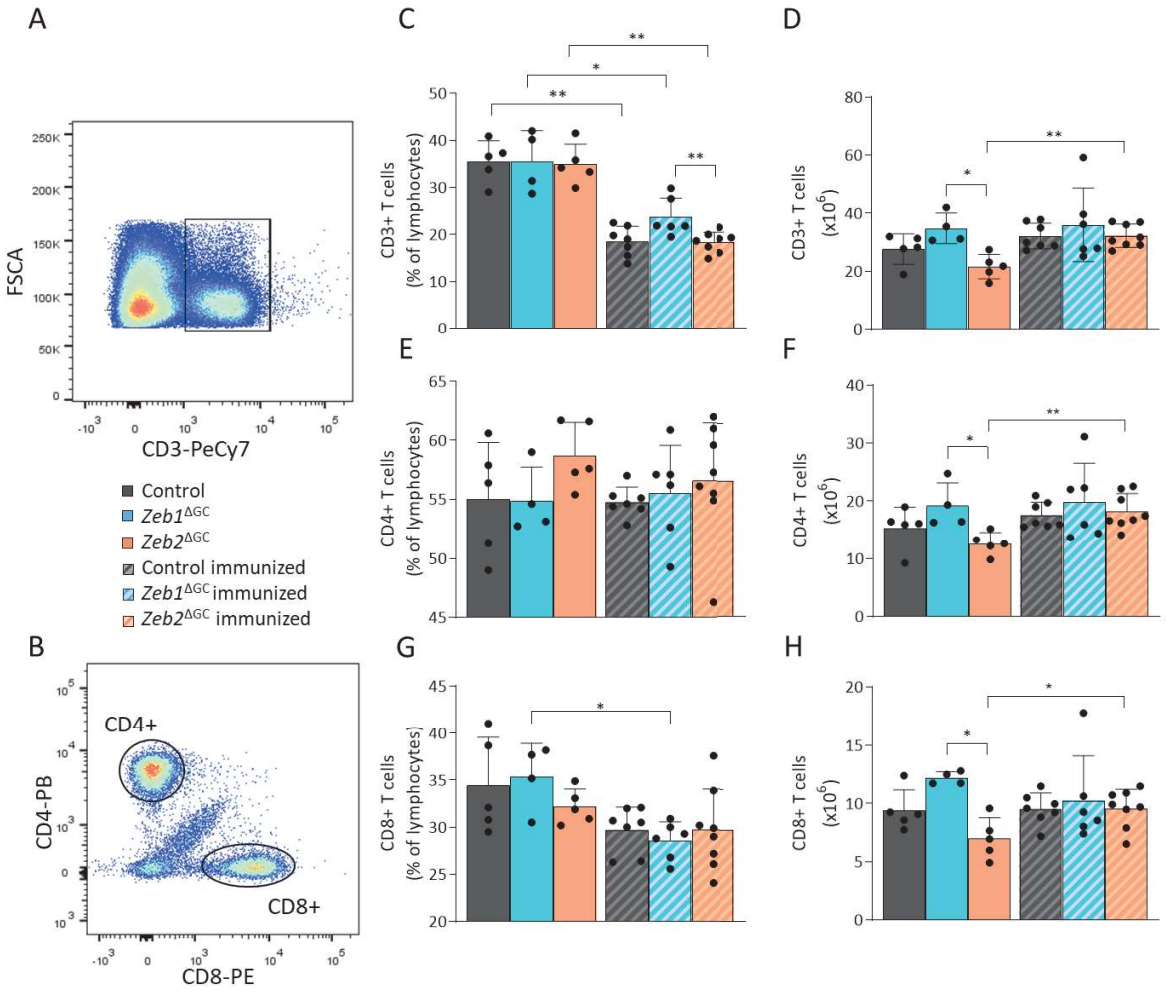

Figure 16. *Zeb1* and *Zeb2* in the T cell population. **(A)** Representative FACS plot of the T cell gating (CD3+). **(B)** Representative FACS plot of the CD4+ T cell and CD8+ T cell gating**. (C)** FACS analysis of the proportion and **(D)** the total number of the T cells. **(E)** FACS analysis of the proportion and **(F)** the total number of the CD4+ T cells. **(G)** FACS analysis of the proportion of and **(H)** the total number of the CD8+ T cells.

When T cells encounter an antigen from a T-dependent response, they will differentiate from naive into effector and central memory, with different degrees of expansion and proliferative capacities; being the central memory T cells more proliferative as they will differentiate to effector memory when they are exposed again to an antigen (Golubovskaya and Wu, 2016).

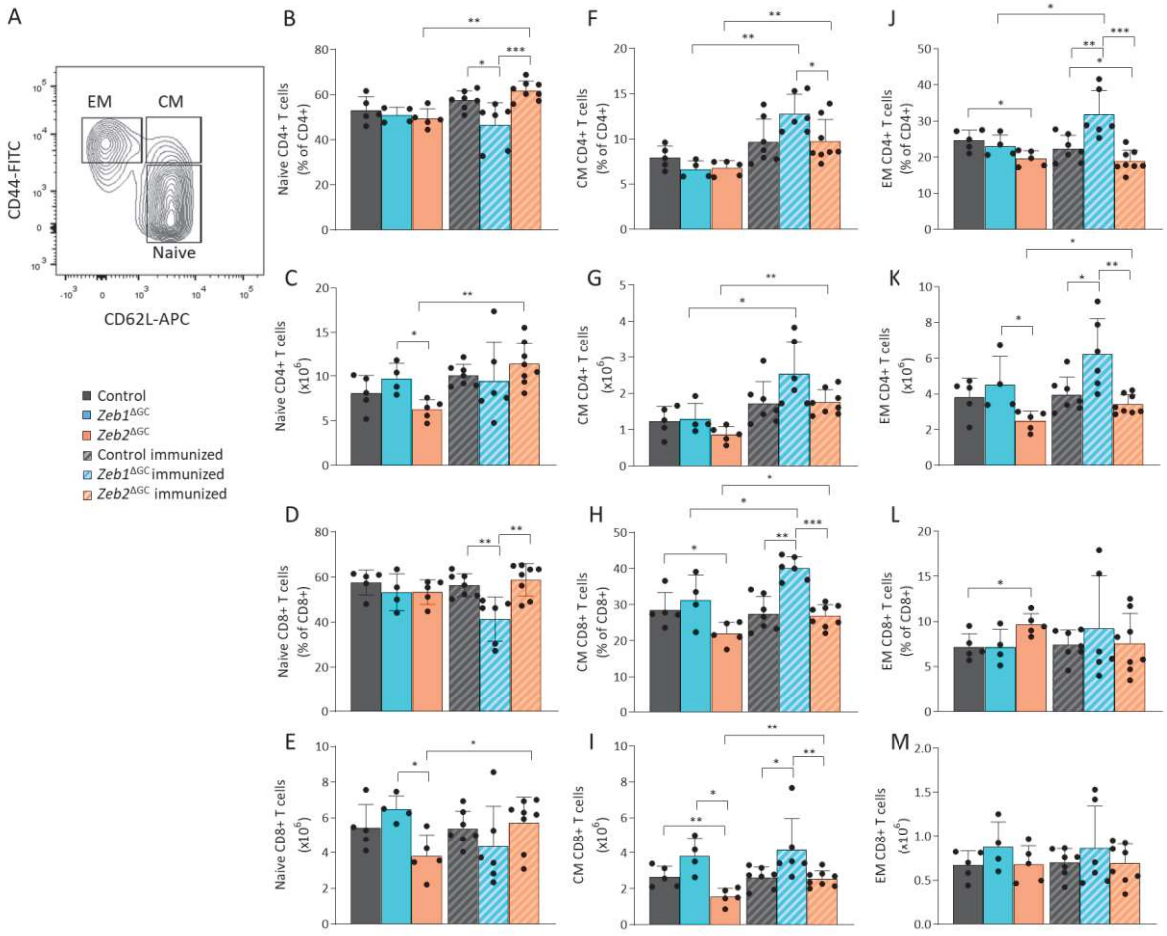

Figure 17. *Zeb1* and *Zeb2* in the T cell subpopulation. **(A)** Representative FACS plot of the T cell gating. **(B)** FACS analysis of the proportion and **(C)** the total number of the CD4 naïve T cells. **(D)** FACS analysis of the proportion and **(E)** the total number of the CD8 naïve T cells. **(F)** FACS analysis of the proportion and **(G)** the total number of the CD4 central memory T cells**. (H)** FACS analysis of the proportion and **(I)** the total number of the CD8 central memory T cells. **(J)** FACS analysis of the proportion and **(K)** the total number of the CD4 effector memory T cells. **(L)** FACS analysis of the proportion and **(M)** the total number of the CD8 effector memory T cells.

Analysis of the different subsets of the T cell subsets showed similar patterns in both CD4+ and CD8+ cells (Figure 17). Upon immunization, the control mice did not vary, but the immunized *Zeb1*$^{\Delta GC}$ increased the number of central memory T cells compared with its non-immunized counterpart. In the *Zeb2*$^{\Delta GC}$ immunized mice, the number of cells also increased in the different subsets.

Within each group, we observed that in the non-immunized conditions, there were more naïve cells in the *Zeb1*$^{\Delta GC}$ and fewer in the *Zeb2*$^{\Delta GC}$, along with a decrease in the effector memory of the *Zeb2*$^{\Delta GC}$. In the immunized subset, we did not observe changes in the *Zeb2*$^{\Delta G}$, but a trend in having fewer naïve cells and more central and effector memory cells in the *Zeb1*$^{\Delta GC}$.

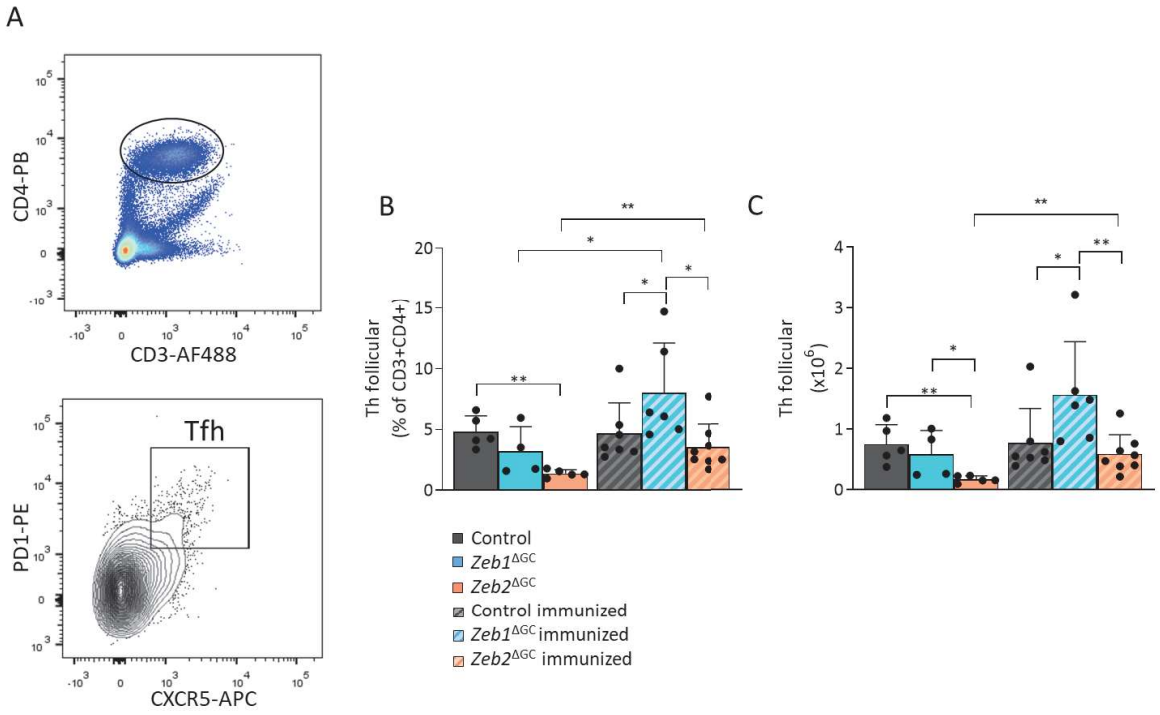

Figure 18. *Zeb1* and *Zeb2* in the Tfh cell population. **(A)** Representative FACS plot of the T subpopulation cell gating. **(B)** FACS analysis of the proportion and **(C)** the total number of the T follicular helper cells.

An important population of T cells in the differentiation and activation of the B cells are the Tfh cells (PD1^{+}CXCR5^{+}). These specialized cells arise from the CD4+ T cells subset and are responsible for starting a crosstalk with the B cells that will initiate the

GC reaction (Crotty, 2014). We found that upon immunization control mice did not vary, but there was an increase in both *Zeb1*$^{\Delta GC}$ and *Zeb2*$^{\Delta GC}$ (Figure 18). In the non-immunized group, *Zeb2*$^{\Delta GC}$ had a decreased population compared with its control, but it was reverted upon activation. On the other hand, *Zeb1*$^{\Delta GC}$ had an increased population of Tfh upon immunization.

Taken together all the data on the splenic B and T populations, we can conclude that under non-immunized conditions, *Zeb2*$^{\Delta GC}$ could not form the GC correctly, but this was reversed upon a humoral response. On the other hand, in non-immunized conditions, a knocked out of *Zeb1* in the GC B cells did not affect the B and T cell populations, but it changed upon immunization. *Zeb1*$^{\Delta GC}$ mice were not able to form GC B cells but had an increased MZ B cells and Tfh, indicating that there could be a bypass of the GC B cells and the MZ B cells could be interacting directly with the Tfh to form a response against TNP.

1.5. Role of ZEB1 and ZEB2 in B cell activation

Changes in the different B cell subsets indicated that ZEB1 and ZEB2 were playing a role in the regulation of the B cell function. Therefore, we studied the antibody production both in non-immunized conditions and upon TNP immunization. From the same mice used in the B and T cell population analysis, we collected serum on days 0, 7, 14, and 21 since the first immunization.

First, we analyzed the total IgG and IgM production in the non-immunized mice and prior immunization and found no statistically significant differences in this process between control and *Zeb1*$^{\Delta GC}$ and *Zeb2*$^{\Delta GC}$ animals (Figure 19).

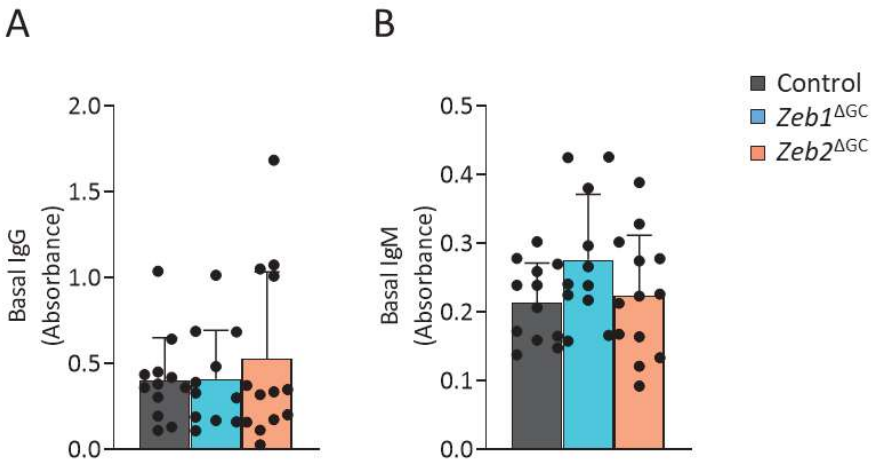

Figure 19. Levels of IgG and IgM production. **(A)** Total levels of IgG and **(B)** IgM in mice serum were measured by ELISA.

Upon TNP immunization, we looked at the specific antibody production against TNP in the serum of the immunized mice at different time points to see if there was an impaired response to T-dependent stimuli (Figure 20.A). We did not observe any statistically significant difference in their IgM production (Figure 20.B). Analyzing the specific IgG production, we could see that from day 0 until day 21, the *Zeb1*$^{\Delta GC}$ had lower levels of TNP-specific IgG compared with its control, indicating an impaired response (Figure 20.C).

These results correlated with the low levels of GC B cells in these mice and confirmed a defect in the GC formation and activation.

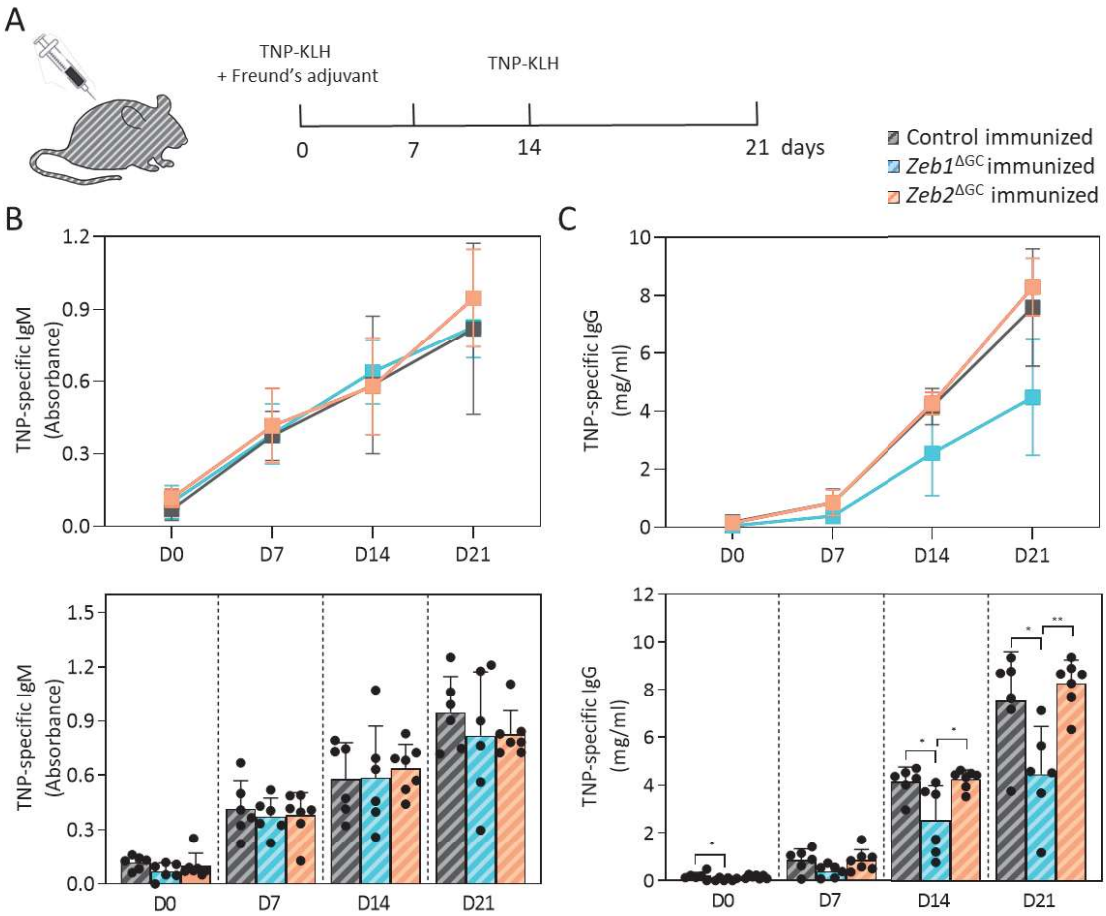

Figure 20. Levels of specific antibody production (IgG and IgM) against TNP. **(A)** Scheme of the protocol used for immunizations. **(B)** Levels of serum TNP-specific IgM and **(C)** IgG were measured by ELISA.

2. ROLE OF ZEB1 AND ZEB2 IN DIFFUSE LARGE B CELL LYMPHOMA

2.1. Characterization of ZEB1 and ZEB2 expression in DLBCL

The role of the transcription factors ZEB1 and ZEB2 has been described in the progression and prognosis of different carcinomas and hematologic malignancies, where they can act either as oncogenic drivers and or tumor suppressors (Goossens and Haigh, 2012; Sánchez-Tilló et al., 2012). ZEB1 has also been described to be associated with a poorer prognosis in DLBCL (Lemma et al., 2013).

We examined the ZEB1 and ZEB2 expression in a series of DLBCL cases (n=66) and found that both factors were expressed in the tumoral B cell with different intensities (Figure 21.A-B). ZEB1 and ZEB2 expression have also been seen in the microenvironment of the lymphomas. Specifically, there was expression of the factors in the macrophages, marked with the CD68 marker and in the fibroblasts, marked with S100A4 (Figure 21.C).

Then we correlated the expression of ZEB1 with different markers commonly used in the classification of DLBCL for its prognosis. B cell lymphoma 6 (BCL6), is expressed in 70% to 95% of DLBCL and has been described as an oncogene, essential for the survival of B Cell Lymphoma cell lines (Basso and Dalla-Favera, 2012). Mice models with deregulated BCL6 end up developing a human-like DLBCL (Cattoretti et al., 2005). In our cases, we saw a correlation wherewith more intense ZEB1 expression; there was more BCL6 expression (Figure 21.D).

B cell lymphoma 2 (BCL2) detection is used as an unfavorable prognostic factor due to its anti-apoptotic and drug resistance mechanism in DLBCL (Tzankov et al., 2010). We observed an apparent concomitant expression of ZEB1 and $BCL2^{high}$ in representative DLBCL cases (Figure 21.D). MYC expression has also been linked to a shorter overall survival (Valera et al., 2013). In our collection of cases, we detected that patients with ZEB1 expression harbored increased expression levels of MYC in comparison with cases with no ZEB1 expression (Figure 21.D).

We also examined the expression of CD10, another factor used for the stratification of the disease (Tzankov et al., 2010), but no differences were found with increasing ZEB1 expression.

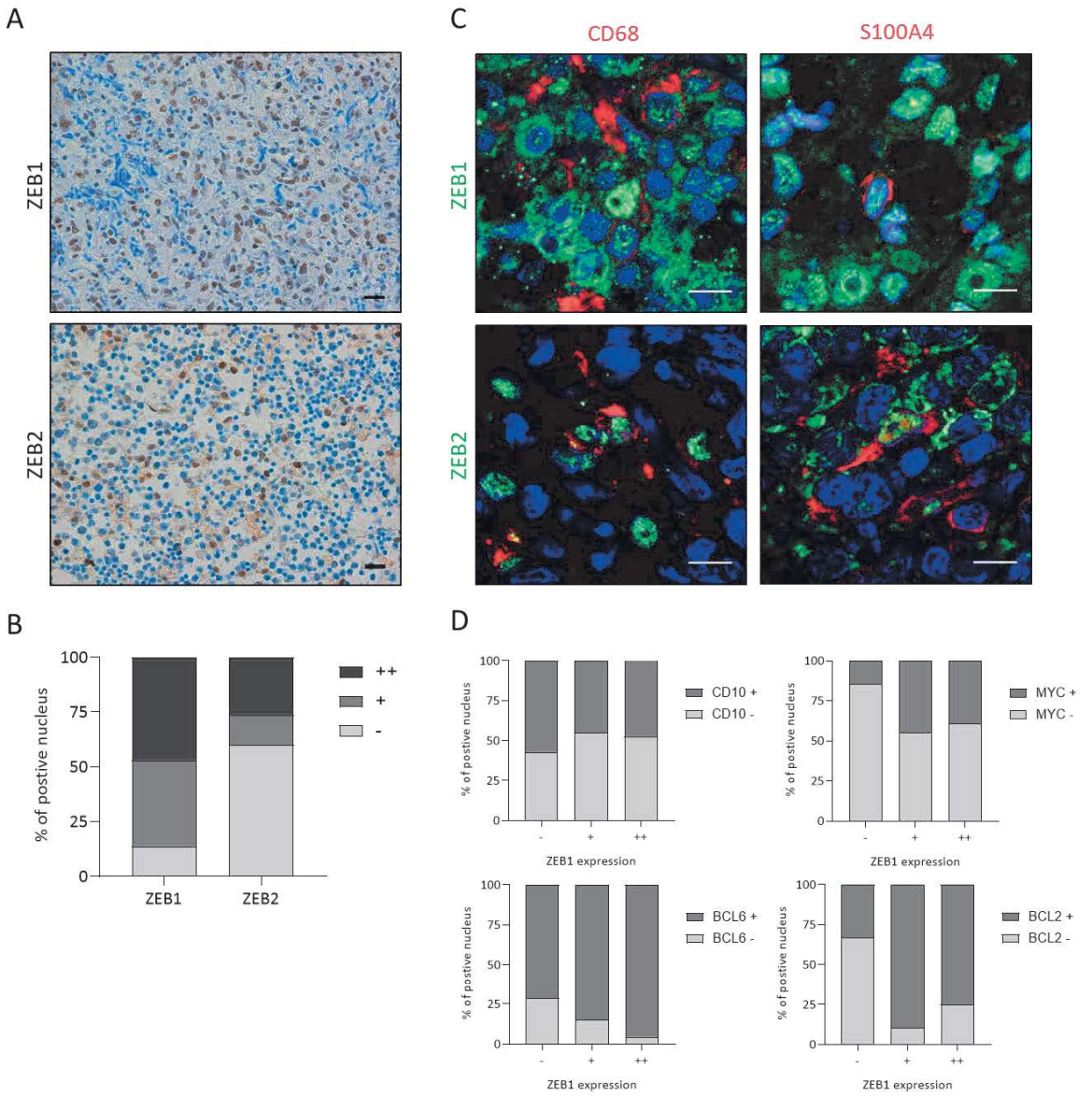

Figure 21. ZEB1 and ZEB2 expression in DLBCL patients. **(A)** Representative images of immunostaining of both ZEB1 and ZEB2 in DLBCL. Scale bar represents 20 μm **(B)** Quantification of the intensity of the staining for all the DLBCL patients.. **(C)** Representative images of immunofluorescence of a DLBCL patient with double staining of ZEB1 (green) or ZEB2 (green) with CD68 (macrophages, red) or S100A4 (fibroblasts, red) counterstained with DAPI. Scale bar represents 50 μm. **(D)** Quantification of different immunostaining of DLBCL markers (CD10, MYC, BCL6, and BCL2) depending on the ZEB1 intensity expression.

We then studied a combined DNA microarray of 472 cases (GSE10846 and GSE34171) and checked the overall survival of the patients with high/low expression of *ZEB1* and *ZEB2*, using optimal probes of both factors. Although not statistically significant, *ZEB1* expression was associated with more reduced survival. In turn, *ZEB2* had an inverse pattern and higher levels were significantly associated with better overall survival (Figure 22).

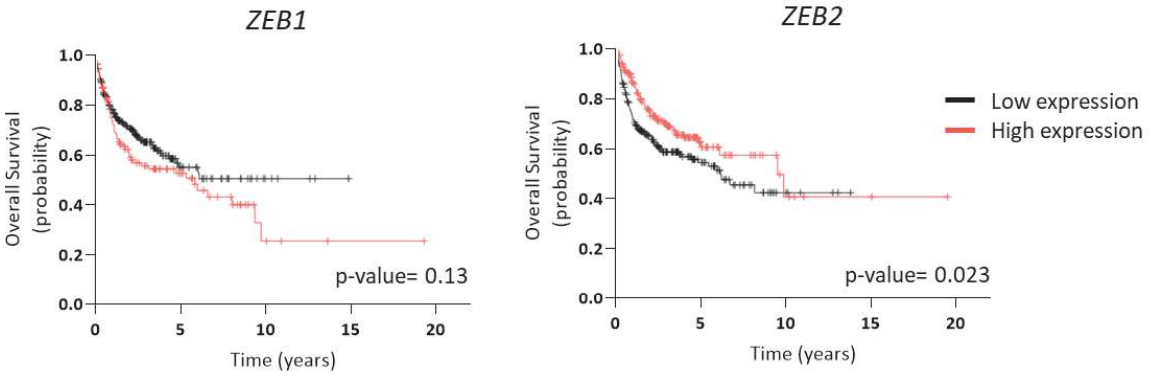

Figure 22. Overall survival of DLBCL cases depending on *ZEB1* or *ZEB2* expression. Overall survival of DLBCL cases depending on high or low levels of *ZEB1* and *ZEB2* expression, respectively (GSE10846 and GSE34171).

2.2. Role of ZEB1 and ZEB2 in DLBCL cell proliferation

ZEB1 regulates genes involved in proliferation and promotes tumor growth *in vivo* in MCL (Sanchez-Tillo et al., 2014). The role of ZEB2 is not clear and since there is a different involvement of these factors depending on the model, we questioned if the expression of ZEB1 and ZEB2 would be regulating the proliferation of DLBCL cells.

To explore this hypothesis, we have been working with different DLBCL cell lines in which we knocked down the expression of either factor through a lentiviral infection by stably interfering with an shRNA against *ZEB1* or *ZEB2*, as described in Materials and Methods.

shRNA expression was under the control of doxycycline, which jointly induced the expression of the shRNA along with that of red fluorescence protein (RFP). When cell

lines were incubated with doxycycline, all cells expressed the RFP (Figure 23.C), confirming a correct incorporation of the plasmid and, therefore, a decrease in the expression of both factors (Figure 23.B).

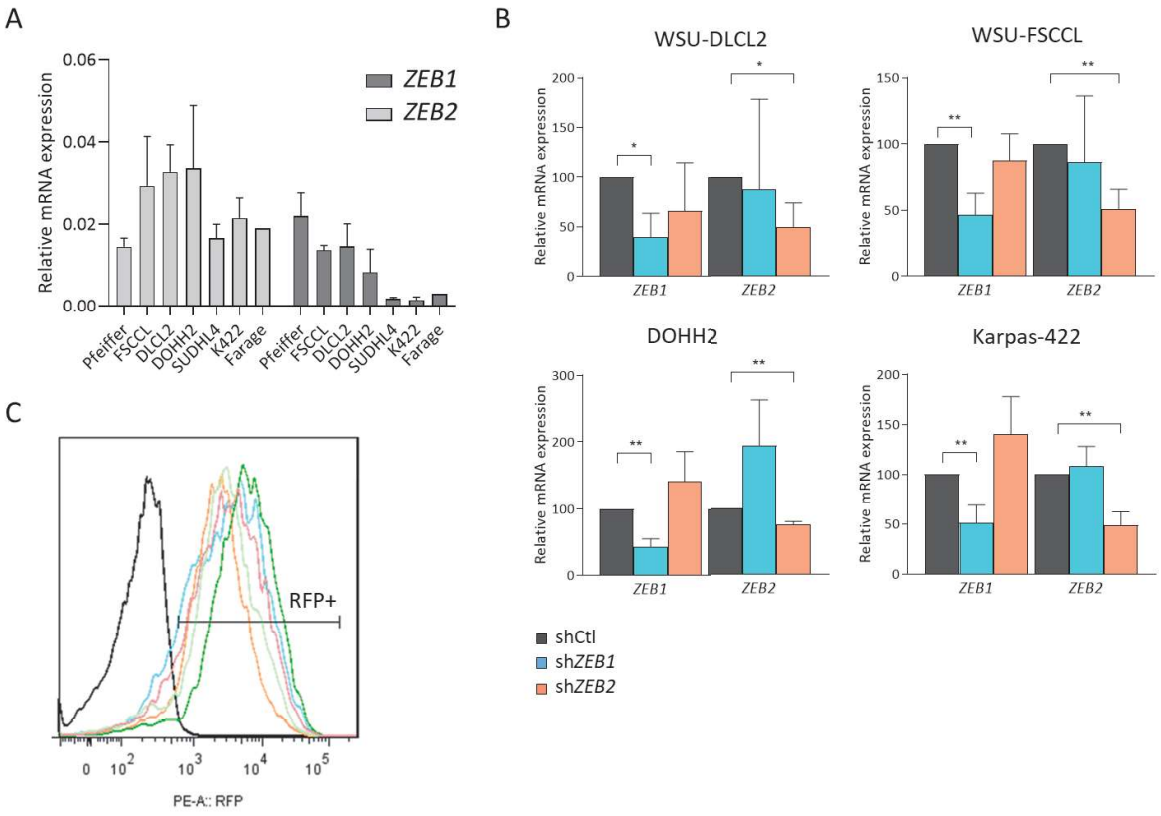

Figure 23. ZEB factors expression in DLBCL cell lines. **(A)** Relative mRNA expression of *ZEB1* and *ZEB2* in different DLBCL cell lines. **(B)** Relative expression of *ZEB1* and *ZEB2* in the knocked down of *ZEB1* (shZEB1) and *ZEB2* (shZEB2) in 4 different cell lines (WSU-FSCCL; WSU-DLCL2, Karpas-422, and DOHH2) after 48h induction with doxycycline. **(C)** Representative FACS of DOHH2 positive stable interference. Cells with stable integration of the shRNA were RFP +.

We tested whether a decrease in *ZEB1* or *ZEB2* expression could affect the proliferation of different DLBCL cell lines. As shown in Figure 24, a knockdown of ZEB1 resulted in a decrease in lymphoma cell proliferation, while that of ZEB2-interfered cells showed increased proliferation and cell growth rates in all our cell lines tested (Figure 24.A-B). We also performed a set of proliferation assays based on EdU uptake. From these experiments, we observed a trend in a decreased proliferation

in *ZEB1*-silenced, although no statistically significant differences were found (Figure 24.C).

These results supported our hypothesis that the ZEB factors have an inversed role in the regulation of DLBCL cell viability and proliferation rate; ZEB1 may promote cell proliferation while ZEB2 may impede this process.

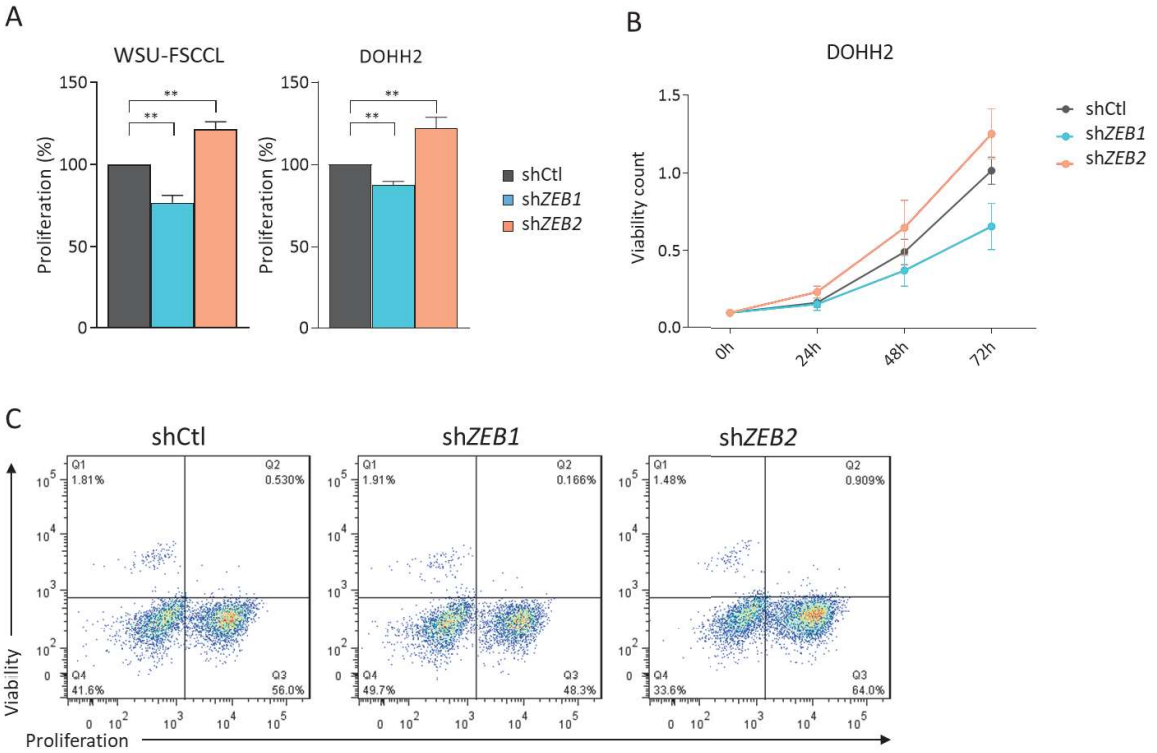

Figure 24. Cell proliferation and growth in DLBCL cell lines. **(A)** Cell proliferation of two representative cell lines (WSU-FSCCL and DOHH2) assessed after 72 h incubation by MTT assay. **(B)** Cell growth assay at different time points. **(C)** Representative FACS of proliferation assay by EdU uptake in DOHH2 cell line.

2.3. Role of ZEB1 and ZEB2 in the response of DLBCL cells to targeted therapies

Although new treatments to increase the overall survival and reduce the percentage of relapsed patients are being studied, DLBCL remains incurable and different therapeutic agents focused on improving the efficiency of R-CHOP have not

shown clinical benefits so far (Liu and Barta, 2019). We decided to evaluate the role of ZEB1 and ZEB2 in some of the novel therapeutic approaches used in DLBCL.

For this aim, we analyzed how ZEB1 and ZEB2 expression modulates the response of DLBCL cells to three different targeted therapies currently used in clinical and preclinical trials. Idelalisib is an inhibitor of the P13K/AKT pathway, which plays a role in cell survival and proliferation and have been proved to be effective in chronic lymphocytic leukemia patients (Cheah and Fowler, 2016). Ibrutinib is a BTK (Bruton's tyrosine kinase) inhibitor that blocks the BCR signaling pathway and has been suggested as a potential treatment for the ABC-DLBCL subgroup. Finally, we have tested R406, SYK inhibitor, an initiator of the BCR signaling pathway, which has shown promising results in BCR-DLBCL tumors *in vitro* (Chen et al., 2008).

As shown in Figure 25 (left panel), we observed a significant decrease in the cell proliferation range upon drug treatment with a standard dose of these different agents. A decreased proliferation in the cell lines with shRNA against *ZEB1* was observed in the cells treated with Ibrutinib and R406. No effects were observed with the Idelalisib treatment or in any of the treatments in the cell lines with shRNA against *ZEB2*.

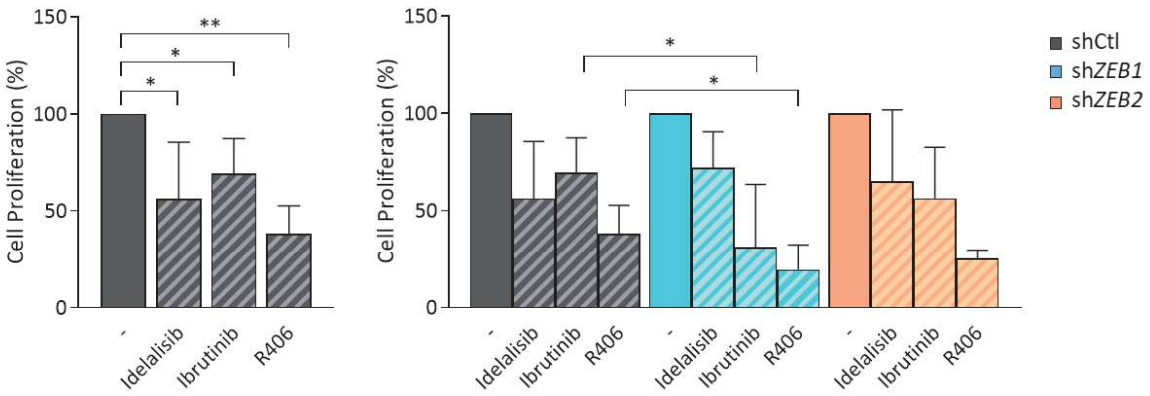

Figure 25. DLBCL cell line response to drug treatment. A representative cell line (DOHH2) was incubated with different concentrations of the drug treatments (Idelalisib, 1 μM; Ibrutinib, 1 μM; and R406, 1 μM) for 72h incubation and the cell proliferation was assessed by MTT assay.

These results indicated that although ZEB1 and ZEB2 had effects in the proliferation of the different DLBCL cell lines, interference of ZEB2 did not affect the acquisition of resistance of novel drug treatments. The knockdown of ZEB1 increased the sensitivity of DLBCL cells to the inhibitor of SYK (R406) and Ibrutinib but did not alter the response of the cells to the other treatment (Idelalisib).

2.4. Role of ZEB1 and ZEB2 in the metabolism of DLBCL cells

As noted in the Introduction, the DLBCL tumors can be classified by the consensus clustering classification, which will rely on the metabolic phenotype of the tumor. The BCR-DLBCL subgroup has a more overall glycolytic capacity and an active BCR signaling, and this will translate into patients that will respond well into treatments using BCR inhibitors. OxPhos-DLBCL cells have a more active mitochondrial oxidative phosphorylation metabolism, as it relies more upon the tricarboxylic acid cycle and the fatty acid oxidation program. This group is characterized by being insensitive to BCR inhibitors as it will not have its pathway activated (Caro et al., 2012; Norberg et al., 2017).

ZEB1 has been described to have a role in the metabolic plasticity of tumoral cells and its metabolic reprogramming (Georgakopoulos-Soares et al., 2020; Krebs et al., 2017) and for this reason, we decided to investigate its role in the DLBCL metabolism.

Using the Caro et al. classification of BCR and OxPhos-DLBCL of the already published array (GSE10846, n=154) we examined the *ZEB1* and *ZEB2* expression in the 2 subsets and found that in BCR cluster, patients had higher levels of *ZEB1*, but no significant difference was observed in *ZEB2* expression between both subtypes (Figure 26).

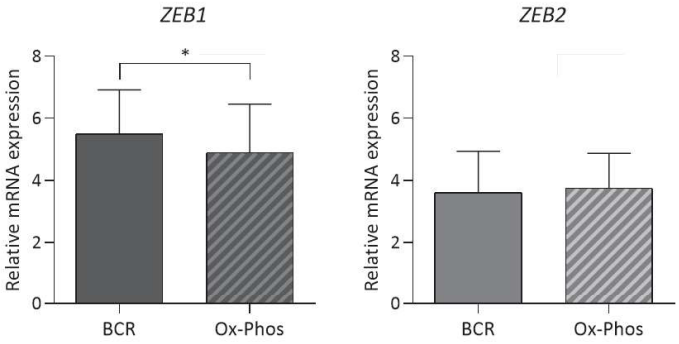

Figure 26. ZEB1 and ZEB2 expression in BCR and OxPhos-DLBCL patients. *ZEB1* and *ZEB2* expression in DLBCL cases classified by their metabolic phenotype (GSE10846).

First, we classified our cell lines depending on their metabolic phenotype. To do so, we incubated the DLBCL cell lines with metabolic inhibitors of the glycolysis and the tricarboxylic acid cycle. The 2-deoxy-D-Glucose (2-DG) is a glucose analog, in which the 2-hydroxyl group is replaced by hydrogen, preventing its use and therefore, stopping the glycolysis. The 2-DG is also a potential therapeutic agent in aggressive breast cancer (O'Neill et al., 2019). In turn, the antibiotic Tigecycline interferes with mitochondrial translation, besides being a promising treatment for AML (Caro et al., 2012).

The 2-DG will inhibit the proliferation of the BCR-DLBCL cell lines but will not affect the OxPhos-DLBCL cell lines, and the Tigecycline will have an opposite effect, decreasing the viability of the OxPhos-DLBCL cell lines and not affecting the BCR-DLBCL ones.

DLBCL cells treated with Tigecycline that exhibited reduced viability were classified as OxPhos-DLBCL cell lines (SC-1, Karpas-422, Pfeiffer, and WSU-FSCCL) and the rest, in which the mitochondrial inhibitor did not affect, were classified as BCR-DLBCL cell lines (Figure 27.A). To confirm these results, we treated a selected group of cells with 2-DG and observed and an inversed phenotype that confirmed our classification (Figure 27.B). We selected the cell lines Karpas-422 and WSU-FSCCL as representative OxPhos cell lines and DOHH2 and DLCL2 as representative BCR cell lines to continue our studies.

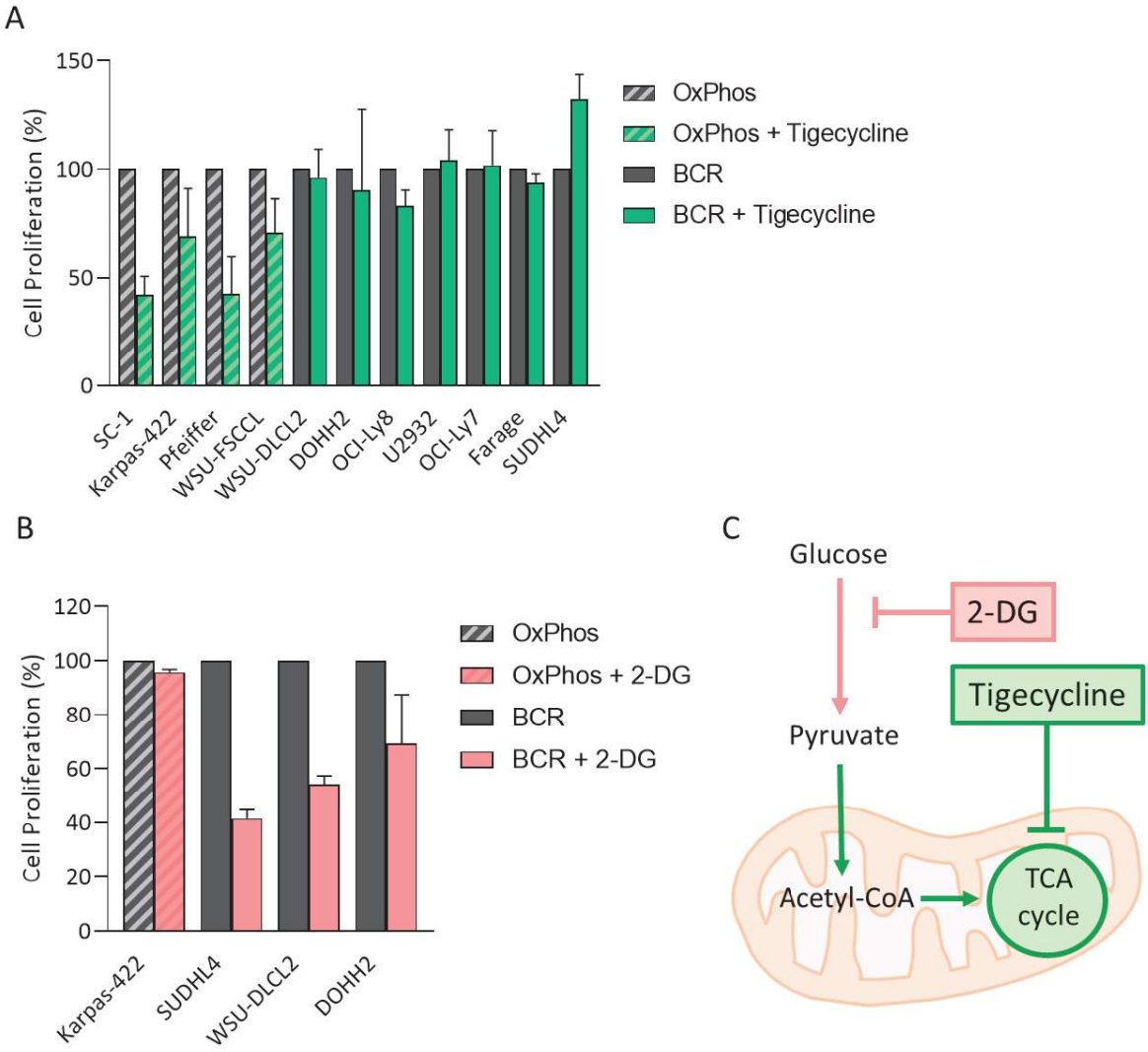

Figure 27. DLBCL cell lines phenotype depending on their metabolism. **(A)** Cell proliferation of different DLBCL cell lines upon Tigecycline (5 µM) treatment assessed by MTT assay after 72h incubation. **(B)** Cell proliferation of different DLBCL cell lines upon 2-DG (12 mM) treatment assessed by MTT assay after 72h incubation. **(C)** Scheme of the mechanism of action of each treatment (2-DG or Tigecycline).

Upon treatment with both metabolic inhibitors, changes in the *ZEB1* and *ZEB2* expression were observed (Figure 28). 2-DG reduced the expression of *ZEB1* in the cell lines and a decreased expression of *ZEB2* in the BCR cell lines but not in the OxPhos cell lines. Upon Tigecycline treatment, a similar phenotype was observed with a decreased expression of *ZEB1,* and also of *ZEB2* in all the cell lines. With these results, we could hypothesize that modulation of the metabolic pathways affected the expression of ZEB1 and ZEB2.

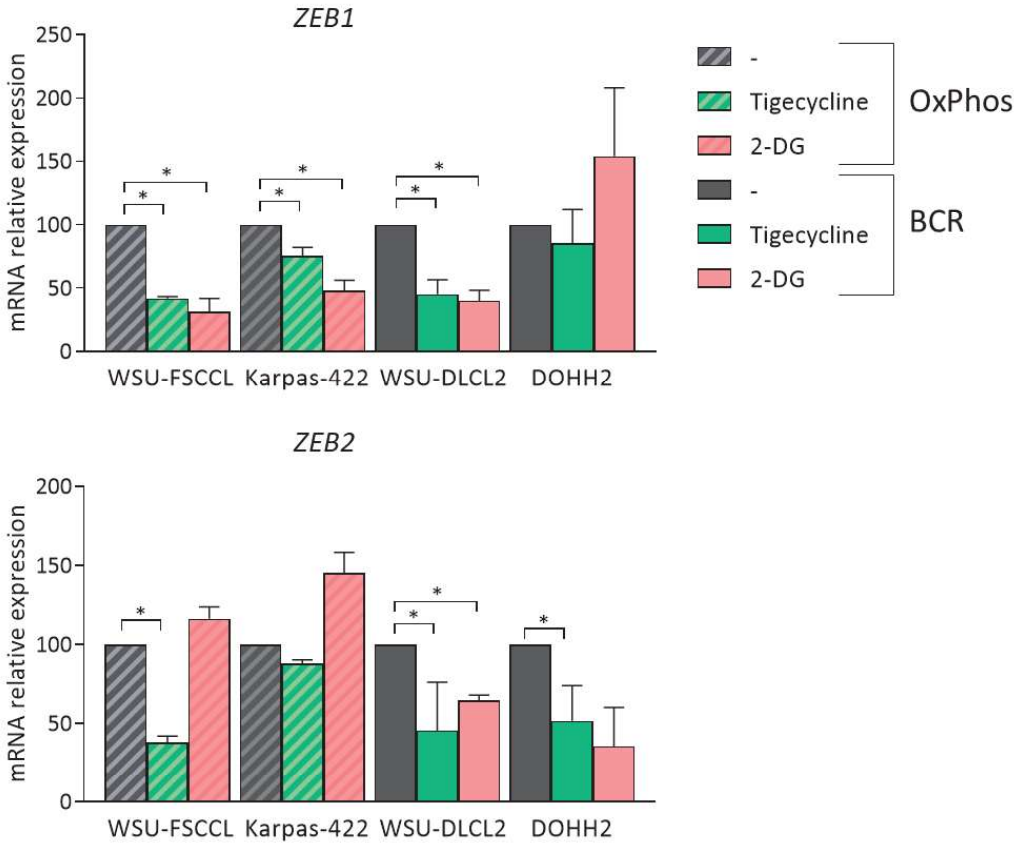

Figure 28. *ZEB1* and *ZEB2* expression upon treatment with metabolic inhibitors. *ZEB1* and *ZEB2* expression of different cell lines treated with Tigecycline (5 µM) or 2-DG (12 mM) after 72h incubation.

Then we analyzed if there were changes in the metabolic phenotype of these cells upon ZEB1 or ZEB2 silencing when treated with the mitochondrial inhibitor, Tigecycline. In the OxPhos cell lines, the ZEB1 knocked down cells were less sensitive to the tigecycline than their controls, resulting in the acquisition of a more glycolytic phenotype and the silencing of ZEB2 resulted in a sensitive cell line, with a similar effect to its control. In BCR cell lines with silenced ZEB1, no effect of tigecycline was detected, similar to the control. In contrast, downregulation of ZEB2 resulted in improved sensitivity to tigecycline, suggesting that these cells may have acquired an OxPhos phenotype (Figure 29).

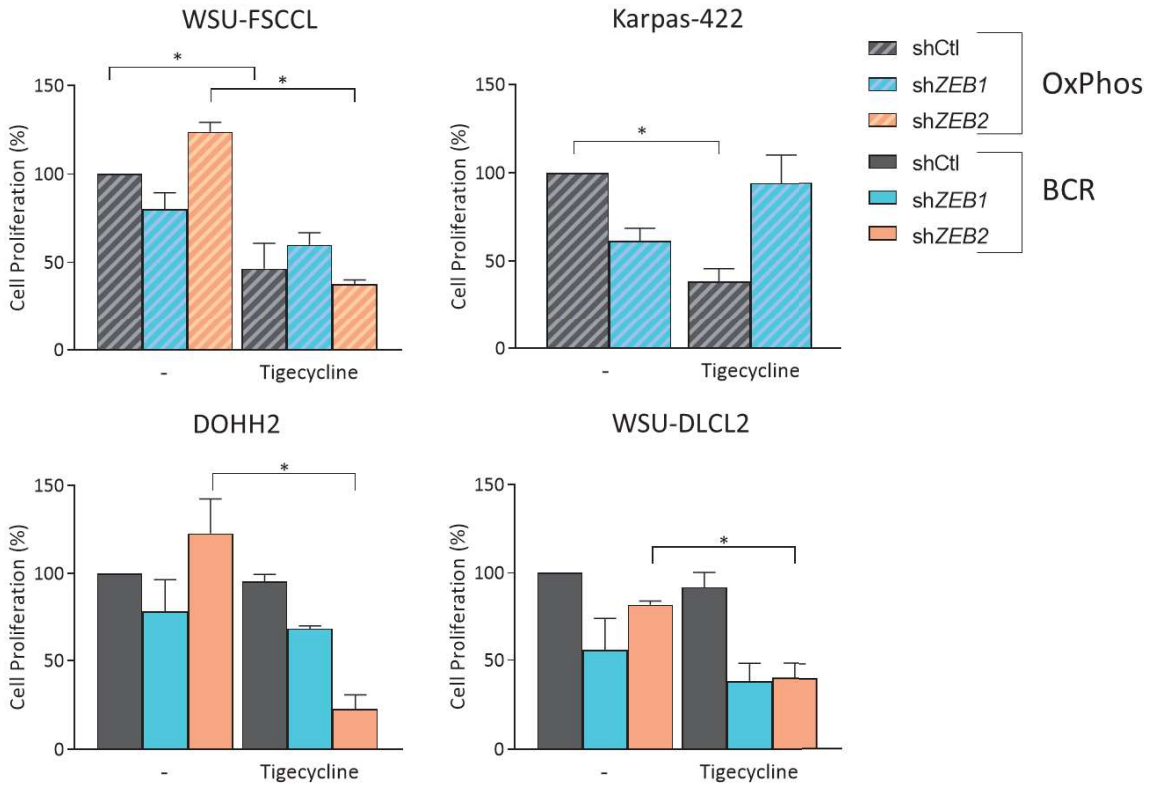

Figure 29. ZEB1 and ZEB2 in DLBCL metabolism Cell proliferation of different cell lines with silenced expression of *ZEB1* or *ZEB2* upon Tigecycline (5 μM) treatment assessed by MTT assay after 72h incubation.

To evaluate the effects of the above treatments on metabolic-associated signaling, we analyzed the expression levels of 1) two glycolytic genes, namely *PDK1* that represses the pyruvate dehydrogenase (*PDHA1*) resulting in shutting down the entrance of pyruvate into the tricarboxylic acid cycle (TCA), and *SLC2A1* (*GLUT1*), a glucose transporter; and 2) three mitochondrial-associated genes, namely *TUFM*, *YARS1,* and *GFM1*, all encoding for mitochondrial translational proteins (Norberg et al., 2017).

We did not observe changes in the metabolic genes between the different DLBCL cell lines in basal conditions (data not shown). When using the cell lines stably expressing *ZEB1* or *ZEB2* shRNA, differential expression of the metabolic-associated genes was observed (Figure 30.A). When the expression of ZEB1 was silenced in the OxPhos cell lines, we observed a decreased expression of the glycolytic genes (*PDK1* and *SLC2A1/GLUT1*) and increased expression of *TUFM* and YARS1. In contrast, in

ZEB1-silenced BCR cell lines, we observed an increased expression of *PDK1* and *SLC2A1*, and also of the mitochondrial-associated factors, *TUFM* and *YARS1*.

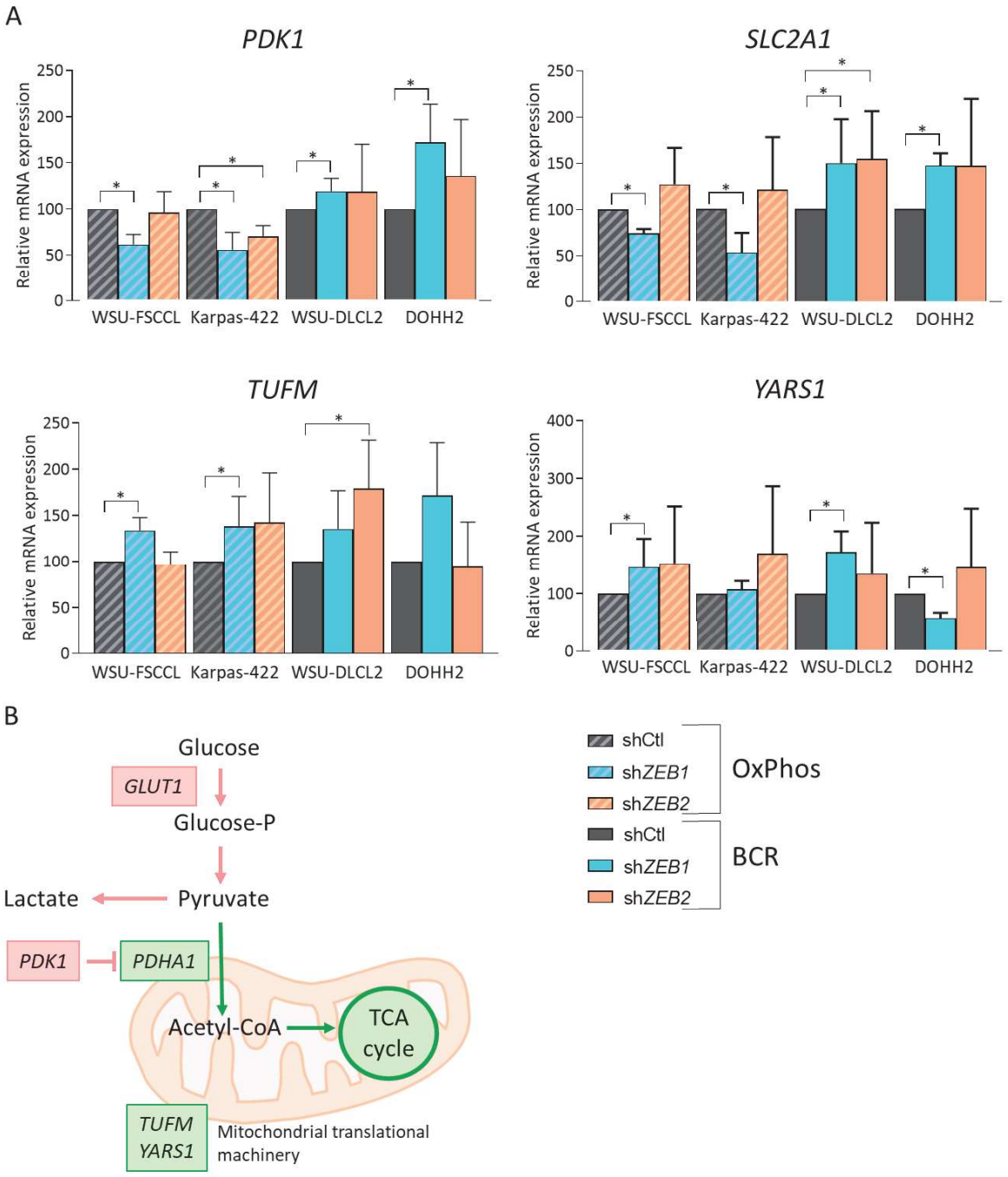

Figure 30. Metabolic genes changes in DLBCL cell lines. **(A)** Relative mRNA expression of different metabolic associated genes in DLBCL cell lines with a knocked down expression of ZEB1 or ZEB2 **(B)** Scheme of the location and function of each gene studied.

When the expression of ZEB2 was silenced, we could not observe a clear pattern in the expression of the chosen genes. In the Oxphos cell lines, SLC2A1 seemed to increase and PDK1 did not vary, while the mitochondrial-related genes also tend to increase. In the ZEB2-silenced BCR cell lines, there was an increase in the expression of *PDK1* and *GLUT1*, but no differences were observed in the mitochondrial genes.

3. ROLE OF ZEB1 AND ZEB2 IN MULTIPLE MYELOMA

3.1. Characterization of ZEB1 and ZEB2 expression in MM

Similar to the canonical metastasis program orchestrated by the EMT factors in carcinoma, the tumoral plasma cells have a high degree of plasticity, undergoing into a less mature phenotype in order to disseminate from one bone, enter to the bloodstream and migrate to another bone (Roccaro et al., 2015). We, therefore, decided to study the roles of the ZEB factors in the malignization of the plasma cells and its hallmarks of progression.

We analyzed the expression of ZEB1 and ZEB2 in few cases of plasmacytoma and corroborated the expression of both factors in the malignant plasma cells (Figure 31). A plasmacytoma is characterized by being a localized one mass of neoplastic monoclonal plasma cells in either bone or soft tissue (extramedullary), and it differentiates from the MM as this last one has the presence of more than one lesion, being more systemic (Dimopoulos et al., 2000).

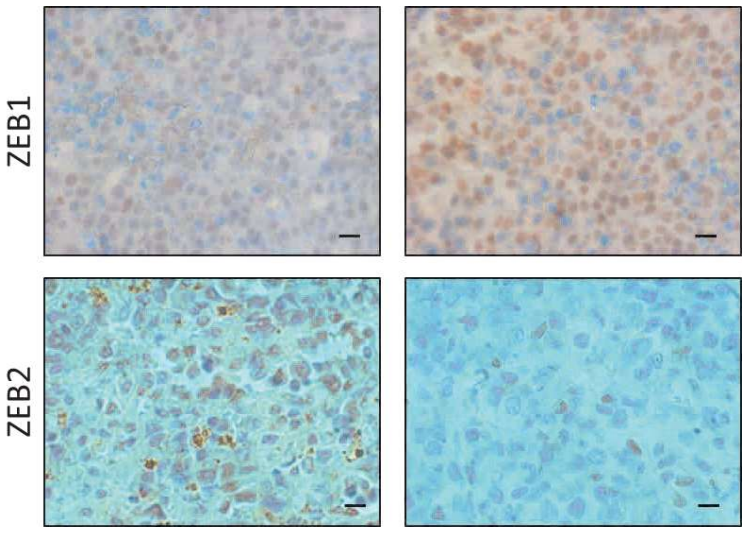

Figure 31. ZEB1 and ZEB2 in plasmacytomas. Representative images of immunostaining of ZEB1 and ZEB2 in two cases of plasmacytoma. Scale bar represents 20 μm.

We then studied the expression of ZEB1 and ZEB2 in primary MM samples. For this aim, we collected MM cases from patients from the Hospital Clinic of Barcelona (n=23) and differentiated the CD38 positive fraction of malignant cells from the negative one. We also analyzed cases of MGUS (n=19) and patients that underwent complete remission (n=10) (Figure 32). The negative fraction had similar levels of *ZEB1* and *ZEB2* with the MGUS and with the complete remission cases. The positive fraction of the MM patients had higher expression of *ZEB1* than in the MGUS, complete remission, or the negative fraction and lower levels of *ZEB2*. Inversely, the negative fraction and the MGUS cases had higher levels of *ZEB2* and lower expression of *ZEB1*.

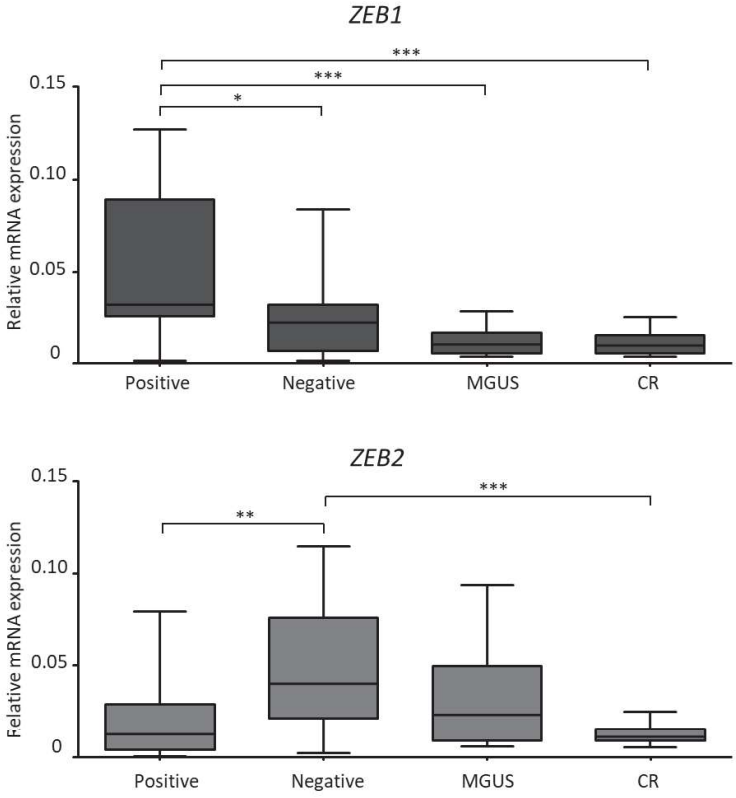

Figure 32. *ZEB1* and *ZEB2* in patients with MM, MGUS, or complete remission. Relative mRNA expression of *ZEB1* and *ZEB2* in the positive and negative fractions of MM patients, MGUS patients, and patients with complete remission (CR).

Analysis of several published arrays confirmed the results observed in the MM patients. The arrays used were from malignant cells isolated from the BM of MM patients (GSE9782, n=265; GSE4204, n=538; GSE24080, n=559), plasma cells from BM

from MGUS patients (GSE5900, n=44), peripheral blood from healthy donors (GSE27838, n=8), and BM from healthy donors (GSE71935, n=9; GSE7307, n=5).

These arrays indicated a differential expression pattern of *ZEB1* and *ZEB2* in MM, where *ZEB1* is more expressed than *ZEB2* (Figure 33.A). Interestingly, we observed the reversed pattern in the MGUS arrays, were *ZEB2* was expressed at higher levels than *ZEB1* (Figure 33.B). We observed that the expression pattern in MGUS was similar to the ones in the bone marrow and peripheral blood of healthy donors arrays (Figure 33.C-D). Altogether, these results indicated that when there was a progression from a non-malignant stage (MGUS) to a malignant one (MM), there was a switch between *ZEB1* and *ZEB2* expression.

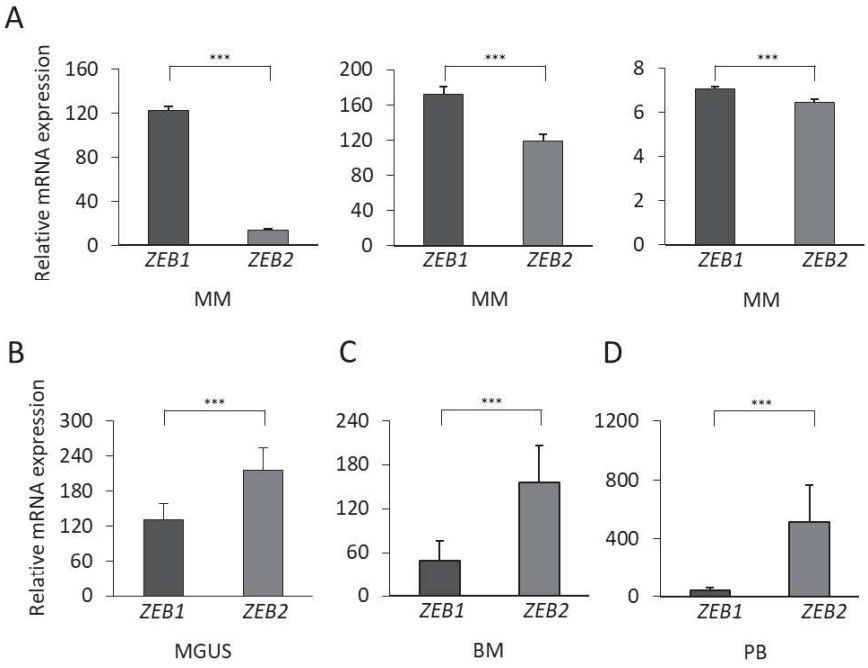

Figure 33. ZEB1 and ZEB2 expression in MM arrays. *ZEB1* and *ZEB2* expression in different published arrays of **(A)** Multiple Myeloma (GSE9782, GSE4204, and GSE54080), **(B)** MGUS (GSE5900), **(C)** bone marrow (BM) from healthy donors (GSE7307 and GSE71935) and **(D)** peripheral blood (PB) from healthy donors (GSE27839).

We then examined the overall survival of MM patients associated with high or low *ZEB1* and *ZEB2* expression in the combined arrays (GSE9782, GSE4204, and GSE24080, n=1359). High expression of *ZEB1* was correlated with a poorer prognosis,

and higher levels of expression of *ZEB2* were associated with a better prognosis (Figure 34.A-B). Combining both factors, we observe that patients with higher *ZEB1* and lower *ZEB2* have the worst overall survival and patients with lower *ZEB1* and higher *ZEB2* were associated with a better prognosis. The patients with high expression of both ZEB1 and ZEB2 or low expression of both factors did not show significant differences in overall survival and for this reason, we grouped them in one cohort in the Figure (Figure 34.C).

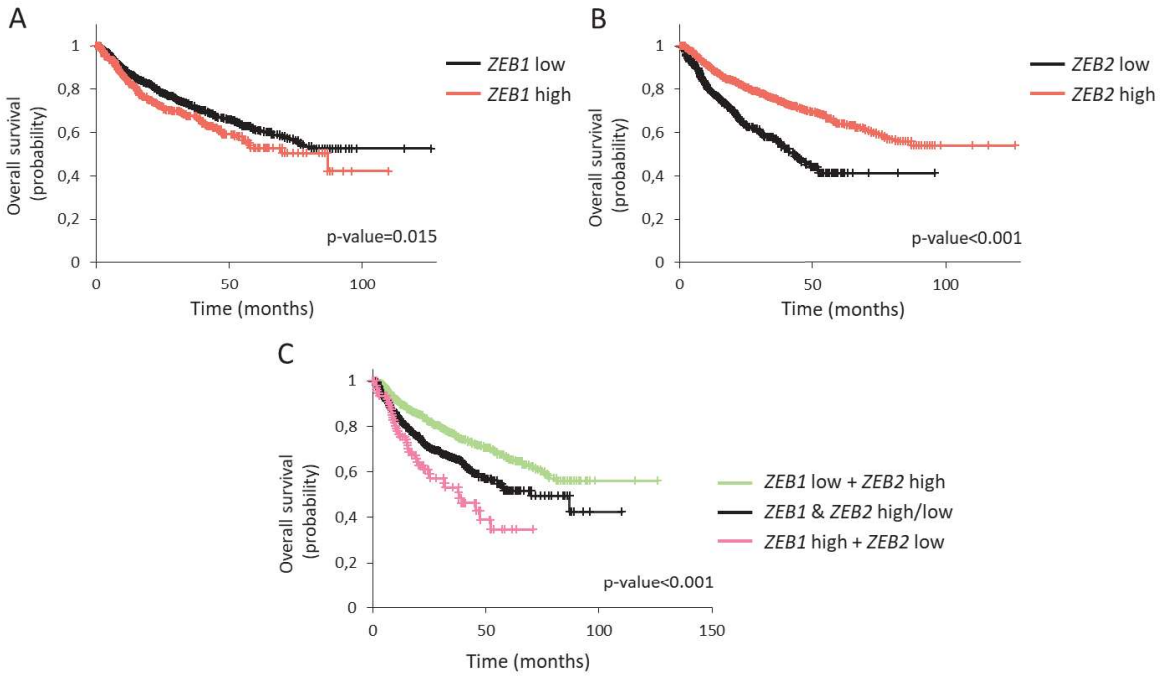

Figure 34. Overall survival of MM patients regarding ZEB1 and ZEB2 expression. **(A)** Kaplan-Meyer survival plot of MM patients segregated by the expression of ZEB1 and **(B)** the expression of ZEB2. **(C)** Kaplan-Meyer survival plot of MM patients with low ZEB1 & high ZEB2, high ZEB1 & high ZEB2 and low ZEB1 & low ZEB2, and high ZEB1 and low ZEB1.

3.2. Role of ZEB1 and ZEB2 in MM cell proliferation

We investigated the effect of ZEB1 and ZEB2 in two different MM cell lines, namely MM1.S and RPMI2886. In agreement with the previous results showing that MM patients have higher levels of *ZEB1* and lower levels of *ZEB2*, we confirmed this differential pattern of expression in our cell lines by mRNA expression (Figure 35.A).

Based on this, we knocked down *ZEB1* with an inducible lentiviral shRNA against *ZEB1* and overexpressed *ZEB2* with an inducible lentiviral pLUT overexpression plasmid containing *ZEB2* cDNA, in both cell lines (Figure 35.B-C), as described in Materials and Methods (Figure 35.B-C).

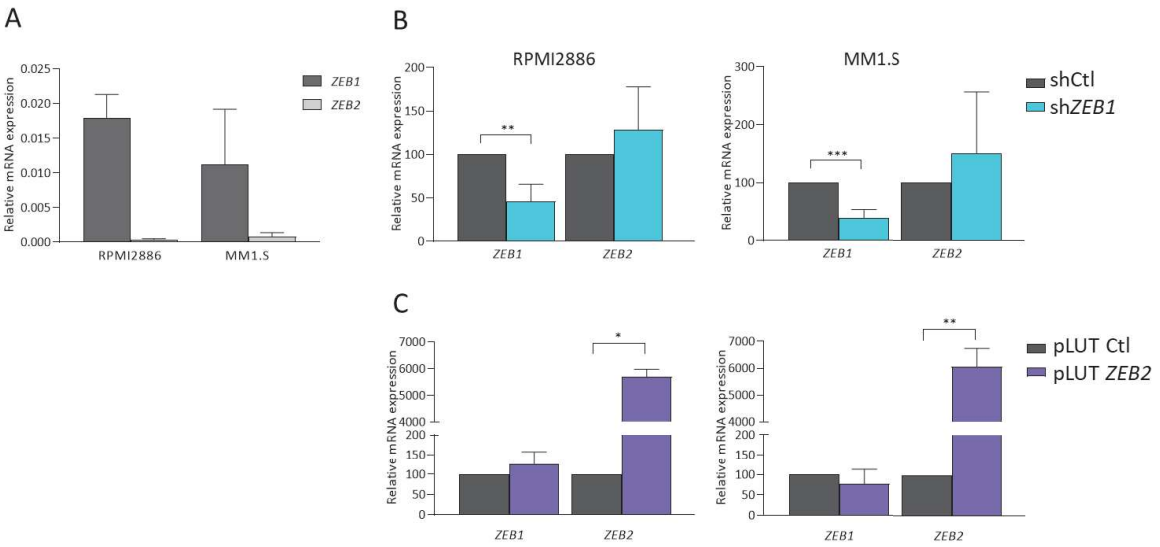

Figure 35. Expression of ZEB factors in MM cell lines. **(A)** *ZEB1* and *ZEB2* relative mRNA expression in RPMI2886 and MM1.S cell line. **(B)** Knockdown of *ZEB1* in MM cell lines after 48h of induction by doxycycline. **(C)** Overexpression of *ZEB2* in MM cell lines after 48h of induction by doxycycline.

Next, we questioned if a poorer prognosis in high-ZEB1 expression patients could be due to highly proliferative tumoral cells. The knockdown of *ZEB1* decreased the proliferation *in vitro* in both RPMI2886 and MM1.S cell lines (Figure 36.A). Therefore, we explored if *ZEB1* expression correlated with a gene proliferation signature associated with MM prognosis. From them, we selected Baculoviral IAP Repeat Containing 5 (*BIRC5*), which is an apoptosis inhibitor found in a gene expression signature for high-risk MM (Kuiper et al., 2012). Another relevant gene in various carcinomas and an indicator for an aggressive outcome is the proliferation marker Ki-67 (*MKI67*), which is also a marker distinguishing MM from MGUS (Miguel-Garcia et al., 1995). Finally, an important modulator of apoptosis is the BCL2 Binding Component 3 (*BBC3/Puma,* which is also relevant in the chemoresistance in MM (Zhao et al., 2015). We selected these genes based on the observation that they are relevant

for the prognosis and overall survival of the MM patients, being *BIRC5* and *MKI67* markers of poorer prognosis and *BBC3* associated with better survival (Figure 36.D).

We observed a decreased expression of pro-survival genes (*BIRC5* and *MKI67*) and a trend in increased expression in pro-apoptotic genes (*BBC3/Puma*) when *ZEB1* was knocked down in both MM1.S and RPMI2886 cell lines (Figure 36.B). When *ZEB2* was overexpressed in MM1.S, we found similar results with a decreased expression of *BIRC5* and *KI67* (Figure 36)

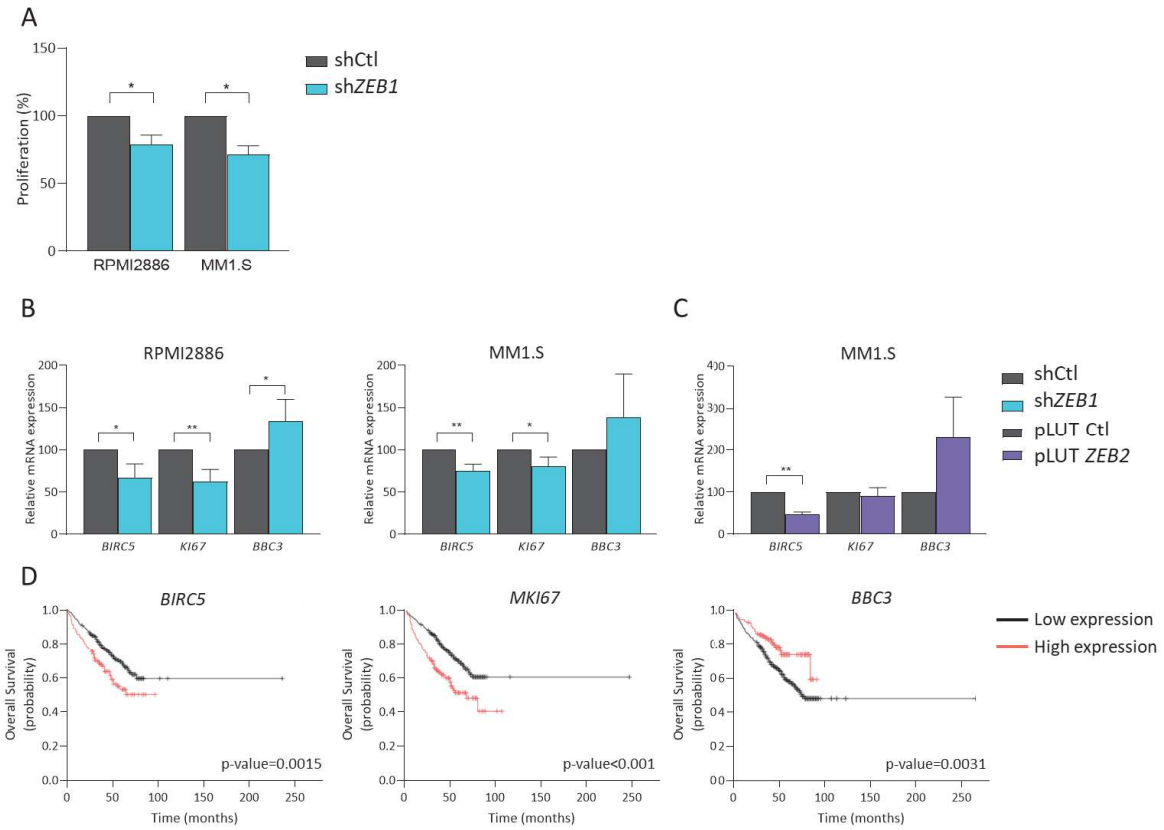

Figure 36. ZEB1 and ZEB2 in cell proliferation and viability. **(A)** Cell proliferation of two representative cell lines (RPMI2886 and MM1.S) assessed after 72 h incubation by MTT assay. **(B)** Relative mRNA expression of *BIRC5*, *KI67,* and *BBC3* in *ZEB1* knockdown cells and in **(C)** *ZEB2* overexpressed cells. **(D)** Overall survival in MM patients from GSE24080 depending on the expression of *BIRC5*, *MKI67,* and *BBC3*.

3.3. Role of ZEB1 and ZEB2 in the response of MM cells to targeted therapies

Although advances have been made in the treatment of MM, it remains incurable. Current strategies comprise a combination of two or three (depending on the patient staging) of these agents: bortezomib, lenalidomide, and dexamethasone, among others. Bortezomib is a proteasome inhibitor, which induces apoptosis of MM cells and also affects its microenvironment (Richardson et al., 2010). Lenalidomide, an analog of thalidomide, is an immunomodulatory drug that alters the cytokine production resulting in increased activity of the Natural Killer cells and T cell activation and also directly targeting the cell cycle of MM cells (Kotla et al., 2009). Finally, dexamethasone is a type of glucocorticoid that has an inhibitory and cytotoxic effect in MM cells by arresting the G1 phase and increasing apoptosis (Richardson et al., 2010). ZEB1 may represent a regulator of chemoresistance in MCL (Sanchez-Tillo et al., 2014) we tested whether such activity could also be observed in MM.

As shown in Figure 37, ZEB1 silencing was associated with increased sensitivity of the MM cells to bortezomib and dexamethasone, but not lenalidomide, thus suggesting a protective role of ZEB1 toward proteasome inhibition and glucocorticoid cytotoxicity.

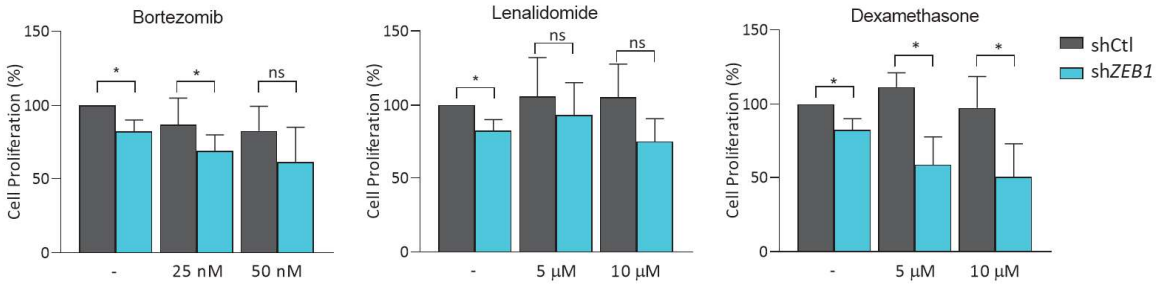

Figure 37. ZEB1 *in vitro* drug resistance. Cell proliferation of MM1.S cell line after 72h incubation with different concentrations of Bortezomib, Lenalidomide, and Dexamethasone assessed by MTT assay.

3.4. Role of ZEB1 and ZEB2 in MM cell migration

MM is characterized by the dissemination of the malignant cells from one BM niche to another one. More than 70% of patients have circulating tumoral MM cells in the bloodstream. For this process, the MM cells are prone to migrate via the CXCR4/CXCL12 axis, as CXCL12 is supposed to act as a chemoattractant required to stimulate the migration or homing of circulating CXCR4+ plasma cells (Alsayed et al., 2007).

We thus investigated whether ZEB1 and ZEB2 were implied in this process, as one of the most widely recognized roles of ZEB1 is its participation in the metastasis and EMT-related processes. Furthermore, in cardiomyocytes, ZEB1 has been described to regulate the activation of the CXCR4/CXCL12 axis by directly targeting CXCR4 (Beji et al., 2017).

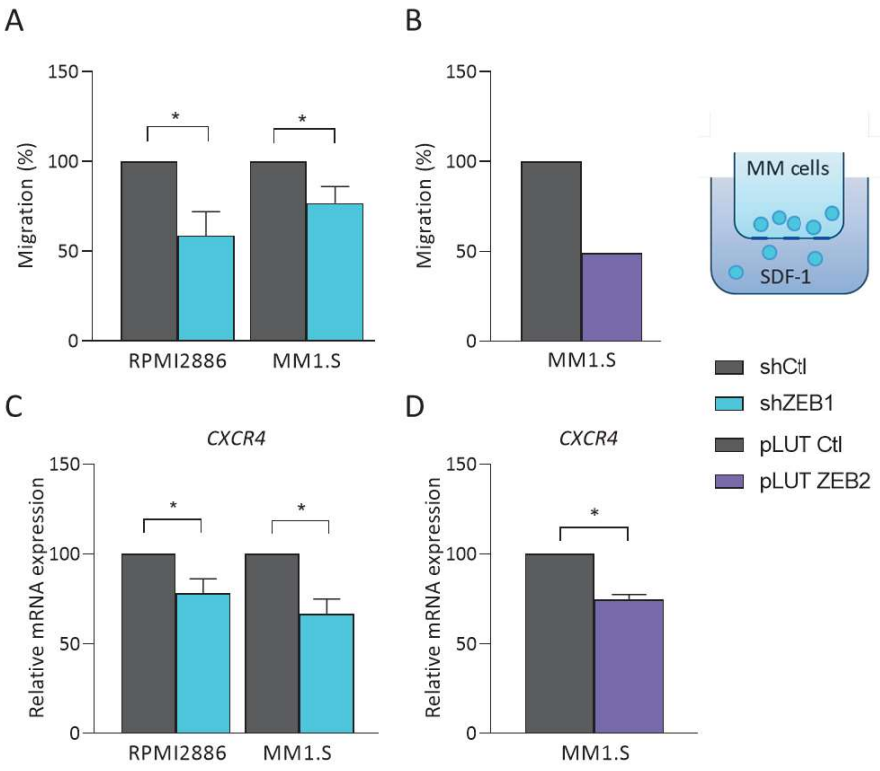

Figure 38. ZEB1 and ZEB2 in MM migration. **(A)** Cell migration in MM cell lines with knockdown of *ZEB1* and **(B)** overexpression of *ZEB2* after 4h incubation with 30 nM SDF-1 (CXCL12). **(C)** Relative mRNA expression of *CXCR4* in MM cell lines with a knockdown of *ZEB1*. **(D)** Relative mRNA expression of *CXCR4* in MM cell lines with overexpression of *ZEB2*.

We cultured the transfected MM cell lines in the upper chamber of a Transwell chamber and analyzed the number of migrating cells into the lower chamber, where we added the chemokine SDF-1 (*CXCL12*), which acted as a chemoattractant. As shown in Figure 38.A, we observed a reduced migration in both MM cell lines with a silenced expression of ZEB1, while the same phenotype was observed when ZEB2 was overexpressed in the MM1.S cell line (Figure 38.B). We analyzed the expression of *CXCR4* in these cell lines and observed that the MM cell lines with the silenced expression of *ZEB1* or overexpressing *ZEB2* were both expressing lower levels of *CXCR4*, suggesting a regulation of the chemokine receptor by ZEB1 and ZEB2 (Figure 38.C-D).

3.5. Role of ZEB1 and ZEB2 in bone formation and osteolysis

Another important hallmark of the MM progression are the bone lesions, which are the primary cause of morbidity (Rajkumar and Kumar, 2016). This process occurs due to impaired osteoblastic bone formation and increased osteoclastic bone resorption.

One of the regulators of this balance between osteoclasts and osteoblasts is DKK1, which is an inhibitor of the Wnt/β-Catenin signaling pathway. DKK1 has been correlated with the presence of bone lesions and the inhibition of osteoblastogenesis via inhibiting the differentiation of osteoblasts precursor cells *in vitro* (Heath et al., 2009; Tian et al., 2003). DKK1 increases the RANKL/OPN ratio in osteoblasts, resulting in a stimulation of the osteoclastogenesis (Terpos et al., 2018). As ZEB1 directly binds and activates *DKK1* in colorectal carcinomas (De Barrios et al., 2017), we decided to evaluate its role in MM. We analyzed *DKK1* mRNA expression in the two MM cell lines and observed that *ZEB1* was required for *DKK1* transcription, while *ZEB2* overexpression did not affect *DKK1* mRNA levels (Figure 39.A-B).

We confirmed these results in the analysis of published arrays (GSE24080, n=559). We classified the cases according to their levels (high/low) of *ZEB1* or *ZEB2* and analyzed the expression of the other two genes. As depicted in Figure 39.C, patients with low levels of *ZEB1* had higher expression of *ZEB2* and lower expression of *DKK1*.

Lower levels of *ZEB2* correlated with higher expression of *ZEB1,* but no differences were observed in the *DKK1* expression.

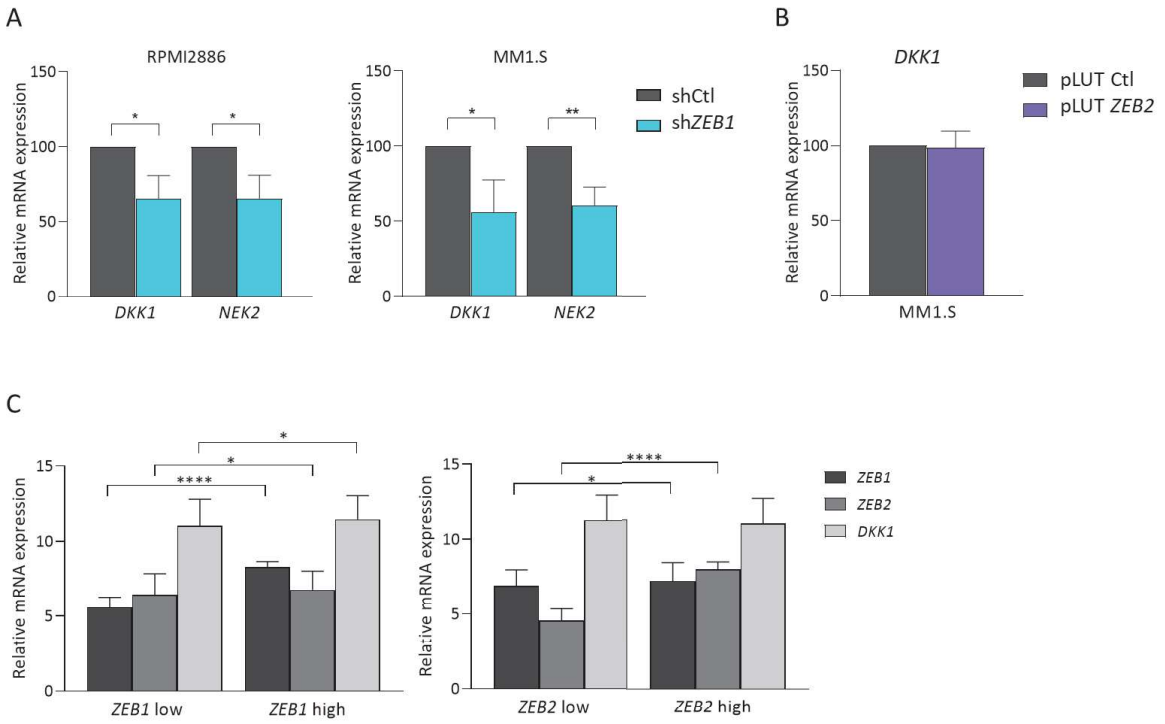

Figure 39. Bone lytic-related genes expression in MM cell lines. **(A)** *DKK1* and *NEK2* relative mRNA expression in MM cell lines with knocked down expression in ZEB1 **(B)** *DKK1* mRNA expression in MM cell lines with overexpression in ZEB2 **(C)** Relative mRNA expression of different genes (*ZEB1*, *ZEB2*, and *DKK1*) in a published array (GSE24080) classified according to ZEB1 or ZEB2 high/low expression levels.

Another driver of the osteoclast differentiation and bone destruction is NIMA Related Kinase 2 (NEK2), which is correlated with bone lesions in MM patients and promotes osteoclasts differentiation *in vitro* (Hao et al., 2017). In our MM cell lines, we observed a decreased expression of *NEK2* upon silencing of *ZEB1* expression, corroborating the relation of ZEB1 expression with bone lesions in MM patients (Figure 39.A).

DISCUSSION

DISCUSSION

The transcription factors ZEB1 and ZEB2 are best known for their role in the epithelial-mesenchymal transition and the carcinoma progression (Dongre and Weinberg, 2019). However, their roles and functions in different tissues and cell types have been largely explored during the last decade. During this dissertation, we have expanded the knowledge of the role of ZEB1 and ZEB2 in GC-derived B cell activation and the progression of two of the most common hematologic B cell malignancies, DLBCL and MM (Figure 40).

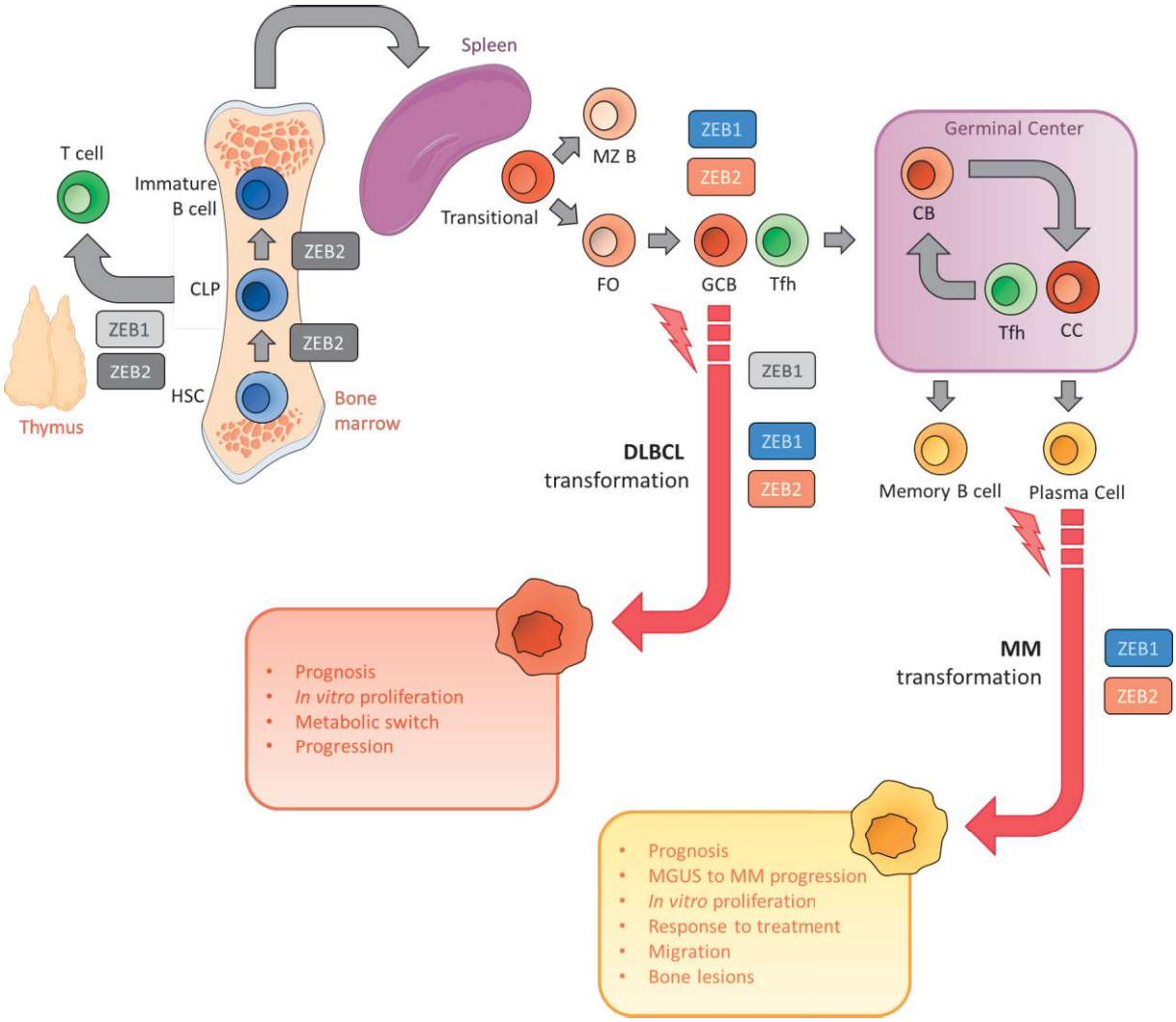

Figure 40. Roles of ZEB1 and ZEB2 in the B cell differentiation and activation and its malignant transformation and progression. In grey boxes, the roles of ZEB1 and ZEB2 already described in the literature. In blue (ZEB1) or orange (ZEB2) boxes, the new functions defined in this dissertation.

ROLE OF ZEB1 AND ZEB2 IN B CELL DIFFERENTIATION AND ACTIVATION

The B cell lineage arises from the hemogenic endothelium, involving a change in the phenotype of endothelial cells, so it is not surprising to find that both ZEB1 and ZEB2 are dynamically expressed in the hematopoietic lineage (Goossens and Haigh, 2012; Yokomizo et al., 2011). In this context, we analyzed the expression of ZEB1 and ZEB2 in human tonsils and mouse spleens. Both factors are expressed in the B cells in the GC, which are the compartments where a T-cell dependent response against an antigen occurs. In order to understand the role of ZEB1 and ZEB2 in the GC activation, *Zeb1* and *Zeb2* flox mice were generated. In order to achieve a similar human-specific T-dependent response, we experimentally induced an immunization of these mice with TNP_{31}-KLH and analyzed their B and T cell populations in both conditions, as an abnormal architecture of the spleen was observed in both models, compared with its control.

In the *Zeb1*$^{\Delta GC}$ mice, the non-immunized had similar B and T cell populations to its control, but when the immunization occurred, the GC reaction failed to respond correctly. In the immunized mice, we observed a decreased population of GC B cells and a reduced expression of BCL6, a master regulator of the GC reaction. ZEB1 forms a transcriptional repression complex along with its cofactor CtBP at the promoter site of *BCL6* in Burkitt's lymphoma cell lines (Papadopoulou et al., 2010) and also to synergize with FOXO transcription factors to activate anti-proliferation-related genes, *Ccng2* and *Rbl2, in vitro* (Chen et al., 2006). According to the literature, we should have expected enhancement of the GC formation in the Zeb1$^{\Delta GC}$ mice, but we observed the opposite. Our results are in the same line than an *in vivo* model, the *Cellophane* mouse, which lacks the C-terminal zinc finger domain of *Zeb1*, resulting in a truncated protein that does not bind to DNA sequences, but probably could still bind to other proteins (Arnold et al., 2012). The *Cellophane* mouse is unable to form the GC and mount a specific antibody-response, but this model had strong limitations, as the mutation was

not cell-type specific but ubiquitous. Thus, it could not be possible to determine the different immune populations responsible for the phenotype of this mouse model.

Along the line of these results, we observed that the *Zeb1*$^{\Delta GC}$ mice did not have the ability to mount a specific IgG antibody response in front of a specific antigen, in this case, TNP-KLH or sRBC. We also studied the IgM antibody production, but no differences were observed in the ability to differentiate into IgM antibody-forming cells. This result was expected because IgMs are associated with a T-independent response and produced by cells that do not enter the GC (Kawabe et al., 1994).

There was an increased number of MZ B cells subpopulations upon immunization in the *Zeb1*$^{\Delta GC}$ mice that go in the opposite direction as in the *Cellophane* model, where there are fewer MZ B cells (Arnold et al., 2012). This controversy could be explained due to the knockout of *Zeb1* is also affecting the MZ B cell differentiation, but our model is specific for GC B, so we would not be able to observe that effect. We also observed an increased number of Tfh cells, which at first sight, along with the increased MZ, would be contradictory to an impaired GC formation. MZ B cells can give rise to antibody-secreting plasma cells either by the canonical T-independent or also by T-dependent pathways (Attanavanich and Kearney, 2004). This last one can be achieved by antigen-presentation to Tfh (Cerutti et al., 2013). We can, therefore, hypothesize that in our *Zeb1*$^{\Delta GC}$ mouse model, there is a blockade in the differentiation into GC B cell that results in a blocked GC reaction and impaired antigen-specific antibody production. However, it tries to bypass it by increasing the MZ B cells interacting with the Tfh to provide a response. Further experiments need to be carried out to confirm these results.

In the *Zeb2*$^{\Delta GC}$ mice, in the absence of a specific immunization, we observed differences in the B and T cell populations compared with control mice. There was an increase of MZ B cells and a decrease of Follicular and GC B cells, accompanied by a decreased number of Tfh. Upon immunization, there was an increased number of T cells, but the mice regained the ability to form GC and to create a response similar to the one observed in the control mice. The antibody production was not altered, either against the specific antigen or in general, the basal antibody production. In published

arrays, we observed that although there was a lower expression of *Zeb2* in the GC B cells, there was an increased expression of it in the centrocytes, compared with the centroblasts. These results could suggest that ZEB2 is important for the entry of the B cells into the light zone and its dynamics. This hypothesis is supported by the fact that when the GC B cells enter the light zone, they undergo a reduction in CXCR4 expression (Bannard et al., 2013) and also by the study of Goossens et al. in which authors described that *Zeb2* knockout mice harbored an increased expression of *Cxcr4* (Goossens et al., 2011). However, further studies are required to confirm this hypothesis as ZEB2 could be having a role in the GC activation, but it might be redundant.

Another aspect that will be important to keep in mind is the cross-regulation between ZEB1 and ZEB2. In this sense, the data obtained from *Zeb1*$^{\Delta GC}$ and *Zeb2*$^{\Delta GC}$ cells may be due to a cross-regulation between the two genes, involving an indirect regulation via the miR200 family members (Guan et al., 2018).

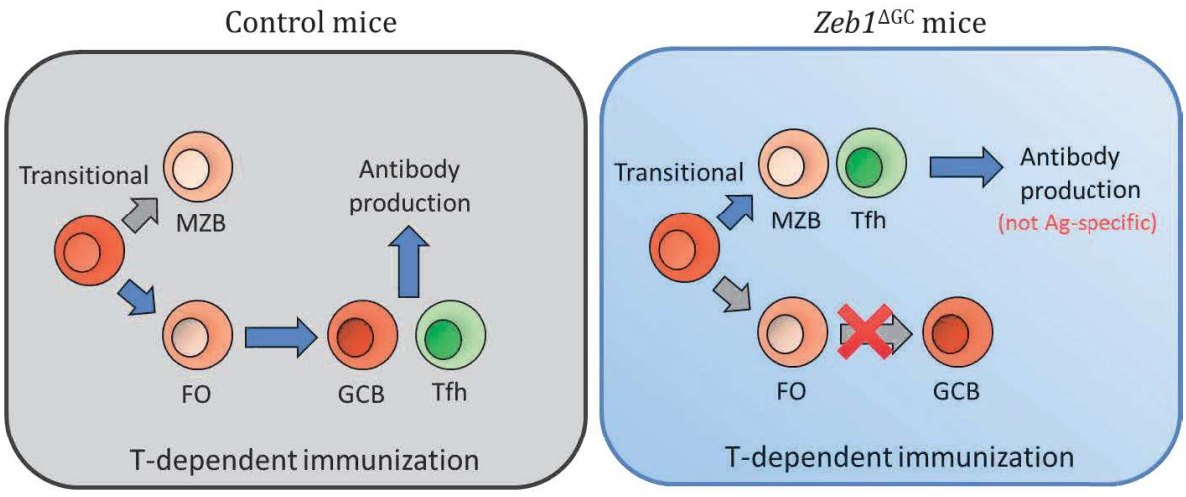

Figure 41. ZEB1 in the GC activation. ZEB1 has a non-redundant role in the GC activation in T-dependent immunizations.

ROLE OF ZEB1 AND ZEB2 IN DIFFUSE LARGE B CELL LYMPHOMA

Due to a process that could take place in many complex steps, the GC B cells could undergo a malignant transformation, evolving into malignant B cells. DLBCL is the most common NHL, and only 60% of patients will have a good response and prolonged survival with current therapies, whereas the remaining patients will eventually relapse (Alizadeh et al., 2000). For this reason, it is crucial to have a better understanding of the molecular pathways involved in its pathogenesis that would lead to new pharmacological strategies aimed at improving the actual outcome and prognosis.

In other lymphomas and leukemias, ZEB1 and ZEB2 are described to have both pro- or anti-tumoral capacities (Soen et al., 2018), but in DLBCL, their role is still not fully understood. Tumoral cells of DLBCL cases, as well as specialized cells from the microenvironment (macrophages and fibroblasts), were expressing both factors, being ZEB1 detected in a more substantial proportion of the cases. We correlated the expression of ZEB1 with prognostic markers, BCL6 and BCL2, both essential markers in the prognosis of DLBCL (Basso and Dalla-Favera, 2012; Tzankov et al., 2010). ZEB2 expression was associated with a better outcome of the patients, as seen in the Kaplan-Meyer survival plots, while ZEB1 tends to be associated with a poorer one. These results are in line with the report by Lemma et al., where authors associated the expression of ZEB1 with an adverse outcome, and SNAI2 (another EMT factor) with a better prognosis (Lemma et al., 2013). Of note, here we add up ZEB2 as a prognostic marker associated to favorable outcome.

In vitro assays with DLBCL cell lines demonstrated, on the one hand, a role for ZEB1 in the promotion of cell proliferation and, on the other one, a repressor role of ZEB2 toward this function and further studies *in vivo* need to be done.

Globally, our results present ZEB1 and ZEB2 as potential candidates for future therapy and prognostic markers. *In vitro* studies showed that ZEB1 increases the resistance of DLBCL cells to specific targeted therapies (Ibrutinib and R406), while ZEB2 did not influence the efficacy of these agents. Not all the DLBCL cell lines

examined in this dissertation responded the same way to different drug treatments, and it could be explained due to belonging to cell lines that could be classified upon their functional phenotype and not only their cell-of-origin.

We classified our cell lines depending on their metabolic phenotype, BCR and OxPhos, to study the role of ZEB1 and ZEB2 in its modulation. From the proliferation assays, ZEB1 might be activating an enhanced OxPhos-like phenotype and ZEB2 might have a role in switching up to a more glycolytic cell line. Examining different metabolic-associated genes, we observed a different behavior of the cell lines depending on their classification. These results indicated that regulation of ZEB1 mostly, but also ZEB2, lead to plasticity in switching between one metabolic phenotype and another, leading to changes in their basic energy pathways.

The lack of consistency among the cell lines in our results might be because there is plasticity between a complete OxPhos cell line and a complete glycolytic cell line. Our cell lines responded with different degrees of severity to the metabolic inhibitors indicating that they may not be comparable and possibly have different mutations. For this reason, a more extensive panel of cell lines needs to be studied. It could also be interesting performing a knock-out of both factors because a partial knockdown might be enough for a partial function of the metabolism.

Krebs et al. also described this shift between pathways was altered in their *Zeb1*-deficient mouse model. They found that *Zeb1* deletion in the pancreatic tumor cells leads to reduced cell respiration, indicating a blocked OxPhos, but also a reduced glycolytic reserve (Krebs et al., 2017). It was also described that the treatment of lung cancer cells with TGF-β (where ZEB1 is an activator of the pathway and ZEB2 a repressor) also leads to a shift from glycolysis to OxPhos (Sun et al., 2014). Controversially, ZEB1 also activates GLUT3, activating the glycolysis in the same type of cancer cells (Masin et al., 2014).

At the same time, modulation of the metabolic phenotype might be affecting the expression of ZEB1 and ZEB2, as seen in the result section. These results are in line with the observation made by Pouyafar et al., that inhibition of the glycolysis pathways

decreased the expression of different EMT factors, one of them, *ZEB1* (Pouyafar et al., 2019).

ROLE OF ZEB1 AND ZEB2 IN MULTIPLE MYELOMA

The cell of origin of Multiple Myeloma is the differentiated plasma cell and its characterized by a clonal expansion of the malignant cells in the bone marrow (Morgan et al., 2012). Other EMT-factors play a pivotal role in the acquisition of a mesenchymal-like phenotype that correlates to an extramedullary disease and a poorer prognosis (Muz et al., 2014), but the role of the ZEB family have not been described. In the case of ZEB1 and ZEB2, we observed expression in the malignant cells in plasmacytomas, a localized one mass of neoplastic cells.

We observed that the analyzed expression of MM patients showed an opposite expression pattern of both factors that were reverted in the MGUS cases. These results were also confirmed in published arrays, where MM patients had higher levels of ZEB1 and lower levels of ZEB2 and MGUS patients and BM of healthy patients showed lower levels of ZEB1 and higher of ZEB2. These expression patterns set *ZEB1* as a pro-tumoral gene expressed in the malignant cells and *ZEB2* as an anti-tumoral gene, expressed in the pre-malignant stage, MGUS. In that line, it has been described that they can have an inversed pattern of expression that could lead to different roles in specific tissues. Guan et al. described that ZEB1 and ZEB2 inhibit each other to decide the cell fate of T cells, and in melanomas, ZEB1 has an oncogenic role and ZEB2 a tumor suppressor function (Caramel et al., 2013).

The expression of both factors correlates with the overall survival of the patients, being ZEB1 a marker of poorer prognosis and ZEB2, a better survival marker. The progression from the pre-malignant stage of the disease, MGUS, into MM is still not fully understood, but both ZEB1 and ZEB2 might be playing a key role in this process.

Silenced expression of ZEB1 in the MM cell lines resulted in decreased proliferation and was associated with reduced expression of proliferation-related genes (*BIRC5* and *KI67*) and an upregulation of the pro-apoptotic gene (*BBC3*). The cell lines used in this dissertation had low levels of ZEB2 expression, so experiments involving the silencing of ZEB2 were discarded. In ZEB2 overexpression assays, similar results to the experiments with silenced ZEB1 were observed. These results support our hypothesis, being ZEB1 more related to a proliferative signature and ZEB2 having an opposite role.

Although current therapies can extend the patients' median survival to 5 to 7 years, MM remains largely incurable. We showed that the downregulation of ZEB1 increased the sensibility of MM cells to targeted therapies (bortezomib and dexamethasone). Of note, ZEB1 has been previously identified to confer resistance to doxorubicin in breast carcinoma and MCL cells (Sanchez-Tillo et al., 2014; Tryndyak et al., 2010).

An essential hallmark in the progression of MM is the cell migration from one bone marrow to another one, a process referred to as homing due to a gradient of chemoattractant (Azab et al., 2012). ZEB1 is best known for its role in promoting metastasis, which could be understood as a similar process to the migration of the malignant plasma cells through the bloodstream. In our experiments, we observed a decreased SDF-1-induced migration in both silenced ZEB1 and overexpressed ZEB2 cell lines. This result was consisting with a reduced expression of the receptor of SDF-1, CXCR4, in the cells.

Finally, another hallmark of MM studied was the ability to produce bone lesions, as is one of the major causes of morbidity in the disease (Rajkumar and Kumar, 2016). We found that ZEB1, but not ZEB2, was activating *DKK1* transcription in MM cells. DKK1 is a master regulator of the balance between osteoclast and osteoblast formation in MM and its expression has been correlated to the formation of bone lesions, as it (Tian et al., 2003). ZEB1 was also activating NEK2, a promoter of the osteoclasts differentiation *in vitro* (Hao et al., 2017). These results suggested that ZEB1 is activating the bone destruction or inhibiting the bone formation via DKK1 activation.

These results set the transcription factors ZEB1 and ZEB2 as possible markers for the prognosis of MM and its transformation from its premalignant stage, MGUS, where ZEB2 is acting as an anti-tumoral factor, to MM, where ZEB1 has the role of a pro-tumoral factor.

These results in both B cell neoplasms, DLBCL and MM, in the functions of ZEB1 and ZEB2 in different oncogenic hallmarks also open the door to modulate ZEB1 and ZEB2 as a potential approach in the treatment of these hematologic malignancies.

CONCLUSIONS

CONCLUSIONS

From the results obtained in this dissertation, we can conclude that:

1. ZEB1 and ZEB2 are required for the formation of the Germinal Center, where ZEB1 is important for a correct T-dependent response and an antigen-specific response.

2. ZEB1 and ZEB2 are implied in the progression of DLBCL. Being ZEB1 a prognostic marker for poorer survival and activating cell proliferation and ZEB2, a marker for better survival, inhibiting cell proliferation.

3. High levels of ZEB1 and low levels of ZEB2 in MM correlate with a poorer prognosis and an inversed pattern determines better survival, as ZEB1 is considered as a pro-tumoral gene expressed in MM, regulating its proliferation, cell migration and response to treatment and ZEB2 as an anti-tumoral gene expressed in MGUS.

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