



An alternative way to look at diffuse large B-cell lymphoma : the impact of frequent engagement of ReIB NF- κ B subunit on cell survival and patient outcome

Davi Moreira Collares

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Université de Paris (Paris Descartes)

École doctorale hématologie, oncogénèse et biothérapies (ED 561)

« NF- κ B, differentiation and cancer » team, EA 7324

An alternative way to look at diffuse large B-cell lymphoma:
the impact of frequent engagement of RelB NF- κ B subunit
on cell survival and patient outcome.

Davi Moreira Collares

PhD thesis on specialty “Hematology”

Directed by Thierry Molina and Véronique Baud

Defended on the 25th of June 2019

Jury composed by:

Christiane Copie-Bergman, PU-PH, Université Paris-Est, Rapporteur

Emmanuel Dejardin, Université de Liège, PhD, Rapporteur

Karen Leroy, PU-PH, Université Paris Descartes, Examineur

Maria Chiara Maiuri, PhD, CR1, Université Paris-Sud, Examineur

Thierry Molina, PU-PH, Université Paris Descartes, Director

Véronique Baud, DR2, Université Paris Descartes, Co-director



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To all trans people who never had a chance.

“Behind every exquisite thing that existed, there was something tragic.”

Oscar Wilde

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ABSTRACT

NF- κ B transcription factors play critical role in cell proliferation, cell survival and the physiopathology of numerous cancers. Deregulation of the classical NF- κ B pathway is known to be involved in at least the ABC subset of diffuse large B-cell lymphoma (DLBCL). However, the activation status of RelB NF- κ B alternative pathway subunit and its role remain unclear. We have demonstrated a frequent engagement of RelB in human DLBCL-derived cell lines. RelB activation protected cells from DNA damage and apoptosis induced by doxorubicin, the main drug in DLBCL treatment. RelB also controlled the expression of the anti-apoptotic protein cIAP2. In a cohort of 66 *de novo* DLBCL patients, we have directly assessed the DNA binding activity of all NF- κ B subunits by EMSA coupled with supershift. RelB activation was present in 66.6% of cases regardless of ABC or GCB classification and was an independent predictor of worse outcome. RelB activation status by EMSA allowed the definition of a RelB gene expression signature that was able to confirm RelB's negative impact on patient outcome when extended to a larger cohort. Altogether, our study indicates that RelB is a potential new biomarker for DLBCL and sheds light on its important role in DLBCL cell biology.

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LIST OF ABBREVIATIONS

(aa)IPI	(age adjusted) International prognostic index
(γ)H2AX	(phosphorylated on serine 139) Histone 2AX
(R)-ACVBP	(Rituximab) doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone
(R)-CHOP	(Rituximab) cyclophosphamide, doxorubicin, vincristine, and prednisone
ABC	Activated B-cell
AICD	Activation-induced death
ASCT	Autologous stem cell transplantation
ATP	Adenosine tri-phosphate
BAFF	B-cell activation factor
Bcl-2/6/10	B-cell lymphoma 2/6/10
Bcl-2A1	BCL2 related protein A1
BCR	B-cell receptor
BLIMP1	B-cell-induced maturation protein 1
BM	Bone marrow
c-FLIP	Casp8 and FADD like apoptosis regulator
CARD11	Caspase recruitment domain family member 11
CAT	Catalase
CCR7	C-C motive chemokine receptor 7
CD	Cluster of differentiation
CI	Confidence interval
cIAP1/2	Baculoviral IAP repeat containing protein 2/3
COO	Cell of origin
CREBBP	CREB binding protein
DAPI	4',6-diaminidino-2-phenylindole
DCF	2'-7' dichlorfluorescein
DD	Death domain
DLBCL	Diffuse large B-cell lymphoma

DNA	Deoxyribonucleic acid
DR4/5/6	Death receptor 4/5/6
DTT	1,4-Dithiothreitol
ECOG	Eastern Cooperative Oncology Group
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
EZH2	Enhancer of zeste homolog 2
FASAY	The p53 Yeast Assay
FasL	Fas ligand
FFPE	Formalin fixed paraffin embedded
GCB	Germinal center B-cell
GEP	Gene expression profile
GFP	Green fluorescent protein
GPX1	Glutathione peroxidase
GRR	Glycine-rich region
HIV	Human immunodeficiency virus
IGH	Immunoglobulin heavy locus
IHC	Immunohistochemistry
IKK $\alpha/\beta/\gamma$	I κ B kinase $\alpha/\beta/\gamma$
IL-1	Interleukin 1
INF- α/β	Interferon α/β
IRF4	Interferon Regulatory Factor 4
LDH	Lactate dehydrogenase
LMP1	Latent membrane protein 1
LPS	Lipopolysaccharide
LT β	Lymphotoxin β
LTR	Long terminal repeats
LYSA	Lymphoma study association
LZ	Leucine zipper

Mdm2	Mouse double minute 2 homolog
MFI	Mean fluorescence intensity
MHC	Major human histocompatibility complex
MnSOD	Mitochondrial antioxidant manganese superoxide dismutase
MYC	Avian Myelocytomatosis Viral Oncogene Homolog
MYD88	Myeloid Differentiation Primary Response 88
NF- κ B	Nuclear factor kappa B
NGS	Next generation sequencing
NIK	NF- κ B inducing kinase
NOS	Not otherwise specified
NOS3	Endothelial nitric oxide synthase 3
NPV	Negative predictive value
NQO1	NAD(P)H dehydrogenase
PCR	Polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PNPP	para-Nitrophenylphosphate
PPV	Positive predictive value
PRDM1	PR domain zinc finger protein 1
RHD	Rel homology domain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Real time
RT-MLPA	Reverse transcriptase multiplex ligation-dependent probe amplification
SDS	Sodium dodecyl sulfate
SE	Standard error
shRNA	Short hairpin RNA
SOD1	Superoxide dismutase 1
TAD	Transactivation domain

TBE	Tris-borate-EDTA
TBS	Tris Buffered Saline
TG-SDS	Tris-Glycine SDS
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor α
TNFAIP3 (A20)	TNF Alpha Induced Protein 3
TNFRSF14	TNF receptor superfamily member 14
TOP2A	Topoisomerase II
TRAF2/3	TNF receptor-associated factor 2/3
TRAIL	TNF-related apoptosis-inducing ligand
ULN	Upper limit of normal
UV	Ultra-violet
WES	Whole-exome sequencing
WHO	World Health Organization
XDH	Endothelial nitric oxide synthase
XIAP	X-linked inhibitor of apoptosis protein

INTRODUCTION

I.1 - NF- κ B family of transcription factors

NF- κ B was first described in 1986 as a nuclear factor binding the kappa light chain enhancer in B-cells (1). NF- κ B transcription factors family plays a crucial role in the inflammatory and immune responses, cell proliferation and survival (2,3). When deregulated, these transcriptional factors can participate in tumor development, maintenance and even initiation, especially in hematopoietic malignancies (4–7). NF- κ B has also been implicated in resistance to cancer treatment (8).

In mammals, this family is composed by five members, RelA (p65), RelB, cRel (Rel), NF- κ B1 (p50 and its precursor p105) and NF- κ B2 (p52 and its precursor p100) (9) (Figure I1) . All NF- κ B family members present a highly conserved region, the Rel Homology Domain (RHD), in the N-terminus domain. It is vital to dimerization, nuclear translocation and DNA binding. Furthermore, RelA, cRel and RelB (but not p105/p50 and p100/p52) possess a transactivation domain (TAD), without which transcriptional activity is not possible. Interestingly, RelB is the only member which has a Leucine Zipper (LZ), a domain in its N-terminal region (10). Cleavage of RelB on the LZ has been described as activation mechanism for RelA-RelB inhibitory complexes (11), however little is known about the functions of RelB LZ.

The precursors p105 and p100 are cleaved in their C-terminal ankyrin repeats to be converted into their active forms p50 and p52, respectively. In their inactive unprocessed forms, they have a crucial inhibition role and behave as I κ Bs (9,10).

These proteins form various homo- and heterodimeric complexes, the only form they can be transcriptionally active. NF- κ B activity is regulated by two main pathways: the first one known as the classical or canonical pathway, mainly applies to RelA and/or cRel containing complexes; the second one, so-called alternative or non-canonical pathway leads to the activation of RelB containing complexes (12).

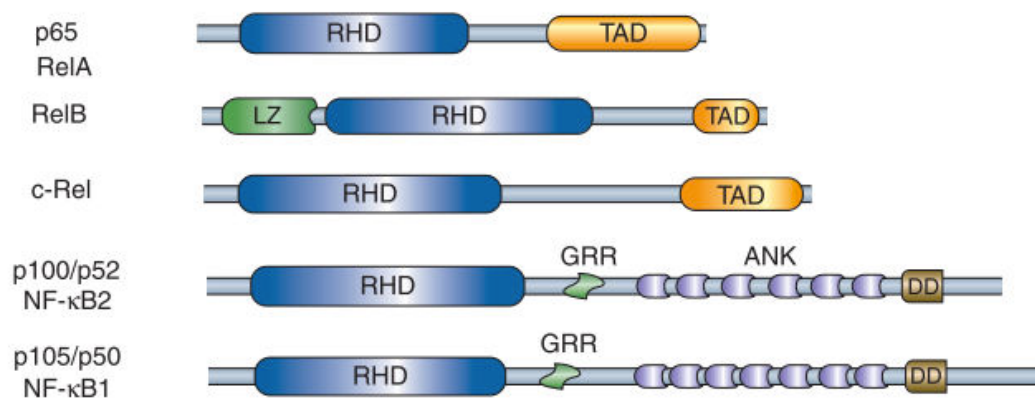


Figure I1: The NF- κ B transcription factor family. The five members of the NF- κ B family are presented with their main domains. Proteins p100/p52 and p105/p50 are shown in their full length. DD, death domain; GRR, glycine-rich region; LZ, leucine-zipper; RHD, Rel homology domain; TAD, transactivation domain. From Oeckinghaus and Ghosh, *Cold Spring Harb Perspect Biol*, 2009.

I.1.2 - NF- κ B pathways

I.1.2.1 - Classical (or canonical)

Inflammatory cytokine tumor necrosis factor α (TNF- α), toll-like receptors (TLR), interleukine-1 (IL-1) and lipopolysaccharide (LPS), are some of the stimuli

capable of activating this pathway. This pathway regulates mainly RelA/p50 heterodimers, but can also regulate c-Rel/p50 and p50/p50 complexes (9,10).

Among activators of NF- κ B classical pathway, TNFR1 signaling pathway is perhaps one of the best characterized. After ligand recognition, TNFR1 cytoplasmic death domain (DD) binds to TRADD (TNFR-associated protein with a DD) and RIP1, which also contains a death domain. TRAF2 is then recruited by TRADD via its TRAF-binding domain, which in turn will recruit cIAP1 and cIAP2. cIAP1/2 act as E3 ubiquitin ligases, recruiting LUBAC (linear ubiquitin assembly complex), which will catalyse linear polyubiquitination of NEMO and subsequent activation of IKK (13). Recruitment of TAK1 results in phosphorylation and activation of IKK β and IKK α . IKK β phosphorylates I κ B α , leading to its polyubiquitination and degradation by the proteasome. In absence of stimulation, the NF κ B dimer is sequestered in the cytoplasm through interaction with inhibitory proteins of the I κ B family. Now released, the active dimer translocates to the nucleus and executes its transcription functions (2,9). Some of targets genes of NF- κ B are its own negative regulators, such as I κ B α and A20. Newly synthesized I κ B α binds to NF- κ B active dimers shutting them back to the cytoplasm. A20 and CYLD deubiquitinate RIP1, NEMO and TRAFs, ending the signal, thus acting as negative regulator of the duration of NF- κ B activity. (Figure I2) (14).

This pathway is typically responsible for a strong and rapid NF- κ B activating signal in response to stress situations and plays a crucial role in the regulation of inflammation and innate immunity (2).

I.1.2.2 - Alternative (or non-canonical)

This pathway is known to be involved in diverse processes such as proliferation and lymphoid organogenesis (15). It is activated by a subset of TNF family members (e.g. lymphotoxin β (LT β), B-cell activating factor (BAFF), CD40 ligand, TWEAK) (9,15,16). It is important to state that, except for BAFFR, all activators of the alternative pathway also induce the classical pathway.

In unstimulated cells, TRAF3 and TRAF2 form a complex with cIAP1/2, which ubiquitin tags NF- κ B inducing kinase (NIK) for its degradation. Stimulation of activator receptors results in the ubiquitination of TRAF3 by cIAP1/2 and its following degradation. Degradation of TRAF3 by the proteasome allows stabilization of NIK, which will activate IKK α that will phosphorylate p100. Phosphorylation will ultimately conduct to either total p100 degradation and liberation of RelB/p50, or partial degradation, producing free RelB/p52 dimers (9,15,17) (Figure I3).

Activated IKK α has been described to destabilize NIK as negative feedback mechanism. TANK-binding kinase 1 (TBK1) also binds and phosphorylates NIK for degradation (16). The alternative pathway induces the protein OTUD7B, a deubiquitinase that deubiquitinates TRAF3, stabilizing it, reducing the signaling and creating a negative feedback loop (16,18). Recently described as a regulator, OTUB1 is a deubiquitinase that controls p100 stability and prevents abnormal alternative pathway activity (19). Additionally, crosstalk between the classical and alternative pathway has been described, being NEMO responsible for NIK negative regulation (16).

The alternative pathway plays a crucial role in innate and adaptive immunological responses, immune system development and maturation and even bone remodeling. It is not surprising that its deregulation is implicated in several inflammatory diseases and cancers (9,16,17,20).

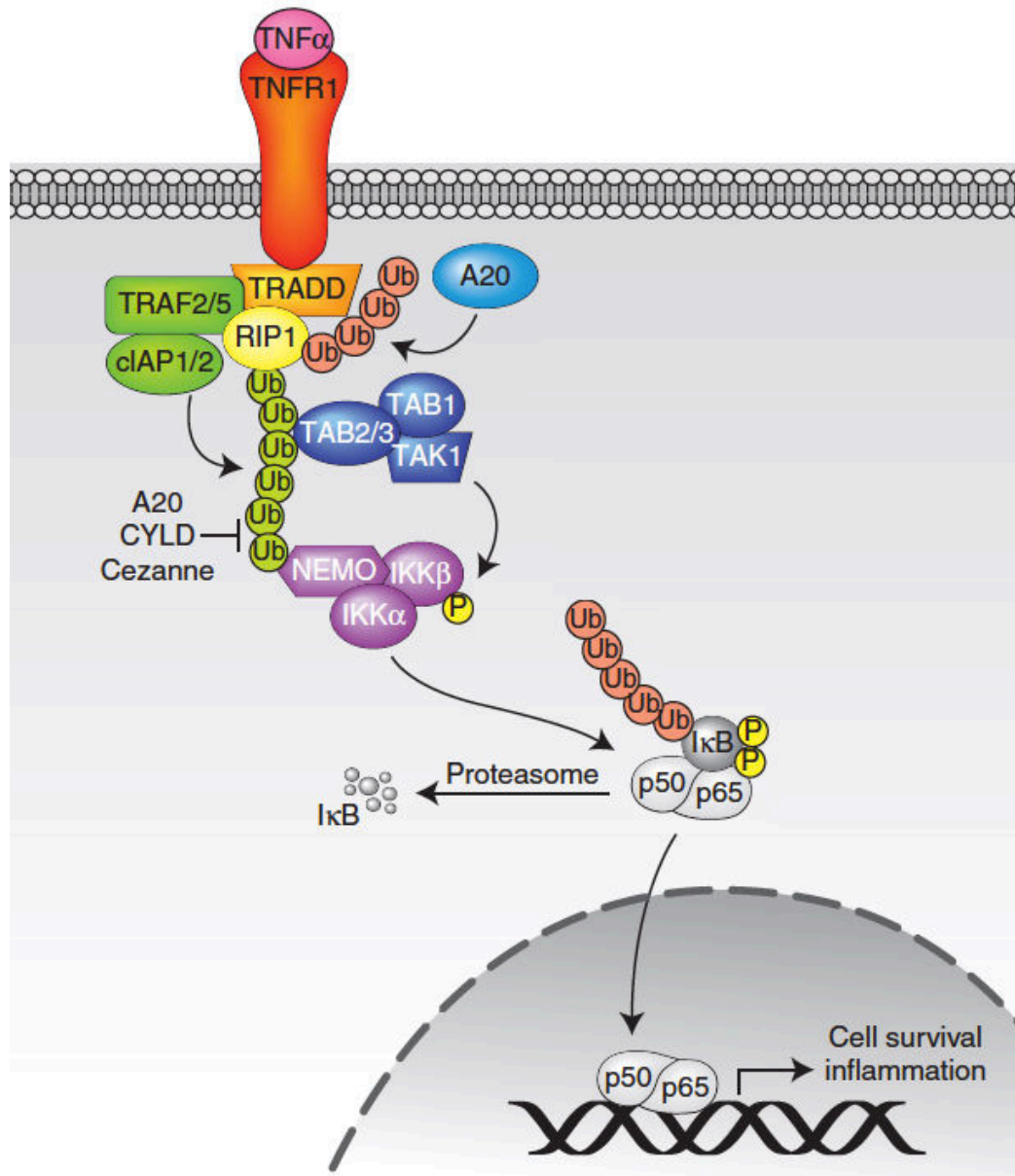


Figure I2: The NF-κB classical pathway. Schematic representation of NF-κB activation cascade by TNFR1. Engagement of TNFR1 results in activation of TRADD and recruitment of TRAF proteins, which will ultimately result in ubiquitination of NEMO and activation of the IKK complex. This complex will

phosphorylate $\text{I}\kappa\text{B}\alpha$ allowing its degradation and liberation of NF- κ B dimer to translocate into the nucleus. Lys-48 linked ubiquitin chains are shown in red, and Lys-63-linked ubiquitin chains are shown in green. Adapted from Wertz et al, *Cold Spring Harb Perspect Biol* 2010.

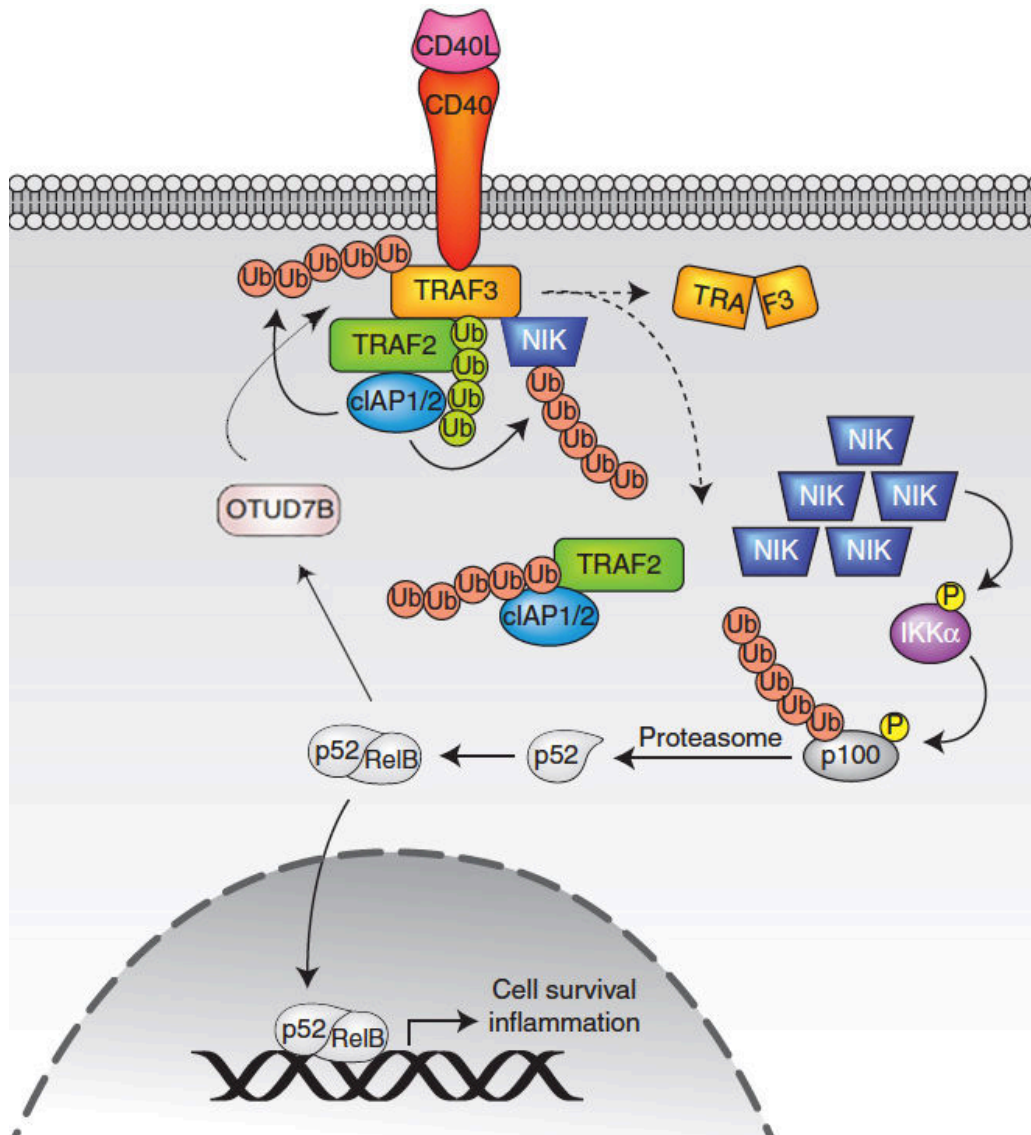


Figure I3: The NF- κ B alternative pathway. Activation of NF- κ B alternative pathway through CD40. Lys-48 linked ubiquitin chains are shown in red, and Lys-63-linked ubiquitin chains are shown in green. See text for additional details. Adapted from Wertz et al, *Cold Spring Harb Perspect Biol* 2010.

11.2.2.1. RelB regulation and functions

RelB gene is located on chromosome 19q13.32 and encodes an mRNA from 11 exons, resulting in a 579-amino acid protein (21). RelB can potentially be regulated by many different transcription factors at its promoter, introns, and exons; among these putative regulatory sites are domains that bind glucocorticoid receptors, c-Jun, c-Fos, sp-1, and B-zip chromatin repressor and insulator CCCTC-binding factor (CTFC), which defines the boundary between euchromatin/heterochromatin and IFN response factor 1 (IRF1).

The best-studied transactivator of RelB is RelA, which can bind proximal promoter NF- κ B sites (22). Interestingly, the same study shows that RelB can bind its own promoter and regulate its own mRNA expression. In presence of cytomegalovirus protein IE1, AP-1 regulates RelB expression. It can be inhibited by $1\alpha,25$ -dihydroxyvitamin D3 blocking dendritic cell differentiation. SIRT1, a cellular bioenergy sensor, can also induce RelB mRNA expression (21).

In addition to the alternative NF- κ B signaling cascade (described before), RelB DNA-binding activity is negatively regulated at the nuclear level by several mechanisms, such as trapping in RelA/RelB or p100/RelB complexes, as well as post-translational modifications (discussed ahead). RelB-containing dimers also display DNA-binding specificity. RelB recruitment to some genes correlates with transcriptional downregulation (IL12-p40), whereas in other cases (EBV-induced molecule 1 ligand chemokine (ELC) and macrophage-derived chemokine (MDC)), it increases transcriptional activity (12).

RelB-p50 heterodimers crystal structure researches have suggested that although DNA-interacting residues are highly conserved among NF- κ B members, RelB can bind a more diverse set of NF- κ B consensus sequences than the other

family members (21).

Studies in knockout mice showed that RelB has important roles in development and function of immunological system that cannot be compensated by the presence of the other NF- κ B subunits (23).

RelB directly controls the transcription of MMP3 on fibroblasts, impacting on cell migration (24); cIAP2 in multiple myeloma cells, influencing cell survival (25); manganese-SOD in prostate cancers (26), as well as EZH2 (27) have also been identified as direct RelB targets. RelB participates in metabolisms and mitochondria biogenesis. RelB-mediated changes in the NADH/NAD⁺ balance, activate the NAD⁺-sensing sirtuin family member, SIRT1. In turn, SIRT1 deactivates nuclear RelA and induces RelB transcription, resulting in an anti-inflammatory phenotype and increased fatty acid flux and transport into mitochondria. RelB can also regulate mitochondrial biomass by regulating PGC-1 β . Additionally, RelB supports expression of SIRT3, which localizes to mitochondria and enhances many mitochondrial functions, having implications for oxidative metabolism (21). RelB was shown to participate in the regulation of the circadian rhythm in murine fibroblasts, by direct binding to BMAL1, one of the main regulators of the cycle (28). The regulation of the pathway is also influenced by SIRT1, which gives RelB a second indirect role (21). It also takes part in the xenobiotic-detoxifying pathway in lung fibroblasts by binding to AhR, a transcription factor involved in the xenobiotic response responsible for detoxifying polychlorinated biphenyls and polycyclic aromatic hydrocarbons via increased expression of the cytochrome P450 family. This interaction induces RelB, which provokes a decrease in COX-2 and PG-mediated inflammation. RelB also plays an important role in RANKL-induced osteoclastogenesis that cannot be

compensated for by RelA (12,21). Some of the main functions of RelB are summarized in figure I4.

RelB can also function as a negative regulator of the classical pathway of NF- κ B. RelB can bind to RelA, forming an inhibitory complex (29), and cleavage of RelB on its LZ can activate the classical pathway of NF- κ B (30).

Post-translational modifications are changes or alterations in a protein occurring after the completion of the translational process that act as a mechanism for the specification of proteins and increase their variety. Reported modifications involving NF- κ B include phosphorylation, methylation, ubiquitinylation, acetylation, SUMOylation, and isomerization of specific amino acid residues, and target the IKKs, the I κ Bs, the NF- κ B subunits, or critical adaptor proteins that feed into NF- κ B. However, until now few of such described modifications have been reported for RelB (see table I1). Remarkably, the best characterized is its phosphorylation on serine 472 by IKK α /IKK β , which has been reported to induce the transcription of MMP3 and influence on cell migration. (24). Enzymes regulating other RelB modifications are not yet identified and no biological functional has been associated to them for the moment. In some cases, it remains to be uncovered if the described phosphorylation happen *in vivo* (12). Other modifications are summarized on table I1.

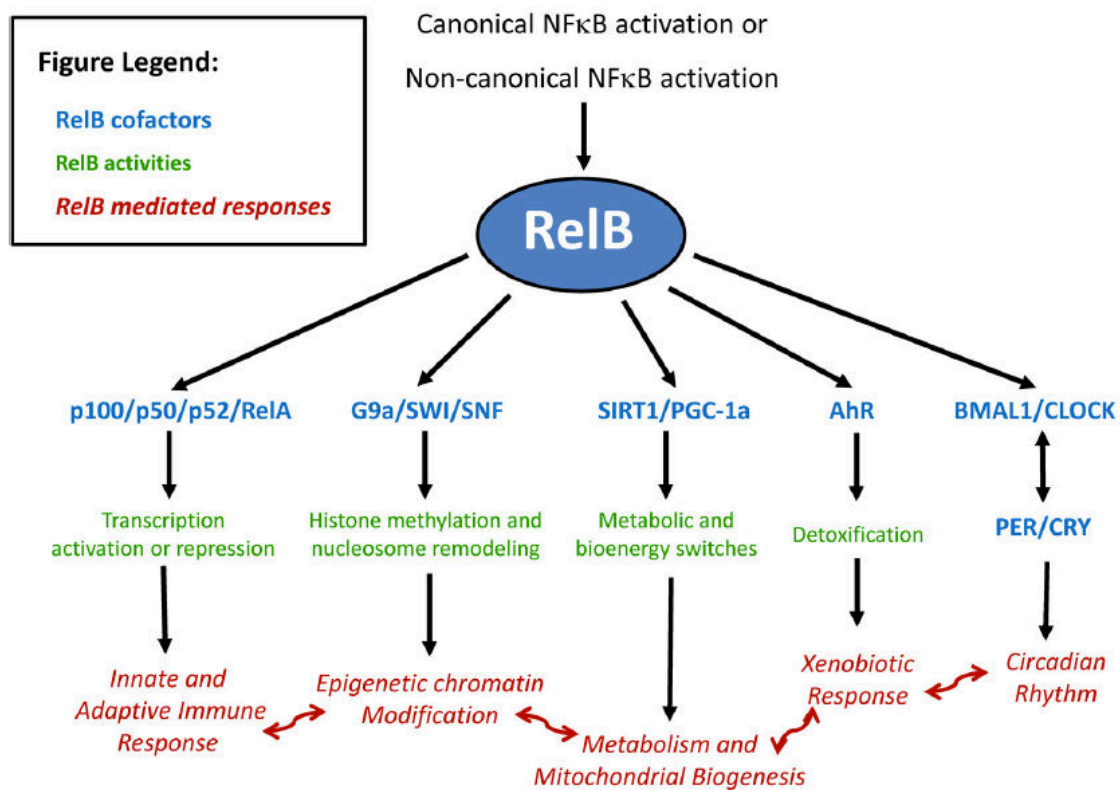


Figure I4: Overview of some of RelB functions. RelB interacts with different proteins and factors (blue), involved in actions described in green, culminating in the execution of diverse functions (red). From Millet et al, *J Leukoc Biol* 2013.

Modification	Site(s)	Enzyme(s)	Effect	Reference
Phosphorylation	Threonine 84, Serine 552	Unknown	Degradation	Marienfeld <i>et al.</i> 2001 [71]
Phosphorylation	Serine 368	Unknown	Dimerization	Maier <i>et al.</i> 2003 [72]
Polyubiquitination	Lysine 273, 274, 305 and 308	Unknown	Transcriptional activity	Leidner <i>et al.</i> 2008 [73]
Phosphorylation	Serine 472	IKK α /IKK β	Cell migration	Authier <i>et al.</i> 2014 [74]
SUMOylation	Lysine 387, 388, 390, 411, 414, 415, and 416	Unknown	Transcriptional activity	Leidner <i>et al.</i> 2014 [75]

Table I1: Post-translational modifications of RelB. From Baud and Collares, *Cells*, 2016.

I.1.3 - NF- κ B and B-cell physiology

Notably, during B-cell generation and maintenance, NF- κ B subunits have many physiological functions and play unique roles in the development and function of mature B lymphocytes (Figure I5) (31).

The primary role of NF- κ B in B-cell development is to guarantee cell survival. In early stages, NF- κ B protects B-cells from TNF α induced apoptosis by induction of Bcl-xL. It also participates in the negative selection in bone marrow (BM) via both alternative and classical pathways (2,32,33).

After leaving the BM, they complete maturation in the spleen. At this point, both survival, mainly by NF- κ B-induced Bcl-2 and A1 expression, and differentiation depend on both NF- κ B pathways. Differentiation of marginal zone B-cells (MZB) depends as well on NF- κ B. Perturbations in NF- κ B, particularly RelB, at this point can result in autoimmunity (2,10).

Activation of the alternative pathway in wild-type immature B cells is likely to occur via BAFF receptor signaling and maturation is at least in part dependent on that pathway. The alternative pathway promotes survival via Bcl-2 expression, and, indirectly, the retention in the cytoplasm of otherwise apoptotic nuclear PKC δ (34).

Classical and alternative pathway activation in stromal cells is crucial for establishing primary and secondary lymphoid organs. These anatomical niches are required for good functioning of the adaptive immune response and RelB seems to have a particularly important role in their establishment (35,36).

Mature B-cells express a variety of NF- κ B dimers. Constitutive, low nuclear activation of both classical and alternative pathways of NF- κ B is thought to mainly serve a survival role in non-activated B-cells (2,33). NF- κ B activation via the B-cell receptor (BCR) protects CD40-stimulated B-cells from activation-induced death and is essential for survival and differentiation (37). Different B-cell receptors activate different NF- κ B pathways by different signaling. The crosstalk among these receptors is crucial for determination of B-cell response and how NF- κ B will influence functionally the B-cell (Figures I6 and I7). NF- κ B signaling is also essential for immunoglobulin isotype switching (32).

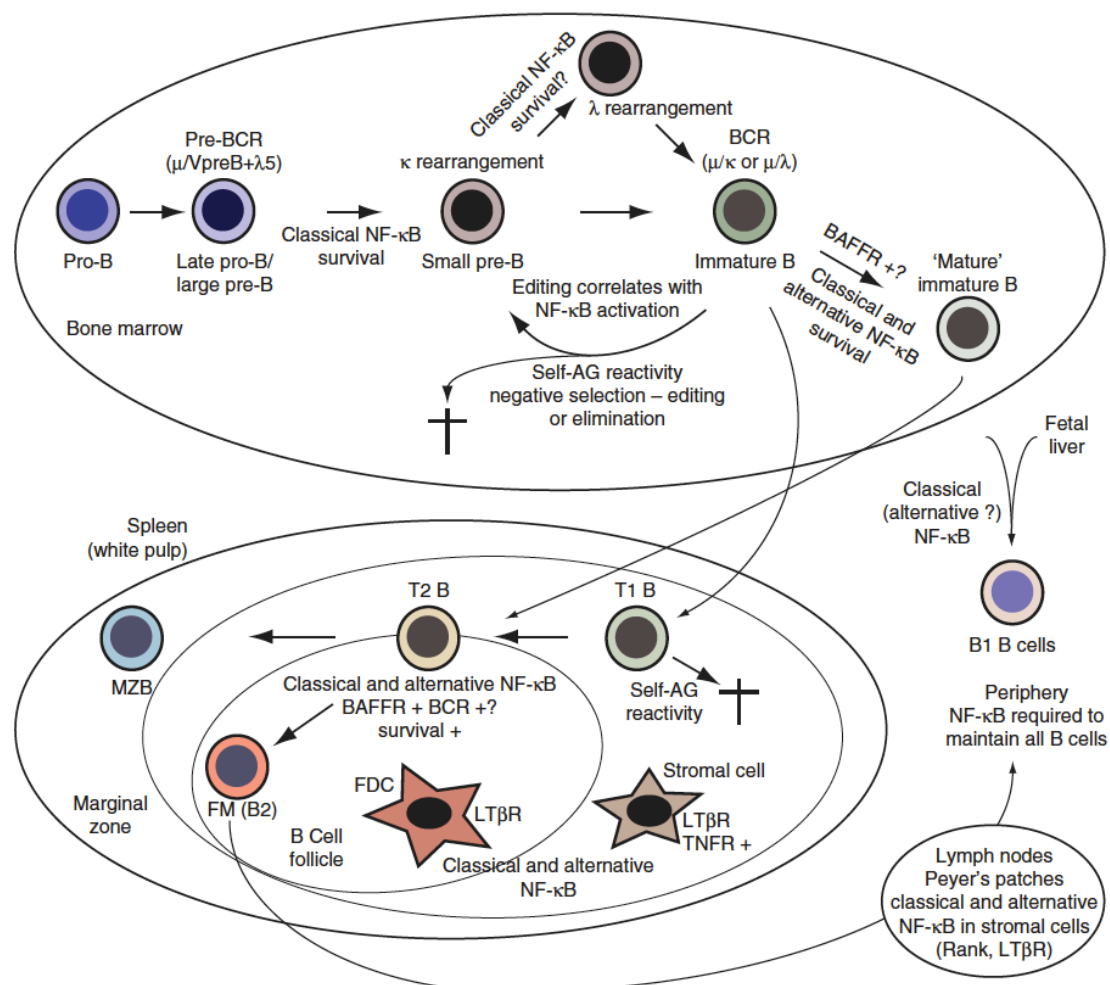


Figure I5: B-cell development and NF- κ B. Simplified representation of bone marrow and splenic B-cell development. NF- κ B contributions in different stages of differentiation, maturation, formation of marginal zone (MZ B) and follicular mature (FM) B cells (B2 B cells) and entry the peripheral circulation are indicated. The roles of NF- κ B in the development of B1 B-cell, a peripherally self-renewing population, are also indicated. Attention must be paid to the participation of NF- κ B in formation of proper architecture in spleen, Peyer's patches and lymph nodes. From Gerondakis et al, *Cold Spring Harb Perspect Biol* 2010.

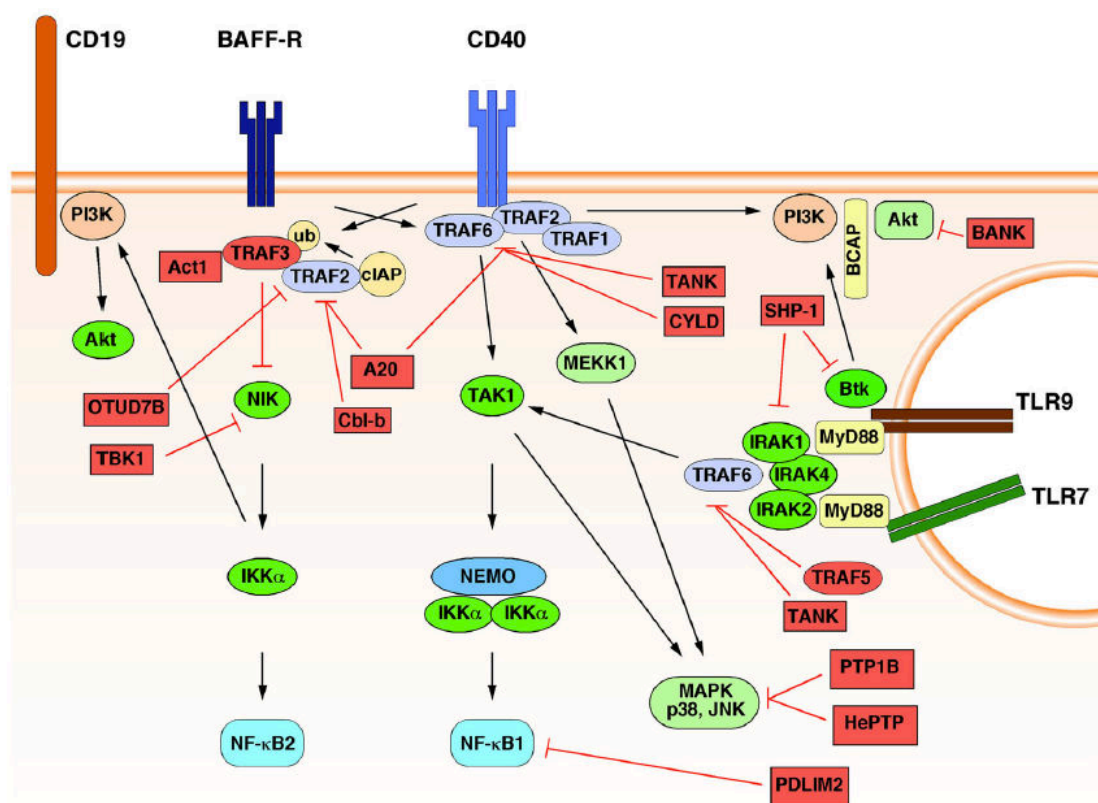


Figure I6: Control of CD40 and BAFF-R signaling. TRAF2 is inhibited by A20 and Cbl-b, preventing the degradation of TRAF3 at either BAFF-R or CD40. This results in the degradation of NIK, which prevents alternative NF- κ B activation. OTUD7B and TBK1 are indirectly regulators, through their activities of TRAF2

deubiquitination and NIK phosphorylation, respectively. CYLD and TANK inhibit the canonical NF- κ B pathway downstream of CD40 through TRAF6. Tyrosine phosphatases PTP1B and HePTP regulate CD40 and BAFF-R signaling by directly dephosphorylating the p38 MAPK. PDLIM2 is a nuclear ubiquitin ligase that induces the degradation of the p65 NF- κ B protein. From Hobeika et al J Mol Med, 2015 (38)

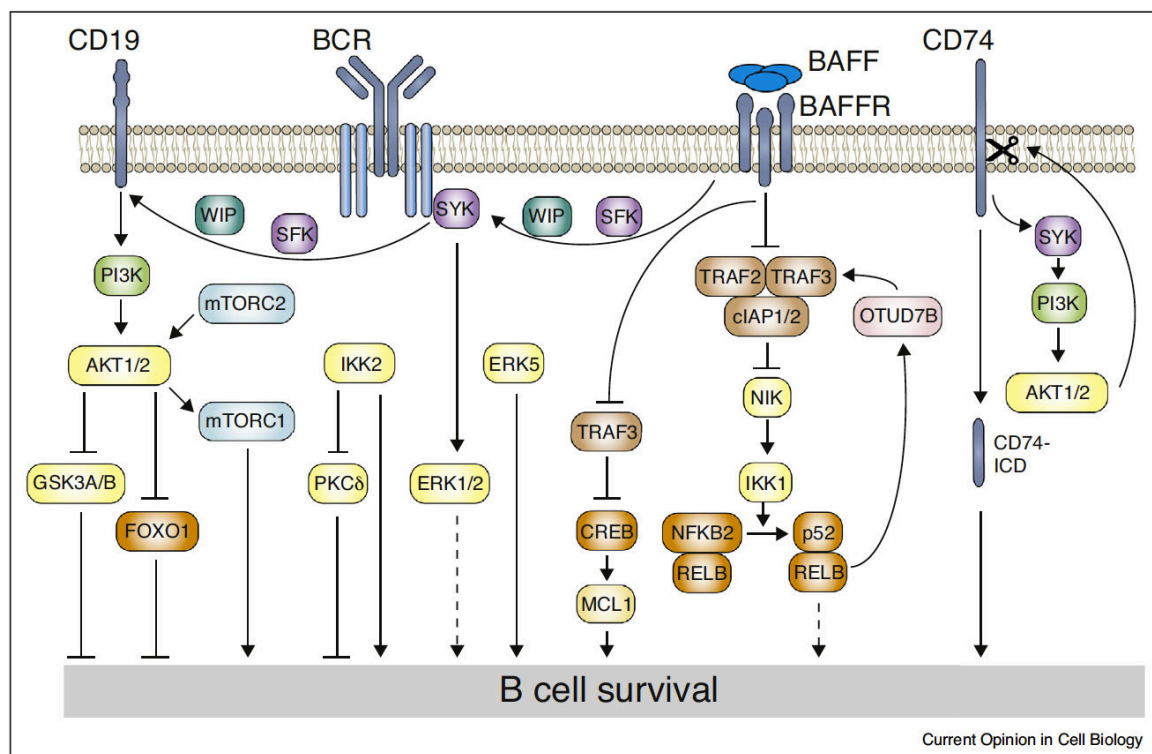


Figure I7: B-cell survival signaling pathways. Signaling from BAFFR to BCR and SYK depends on SRC-family kinases (SFK). Further signaling to CD19 requires either SFK or SYK. Regulation of the actin cytoskeleton by WIP is required for signal transduction from BAFFR to CD19. Signals from the BCR via SYK lead to activation of ERK1 and ERK2, contributing to cell survival in unclear ways. BAFFR transduces signals via the TRAF2-TRAF3-cIAP1/2 E3 ligases to

NIK, leading to processing of p100. TRAF3 also directly enters the nucleus and associates with CREB, leading to its degradation. CREB induces expression of MCL1. The alternative NF- κ B pathway induces OTUD7B deubiquitinase, which stabilizes TRAF3 in negative feedback. From Schweighofer et al, *Curr Opin Cell Biol* 2018. (18)

I.1.4 - NF- κ B and apoptosis

Apoptosis is a physiological process of organized programmed cell death, fundamental for organ development, homeostasis, and elimination of defective or potentially dangerous cells. It can be triggered by a great variety of stimuli and can be divided in two major subtypes: extrinsic and intrinsic (39,40).

Extrinsic apoptosis is mediated by death receptors (e.g., fas cell surface death receptor [FAS] and TNFR1), initiated by caspase 8 and caspase 10; or the withdrawal of dependence receptor ligands, initiated by caspase 9 (Figure I8) (40).

In response to cellular stress, BH3-only proteins promote mitochondria outer membrane permeabilization (MOMP), releasing mitochondrial proteins, such as cytochrome C into the cytosol. MOMP is tightly controlled by the BCL2 family, including pro-apoptotic (e.g., BCL2 associated X, apoptosis regulator [BAX], BCL2 antagonist/killer 1 [BAK1, also known as BAK]), and anti-apoptotic (e.g., BCL2 and BCL2 like 1 [BCL2L1, also known as BCL-XL]) members. Cytochrome C will interact with apoptosis protease activating factor-1 (Apaf-1), recruiting caspase 9, for the assembling of the apoptosome, culminating in cleavage of caspase 3, which will cleave substrates in order to assure cell death.

DNA damage, hypoxia, metabolic stress, are some of the inducers of intrinsic apoptosis (Figure I8) (39–41).

In this context, activation of NF- κ B (through TNFR for instance) can activate the transcription of regulators of intrinsic and/or extrinsic death signaling cascade (Figure I9) (42). The main targets are inhibitor of apoptosis proteins XIAP, c-IAP1 and c-IAP2, that prevent activation of pro-caspase-9 and blocking caspase-3 and -7 activity; and the antiapoptotic Bcl-2 family proteins Bcl-xL, Bcl-2A1, NR13 and Bcl-2, all of which will ultimately prevent mitochondrial cytochrome c unloading (39,42–44). Other upregulated genes are A20, that prevents recruitment of TRADD and RIP1, and c-FLIP, which regulates caspase 8 (4).

NF- κ B can also induce the expression of proapoptotic genes. These include mainly death receptors Fas, TRAIL receptors DR4, DR5 and DR6, and their death-inducing ligands FasL, TNF α and TRAIL; tumor suppressor p53, the proapoptotic Bax and the proapoptotic alternatively spliced form of Bcl-xL, Bcl-xS (42,44). In ultra violet (UV) exposure induced- and chronic exposure to hydrogen peroxide induced-cell death, NF- κ B represses prosurvival genes (4).

NF- κ B's role in apoptosis is critical for development and homeostasis in diverse cell systems (42). As an example, it is required for developing B-cells survival, but also for activation-induced cell death (AICD), important mechanism through which immune system homeostasis is maintained (2).

Context can interfere in the role of NF- κ B in apoptosis: c-Rel upregulates the expression of MnSOD to protect cells against cell death by converting toxic superoxide anions to H₂O₂. However, the accumulation of hydrogen peroxide itself can trigger apoptosis (45). In another example, atypical activators of NF- κ B

can turn RelA into a transcriptional repressor of antiapoptotic target genes (42,46). Fas-L-induced NF- κ B activation can induce c-FLIP, which will in turn downregulate NF- κ B and promote FasL-induced cell death (4).

Importantly, *TP53* gene, a major regulator of apoptosis and cell cycle, has a κ B site in its promoter, and can have its expression induced through this site through TNF α stimulation (47). NF- κ B and p53 are also both induced by certain forms of stress that cause DNA damage, such as UV radiation (43). p53 and NF- κ B can function together to promote transcription of certain genes, but can also compete for co-activators. p53 activation can lead to NF- κ B DNA binding, however suppressing its transcriptional activation (4). NF- κ B's transcriptional activity can result in enhanced degradation of p53 caused by NF- κ B-dependent upregulation of Mdm2, culminating in escape from cell death (42). TNF-induced NF- κ B protects against UV-induced cell death in p53-expressing cells. In another example, activation of NF- κ B by p53, not mediated by TNF α , has been reported to suppress p53-induced cell death. In this context, inhibition of NF- κ B in presence of wild-type p53 might enhance cell survival (48).

NF- κ B also participates in several non-apoptotic programmed cell death processes. Since RIPK1 phosphorylation prevents apoptosis or necrosome formation with following necroptosis, activation of NF- κ B by TNFR1 participates in the regulation of this pathway. In another example, induction of inflammasome components through NF- κ B, triggered by INF or TLR signaling participate in pyroptosis (40).

In conclusion, regulation of apoptosis through multiple different mechanisms is with no doubt one of the main roles of NF- κ B activation.

Dependence on NF- κ B to escape programmed cell death and resist conventional therapy is a trait shared by numerous human cancers. Therefore, it remains a central topic for understanding cancer genesis and development of cancer therapy.

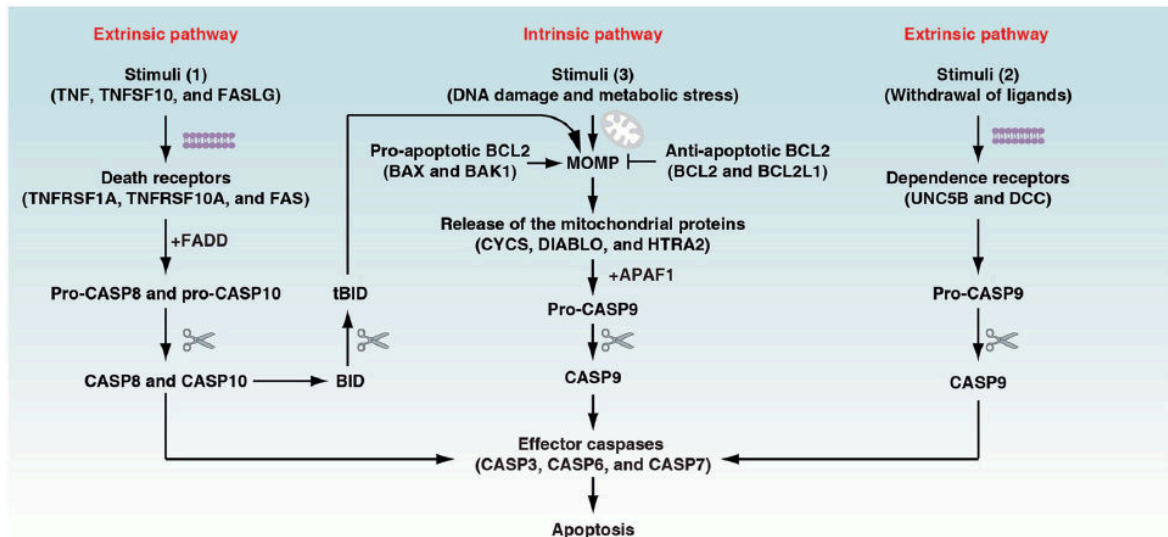


Figure I8 : Extrinsic and intrinsic apoptosis. Representation of the main steps in extrinsic and intrinsic apoptosis pathways, and interaction between them *via* BID. From Tang et al, *Cell Research* 2019.

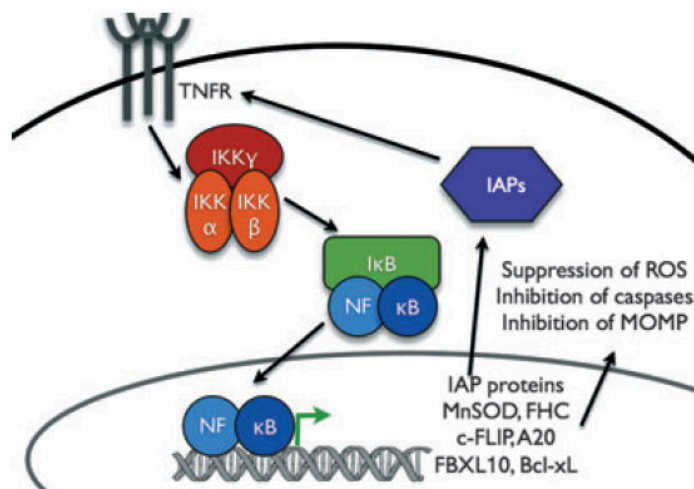


Figure I9: NF- κ B target genes involved in programmed cell death. Activated NF- κ B induces transcription of multiple genes (e.g. IAP proteins, antioxidant proteins such as MnSOD and ferritin heavy chain (FHC), c-FLIP, A20, FBX10, and Bcl-xL) to suppress apoptosis, both in the intrinsic and extrinsic pathways. Adapted from Baldwin, *Immun Rev* 2012.

I.2 - Diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin lymphoma. The median age of incidence is the 7th decade of life, nevertheless, it can occur in young adults and more rarely in children. There is a slight male preference. Its most usual clinical presentation is one or multiple fast growing nodal and/or extra-nodal masses (up to 40% of cases), being the gastrointestinal tract the most frequent site (49,50). The most common type of DLBCL, representing 80-85% of all cases, is designated as “not otherwise specified” (NOS) (50). It is the aim of this work and will be referred henceforth simply as “DLBCL”.

I.2.1 - Classification

Gene expression profiles (GEP) allowed cases to be separated in two main groups, based on their cell of origin (COO): germinal center B-cell-like (GCB) and activated B-cell-like (ABC) (51). Shortly after the publication of this study a third group was added. Since its gene expression profile according to Wright's algorithm does not fit neither within germinal center nor activated B-cell-like profiles, it has been called “type 3” (52), and later “unclassified”. This

classification revealed that the ABC group presented a significant worse prognosis among patients treated with R-CHOP (51–54).

Even though the classification brought precious information, GEP is not applicable in routine, thus many different groups tried to develop immunohistochemistry (IHC) surrogates. The most widely used was the Hans algorithm, which is based on the combination of CD10, BCL6 and MUM1 immunohistochemistry markers to differentiating DLBCL into two groups: GCB (germinal center B-cell like) and non-GCB (55). Recently, new technology of GEP using a smaller panel of genes, applicable in formalin fixed paraffin embedded (FFPE) tissue has been developed with higher concordance with the transcriptome (i.e. RT-MLPA (56) and nanostring (57)) and might probably be increasingly used. Nonetheless, there is considerable heterogeneity within each group (58), pointing out for the necessity for further stratification.

For these reasons, many efforts were made to better stratify DLBCL patients by gene expression in order to better treat, predict response to treatment and have a better understanding of the pathogenesis of the disease.

Further characterization, with gene clustering based on the clinical outcome of patients defined four groups: “germinal center B-cell”, associated with better prognosis; “proliferation”, associated with poor outcome, *MYC* and its target genes; “major histocompatibility complex (MHC) class II”, associated with silencing of MHC II expression and inferior survival; and “lymph node”, which presented a better prognosis and was suggested to be a microenvironment-related signature (52). The “proliferation” profile correlated fairly with the ABC subtype, while not surprisingly, “germinal center B-cell” correlated with GCB

subtype. Interestingly, “MHC II” and “lymph node” profiles did not correlate with any of the COO groups.

In 2005, Monti et al. have established new genetic signatures that separated cases in 3 clusters (59). The first one showed enrichment in mitochondrial function and oxidative phosphorylation-related genes. It was called “OxPhos”. The second cluster had increased expression of cell cycle regulator, DNA repair genes and elements of the BCR signaling cascade. It was named “BCR/proliferation”. Finally, the third one was called “Host response cluster”, for it presented enhancement in T-cell mediated response and complement marker, connective tissue elements, among other host response related genes.

All 3 clusters had similar 5-year survivals and did not overlap with the previous COO classification, suggesting their value is more related to pathogenesis mechanisms and possible development of specific targeted therapy.

In a different approach, Lenz et al. have described two different gene expression signatures of the microenvironment of the tumor (60). The so called “stromal 1” is characterized by monocyte host-reaction and extracellular matrix deposition related genes and is associated with a better prognosis. “Stromal 2” is associated with tumor blood vessel density and angiogenesis and is indicative of a worse outcome.

More recently, Schmitz et al. have analyzed 574 patient samples by means of exome and transcriptome sequencing, deep amplicon resequencing of 372 genes, and DNA copy-number analysis (61). Based on the co-occurrence of genetic abnormalities, they have divided cases in 4 main groups: MCD, BN2, N1

and EZB. MCD was defined by *CD79B-MYD88* double mutation; BN2, by *NOTCH2* mutation or *BCL6* fusion; N1 by *NOTCH1* mutation; and EZB was based on *EZH2* mutation or *BCL2* translocation. MCD and N1 represented predominantly ABC cases, EZB had a majority of GCB, and BN2 was represented by the three subtypes. They were able to classify only 44% of cases, suggesting the others may share features. Regarding prognosis, the 4 molecular types had significantly different outcomes, being EZB and BN2 of more favorable prognosis than MCD and N1. Additionally, they have adapted this classification for feasibility with next generation sequencing (NGS) in clinic.

Later on, Chapuy et al. (58) have performed whole-exome sequencing (WES) capturing identified low-frequency alterations, recurrent mutations, somatic copy number alterations and structural variants in 304 *de novo* DLBCL patients, to assess their prognostic value. By consensus clustering of the 158 genetic driver alterations found they were able to define 5 subsets of tumors and a sixth group with no defining genetic alterations. Four of the groups represented two different subsets of ABC and GCB tumors, respectively, with different outcomes and potential targets for therapy, probably reflecting different pathogenesis. The finding reinforces the heterogeneity within COO subgroups. The 2 remaining groups could not be classified in one single COO subgroup.

In the light of recent knowledge, DLBCL complexity in terms of physiopathology and genetic alteration spectrum is evident. In spite of all the outstanding advances made in the past decades, we still lack optimal classification, risk stratification for patients and therapy selection.

I.2.2 – Frequent genetics alterations

As stated before, DLBCL is a disease characterized by a complex genetic profile of numerous mutations, translocations, amplifications, deletions and others (58,61,62). In a recent series, a tumor harbored a median of 17 (range: 0–48) different genetic driver alterations (58). With increasing development of targeted therapy, a best characterization of frequent and less frequent genetic alterations is in our best interests to better understand and cure this disease.

Because of immunoglobulin rearrangements intrinsic to B-cell development, these cells are particularly prone to translocations. Alterations involving *BCL6* and its control of *IRF4* Blimp1 in the progression to plasma cell differentiation have been implicated in the genesis of DLBCL. As additional oncogenic processes, other alterations involving *MYC* and *BCL2* are reported in DLBCL (62,63).

Frequency of common and less frequent mutations may vary depending on the study. With the improvement of sequencing technologies new alterations are being uncovered. Frequent mutations and their frequencies may be seen in figure I8A.

Some frequent mutations such as *MYD88*, *CARD11*, *CD79B*, *PRDM1* and *IRF4* are preferentially encountered in ABC tumors. Mutations in *EZH2*, *CREBBP*, *TNFRSF14*, *BCL2* and *MYC* are most commonly found in GCB cases (64).

TP53 mutations are present in about 20% of cases of DLBCL and have been shown to be an independent predictor of poorer prognosis (50,63). They occur in both the GCB and ABC/non-GCB subtypes of DLBCL in approximately

equal frequency (50,58,61,63). *TRAF3* is also equally mutated in both GCB and ABC DLBCL in up to 15% of cases (65).

In terms of rearrangement, *IGH*, *BCL2*, *BCL6*, and *MYC* are the most frequently found (58). DLBCLs harboring translocations involving *MYC*, *BCL2* and/or *BCL6* are associated with worse prognosis and were recognized in the latest WHO classification as “high grade B-cell lymphoma with rearrangements of *MYC* and *BCL2* and/or *BCL6*” (49,66). *MYC* and *BCL2* protein expression assessed by IHC has also been shown to identify a subset of patients with poor outcome (66).

Concerning NF- κ B, known alterations involve mainly the BCR signaling, an important activator of the classical NF- κ B pathway in the B-cell (2,67). Loss of function of inhibitors of the cascade, like A20, or gain of function of activators, such as *CD79B*, *MYD88* or *CARD11*, results in signaling autonomous from the actual BCR activation and constitutive NF- κ B activation (62,68–70). Recently, a multiprotein supercomplex formed by MYD88, TLR9 and the BCR has been described to coordinate BCR oncogenic signaling that results in NF- κ B pro survival activation (71). In addition to being implicated in the pathogenesis of DLBCL (62,72,73), NF- κ B constitutive activation results in survival and proliferation signaling, and also in resistance to treatment, as discussed before. These genetic alterations are found mainly in ABC DLBCL (64,69) (Figure I8B). Nevertheless, recent studies found several different alterations resulting in abnormal BCR signaling-dependent NF- κ B activation in nearly 45% of cases. Events that may affected other NF- κ B regulators were present in 66,2% of all cases. Most importantly, these features were not exclusive of ABC DLBCL (61).

I.2.3 - Prognosis and treatment

Classical therapy for DLBCL patients is based on CHOP regimen (CHOP: cyclophosphamide, doxorubicin, vincristine, and prednisone). The introduction of rituximab, a monoclonal antibody against CD20, in the late 90's improved significantly the outcome of these patients (74,75). Nevertheless, refractory/relapse cases can still reach up to 40% (75).

Alternative treatments, have been proposed, all of them with their advantages and flaws. More intensive ones, as ACVBP-based chemotherapy (ACVBP: doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone) in association with rituximab, are more effective, particularly in non-GCB patients, however have more frequent adverse effects and are therefore frequently restricted to younger patients (76,77).

Because DLBCL remains a heterogeneous disease in clinical presentation, genetic findings and response to therapy, it is difficult to stratify patients and define the best treatment. Rituximab in association with CHOP remains the classic first line therapy for DLBCL. Even though it might not be the best option for all patients, we lack markers for more precise and personalized choices of treatments in most patients.

I.2.3.1 – Doxorubicin

Doxorubicin is the main drug in DLBCL first line chemotherapy (75). It is an anthracycline first extracted from the bacterium species *Streptomyces peucetius* in the 1970's (78). It has two main mechanisms of action: the first one is poisoning of topoisomerase II, with following replication collapse and DNA

strain breaks; Doxorubicin has also been described to produce DNA adducts provoking transcriptomic and epigenetic changes, and even crosslink lesions (79–81).

The second is the generation of reactive oxygen species (ROS) through multiple mechanisms (78): doxorubicin is metabolized into a semiquinone, an unstable metabolite, being reduced back to doxorubicin in a cycle that generates ROS (78). It also increases iron accumulation in the mitochondria, increasing ROS production (82). ROS can lead to lipid peroxidation and membrane damage, DNA damage, oxidative stress, and triggers apoptotic pathways of cell death (78,83).

Doxorubicin can also interfere in the iron metabolism and ultimately provoke ferroptosis (84,85). Ferroptosis is a singular non-apoptotic process of iron-dependent ROS accumulation that results in lipid peroxidation and ultimately cell death (86). Although relatively well characterized as an actor in cardiotoxicity secondary to doxorubicin (84,85), its importance for doxorubicin anti-cancer action remains to be further explored (85,86).

It is important to notice that different doses and times of exposure will result in different consequences regarding compartment distribution (nucleus, mitochondria, cytosol), ROS production, growth arrest and cell death (87). Also, that doxorubicin acts through several other minor mechanisms, once again highlighting its complex mechanism of action, which is briefly presented in figure 19.

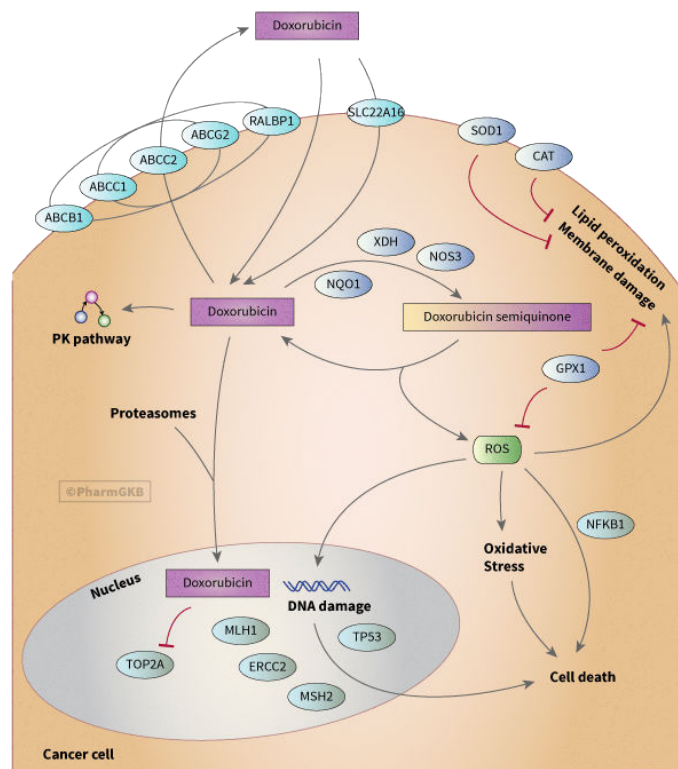


Figure I11: Schematic representation of Doxorubicin mechanisms of action.

Doxorubicin was shown to cause DNA breaks to interfere with DNA synthesis and poison topoisomerase II. Its translocation into the nucleus is thought to occur via binding to proteasomes. Doxorubicin undergoes a one-electron reduction by several oxidoreductases to form a semiquinone radical. Re-oxidation of semiquinone back to doxorubicin leads to the formation of ROS and hydrogen peroxide. ROS, causing oxidative stress, can be deactivated by glutathione peroxidase, catalase and superoxide dismutase. Topoisomerase II, TOP2A; NAD(P)H dehydrogenase, NQO1 ; xanthine oxidase, XDH ; endothelial nitric oxide synthase, NOS3 ; glutathione peroxidase, GPX1 ; catalase, CAT ; superoxide dismutase, SOD1. From Thorn, CF et al. *Pharmacogenet Genomics*.

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I.2.3.2 – Rituximab

Rituximab is a chimeric human/murin anti-CD20 monoclonal antibody, approved for clinical use around 20 years ago that has revolutionized the treatment of B-cell malignancies. Through its affinity with the CD20 transmembrane protein, present in most malignant B-cells, it engages cell death via at least 4 different mechanisms: antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis, complement-dependent cytotoxicity (CDC), and direct antitumor effects via either apoptosis or other cell death pathways (Figure I10) (88).

Despite of its remarkable success and undeniable importance in the treatment of B-cell malignancies, a proportion of patients eventually face resistance and treatment failure (88,89). For this reason new anti-CD20 monoclonal antibodies, such as Obinutuzumab, so called “type II”, are being developed. They present different epitope affinity and different special conformation, serving as promising alternatives for refractory/relapsed patients (89).

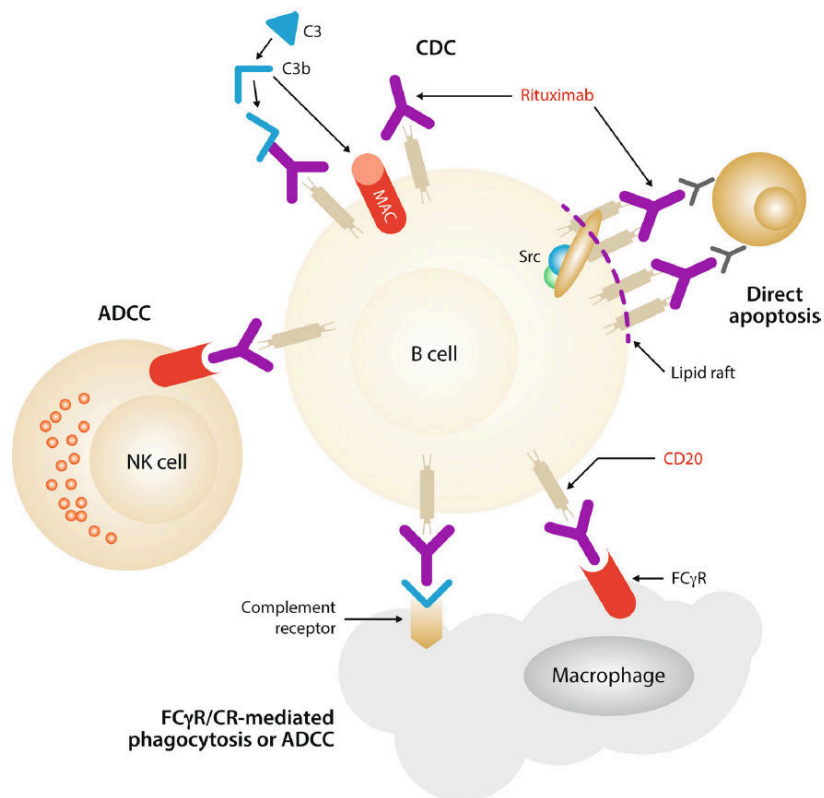


Figure I12: Mechanism of action of Rituximab. Rituximab binds to CD20 on the B-cell surface activating the complement cascade, which generates the membrane attack complex (MAC) and induces B-cell lysis by complement-dependent cytotoxicity (CDC). Rituximab also favors interaction with natural killer (NK) cells through Fc receptors (FcRs), which leads to antibody-dependent cellular cytotoxicity (ADCC). The Fc portion of rituximab and binded complement fragments are recognised by macrophages, which leads to phagocytosis and ADCC. Lastly, interaction of several molecules of rituximab and CD20 in the lipid raft with elements of a signaling pathway involving Src kinases mediates direct apoptosis. FcR Fc receptor, FcCR Fcc receptor. From Salles et al, *Adv Ther* 2017.

I.3 - DLBCL and NF- κ B

NF- κ B has been reported to participate in the pathogenesis of DLBCL through multiple mechanisms (65,72,73). In addition to NF- κ B downstream targets being involved in prevention of apoptosis and proliferation, Calado et al. have described that canonical NF- κ B pathway cooperates with disruption of *BLIMP1* in the development of ABC-DLBCL-like lymphoma in mouse models (73).

The ABC DLBCL subtype was characterized by a GEP associated to BCR activation in peripheral blood B-cells (51,54) and BCR is one of the main upstream activators of NF- κ B classical pathway (2,67). In this context, a small set of previously reported NF- κ B target genes were found to be preferentially overexpressed in ABC subtype (54), defining a NF- κ B-related gene expression signature in DLBCL. However, it is important to stress that BCR activation engages mainly the canonical pathway (90).

Inhibition of the classical NF- κ B pathway by means of either an overexpression of a non-degradable form of the inhibitory protein I κ B α (super repressor SS32/36AA I κ B α), or an IKK β kinase-dead mutant, or the use of a chemical inhibitor of the IKK complex was found to induce cell death in ABC cell lines (54,91). Later works have linked frequent mutations in regulators of the classical NF- κ B pathway (i.e. *MYD88*, *TNFAIP3*, *CD79A*, *CARD11*) with the ABC DLBCL (53,92).

Taken together, these findings led to the concept of NF- κ B addiction in the ABC DLBCL, mainly through the activation of the classical NF- κ B pathway.

However, it is not known if the presence of genetic mutations in regulators of NF- κ B or the expression of the described NF- κ B target genes correlate with the DNA binding activity *per se* of the classical pathway of NF- κ B (i.e. RelA and cRel). Further, the activation status of the alternative NF- κ B pathway (i.e. RelB) subunits remains unclear.

Several attempts have been made in the past 10 years to determine the actual NF- κ B activation status, and their impact in DLBCL (65,70,93–101). However, these attempts to evaluate NF- κ B in DLBCL patients differed largely in methodology, criteria, technology available at the time and aim of the study. Therefore, there are numerous discrepancies, making them very hard to compare with one another. The great majority of such studies present evaluations of NF- κ B status by immunohistochemistry (IHC). In this technique, the positivity is defined by nuclear accumulation of the protein. One of the main limitation of this method is that nuclear localization does not necessarily correspond to the activation of NF- κ B family members (102), neither the amount of protein accumulation is proportional to the activation (as observed in our cell lines, discussed ahead). Another issue is that it is not known how significant levels of activity correlate to protein levels detectable by IHC. There is no consensus about the criteria of IHC positivity either.

Electrophoretic mobility shift assay (EMSA) remains the gold standard assay to evaluate NF- κ B activity (103). This technique allows the qualitative and quantitative determination of direct DNA binding of NF- κ B subunits. In addition to being extremely sensitive, with aid of supershift with specific antibodies, it is possible to precisely define the dimers involved. It also allows the appreciation of

the relative intensities of each subunit as they are all seen at the same time in each case. The main disadvantages are the need of radioactive isotopes and that the technique requires a good level of expertise both for execution and interpretation. Further details are presented in the section “Materials and Methods”.

EMSA has never been largely used in the study of NF- κ B status in DLBCL patients and our study is the first to analyze the NF- κ B DNA binding status using EMSA combined with supershift in a cohort of *de novo* DLBCL patients.

I.3.1 - Alternative pathway and RelB

There is growing interest in the alternative NF- κ B pathway, whether it is activated and its role in pathogenesis of DLBCL (65,70,93–96,104). However, in spite of being demonstrated to have crucial roles in other B-cell lymphomas (25,105–107), the importance of the alternative pathway of NF- κ B in DLBCL remains largely unknown.

Ramachadran et al. have associated the alternative NF- κ B pathway to the control of chromosome stability and DNA repair in p100 knockdown models of DLBCL (104). Recently, Zhang et al. (65) have shown that enforced alternative NF- κ B pathway activity pushes the B-cell towards plasma cell differentiation. When associated with Bcl-6 dysfunction this differentiation is blocked resulting in ABC DLBCL-like tumors in mice. They suggest that frequent TRAF3 mutations described before, for abolishing the negative regulation of NIK, might result in constitutive activation of the alternative pathway of NF- κ B in DLBCL.

Studies concerning RelB in DLBCL face the same technical and methodological problems described in the previous section. Perhaps because of the lack of convincing evidence of RelB constitutive activation in DLBCL so far, its role in this disease remains poorly understood. The status *per se* of RelB DNA-binding activity in DLBCL patients and cell lines remains an open question.

I.3.2 - NF- κ B as therapeutic target

Given the central importance of NF- κ B in DLBCL pathogenesis and maintenance, it is not surprising that it is one of the main candidates for development of target therapy. All alterations described downstream the BCR are potential targets for indirect anti-NF- κ B therapy. Due to the mentioned evidence pointing to ABC DLBCL depend on BCR signaling, up to this point most of the trials with new drugs targeting upstream of NF- κ B have been performed exclusively in non-GCB DLBCL patients.

Proteasome inhibitors, such as bortezomib, block the degradation of classical NF- κ B inhibitors. Bortezomib is ineffective alone, but in a phase II trial has shown benefits in non-GCB patients (31). Nevertheless, a large scale randomized phase III trial recently published showed no benefit in the progression free survival in DLBCL patients, both GCB and ABC subgroups, receiving bortezomib in addition to R-CHOP (108)

Lenalidomide is an oral immunomodulatory drug that acts through multiple mechanisms and it is believed to selectively kill ABC DLBCL cells (92). In a study with 40 refractory relapse patients it has shown efficacy (109). However, phase III trials showed no event free survival benefit of lenalidomide in combination with R-

CHOP in ABC type DLBCL (ROBUST trial, Celgene, press release, April 25, 2019) and no overall survival benefit as maintenance therapy in DLBCL patients (110)

Ibrutinib is an inhibitor of the BTK complex, which is immediately downstream to the BCR. Patients bearing *CARD11* mutations did not respond to ibrutinib and the status of *CD79B* did not seem to interfere. *MYD88* mutated patients bearing a wild type *CD79B* showed no response to the drug, however *MYD88* and *CD79B* double mutated patients were the group with best response. This indicates that ibrutinib requires specific selecting of patients, which depends on the mutational profile of their tumors (31,92,111). However, in a recent phase III trial (Phoenix), ibrutinib did not improve the event free survival of patients with non-GCB DLBCL. Additionally, in elderly patients (>60 year old), and added in serious adverse events, hindering them from receiving complete R-CHOP therapy (112).

Inhibitors of other BCR associated kinases, such as PI3K, SRC and SYK have been tested, especially in combination with ibrutinib in order to prevent and overcome resistance (113–115). Most studies haven't reached clinical testing yet. Observation in vitro and using patient-derived xenografts in mice suggest they might be an effective alternative. However, a phase II clinical trial for the use of SYK inhibitor fostamatinib showed poor response in DLBCL patients (116).

SMAC mimetics are IAP antagonists that prevent activation of canonical NF- κ B pathway. However, IAPs also control negatively NF- κ B alternative pathway. Because of ABC DLBCL addiction to BCR signaling, SMAC mimetic birinapant showed to be beneficial in ABC, but not GCB, DLBCL cell lines.

However, authors conclude care has to be taken in the use of the drug in patients with constitutive activation of NF- κ B alternative pathway (117). The use of this class of drugs still lacks further studying and robust clinical trials.

Other drugs targeting NF- κ B are facing preliminary tests and new ones are being developed. The main concerns involving this modality are toxicity, side effects in long-term administration and clinical criteria for selecting patients (31,118). The results so far showed that, at least for the moment, addition of targeted therapy has not been successful in demonstrating superiority to R-chop alone in any phase III DLBCL trial. The design of diagnostic biomarkers is required to better define clinically relevant subgroups in DLBCL.

OBJECTIVE

We aimed to define the activation status of the RelB NF- κ B subunit in DLBCL and evaluate its prevalence and impact. To do so, we used two different strategies: human-derived DLBCL lines and DLBCL patient samples.

In cell lines we planned first to determine the activation status of NF- κ B subunits. With aid of RNA interference, we aimed to knockdown RelB protein expression on cell lines with constitutive activation of RelB to study its importance on DLBCL cell survival and resistance to treatment.

In patients, our aim was to determine the NF- κ B subunits activation status by EMSA in a group of newly diagnosed DLBCL patients included in clinical trials and determine the frequency of RelB constitutive activation. Following, we wished to establish, the impact of RelB activation in patient outcome. In the next step, we defined a gene expression signature linked to RelB activation status to study larger groups of patients.

MATERIAL AND METHODS

M.1 - Patient selection and biopsies

Patients were selected from the GHEDI (Deciphering the Genetic Heterogeneity of Diffuse large B-cell lymphoma in the rituximab era) study program of the LYSA group, previously published and described (119). Patients were enrolled in previous trials summarized on table M1, with available frozen tumor samples, centralized histopathological review, adequate DNA/RNA quality and complete clinical information (120). COO molecular classification was obtained with HGU133+2.0 Affymetrix GeneChip arrays (Affymetrix), grouping patients into ABC, GCB and “unclassified”. We had access to 70 frozen samples from de novo DLBCL patients, as well as complete clinical and transcriptomic data of 202 patients. Patients included in each part of the study are listed on table M2.

For all the analyses patients who received R-CHOP 14, R-CHOP 21 and mini-R-CHOP were grouped as “R-CHOP”. Patients who received R-ACVBP+conso, ACVBP+ASCT and ACVBP were grouped as “R-ACVBP”.

Cohorts evaluated by EMSA					Cohorts evaluated by GEP							
Total evaluated (n=66)			R-CHOP (n=40)		R-CHOP/GCB/ABC (n=98)					R-ACVBP/GCB/ABC (n=63)		
GHE0001	GHE0534	GHE0909	GHE0050	GHE0837	GHE0050	GHE0436	GHE0810	GHE1035	GHE1560	GHE1385	GHE2127	GHE0776
GHE0024	GHE0535	GHE0988	GHE0061	GHE0852	GHE0057	GHE0440	GHE0811	GHE1036	GHE2005	GHE0393	GHE0001	GHE0777
GHE0050	GHE0562	GHE0992	GHE0150	GHE0856	GHE0061	GHE0446	GHE0822	GHE1066	GHE2019	GHE0395	GHE0602	GHE0834
GHE0061	GHE0602	GHE1017	GHE0242	GHE0860	GHE0134	GHE0454	GHE0837	GHE1069	GHE2023	GHE0659	GHE0877	GHE0908
GHE0150	GHE0604	GHE1023	GHE0258	GHE0865	GHE0150	GHE0459	GHE0849	GHE1071	GHE2026	GHE0717	GHE0714	GHE0909
GHE0218	GHE0622	GHE1028	GHE0275	GHE0885	GHE0197	GHE0469	GHE0852	GHE1209	GHE2030	GHE0024	GHE0721	GHE0982
GHE0242	GHE0629	GHE1034	GHE0300	GHE0988	GHE0206	GHE0470	GHE0855	GHE1222	GHE2036	GHE0028	GHE0015	GHE1018
GHE0258	GHE0659	GHE1035	GHE0397	GHE0992	GHE0236	GHE0495	GHE0856	GHE1225	GHE2096	GHE0515	GHE0025	GHE1023
GHE0275	GHE0685	GHE1192	GHE0400	GHE1028	GHE0241	GHE0522	GHE0857	GHE1229	GHE2106	GHE0534	GHE0202	GHE1046
GHE0300	GHE0690	GHE1349	GHE0414	GHE1034	GHE0242	GHE0562	GHE0860	GHE1353	GHE2121	GHE0535	GHE0216	GHE1053
GHE0393	GHE0701	GHE1353	GHE0415	GHE1035	GHE0258	GHE0563	GHE0865	GHE1369		GHE0629	GHE0218	GHE1311
GHE0395	GHE0705	GHE1379	GHE0436	GHE1353	GHE0262	GHE0685	GHE0885	GHE1373		GHE0842	GHE0219	GHE1352
GHE0397	GHE0771	GHE1380	GHE0454	GHE1380	GHE0275	GHE0689	GHE0923	GHE1380		GHE1017	GHE0292	GHE1379
GHE0400	GHE0807	GHE1385	GHE0459	GHE1437	GHE0300	GHE0690	GHE0925	GHE1413		GHE1099	GHE0294	GHE1392
GHE0414	GHE0837	GHE1392	GHE0470	GHE1440	GHE0397	GHE0701	GHE0955	GHE1423		GHE1192	GHE0358	GHE1393
GHE0415	GHE0842	GHE1393	GHE0495	GHE1536	GHE0400	GHE0705	GHE0984	GHE1424		GHE1302	GHE0371	GHE1450
GHE0436	GHE0852	GHE1437	GHE0562	GHE1553	GHE0414	GHE0733	GHE0986	GHE1437		GHE1349	GHE0506	GHE1537
GHE0454	GHE0856	GHE1440	GHE0685	GHE1558	GHE0415	GHE0743	GHE0988	GHE1438		GHE1360	GHE0519	GHE1554
GHE0459	GHE0860	GHE1536	GHE0690		GHE0423	GHE0745	GHE0992	GHE1440		GHE2012	GHE0604	GHE2003
GHE0470	GHE0865	GHE1537	GHE0701		GHE0427	GHE0807	GHE0993	GHE1498		GHE2017	GHE0615	
GHE0495	GHE0877	GHE1553	GHE0705		GHE0429	GHE0808	GHE1028	GHE1553		GHE2037	GHE0622	
GHE0519	GHE0885	GHE1558	GHE0807		GHE0430	GHE0809	GHE1034	GHE1558		GHE2119	GHE0771	

Table M1: Patients from GHEDI cohort included in the study. Patients' reference numbers are listed in the table accordingly to the part of the study where they were included.

Trial name Clinicaltrial.gov	Number of patients	Condition (age, aalPI)	Treatment arms	Reference
LNH03-1B NCT00140660	223	<66 years aalPI = 0	ACVBP +/- R	<i>Ketterer N et al Ann Oncol. 2013;24:1032-7.</i>
LNH03-2B NCT00140595	379	18-59 years aalPI = 1	R-CHOP vs R-ACVBP	<i>Récher C et al Lancet. 2011;378:1858-67.</i>
LNH03-3B NCT00144807	210	18-59 years aalPI = 2, 3	R-ACVBP + ASCT	<i>Fitoussi O et al Haematologica. 2011;96:1136-43.</i>
LNH03-6B NCT00144755	602	60-80 years aalPI >= 1	R-CHOP 14 vs R-CHOP 21	<i>Delarue R et al, Lancet Oncol. 2013;14:525-33.</i>
LNH03-7B NCT01087424	150	>=80 years	R-miniCHOP	<i>Peyrade F et al, Lancet Oncol. 2011;12:460-8.</i>
LNH01-5B NCT00135499	<i>Expected 150</i>	60-65 years	R-CHOP vs R-ACVBP	<i>Premature closure</i>

Table M2: Clinical trials used to compose the GHEDI cohort. The GHEDI cohort included selected patients from the trials listed in the table. aalPI: age adjusted International Prognostic Index. ACVBP: doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone, CHOP: cyclophosphamide, doxorubicin, vincristine, and prednisone, R: rituximab, ASCT: autologous stem cell transplantation. From Jais et al. *Hematologica* 2017.

M.2 - Human DLBCL cell lines

DLBCL cell lines were obtained from José Angel Martinez-Climent (Pamplona, Spain). Cells were grown in RPMI-1640 medium (Gibco glutamax®) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 100 U/mL penicillin, and 100mg/mL streptomycin (Invitrogen). Cells are non-adherent, thus incubated in suspension, in a 37° C humid atmosphere. Periodic counting for viability was realized with aid of trypan blue (Thermo Scientific) and a Malassez cell.

M.3 - Protein extraction

Cell lysates of both cell lines and frozen samples were obtained in High Salt buffer (NaCl 0,4 M, Hepes 25 mM pH 7,7, MgCl₂ 1,5 mM, EDTA 0,2 mM, NP4O 1%), along protease and phosphatase inhibitors (glycerol phosphate 20 mM, Na₃VO₄ 0,2 mM, PNPP 10 mM, DTT 2mM, PMSF 0,1 M and a Boehringer inhibitor cocktail).

The process takes place at 4°C (on ice) and lasts 20 minutes for cell lines and 30 for patient samples. Afterwards the extracts obtained (total protein extracts) are centrifuged during 10 minutes at 13000 rpm, also at 4°C. Proteins are dosed using the Bio-Rad DC protein assay kit (Bio-Rad).

M.4 Electrophoretic mobility shift assays for NF-κB

NF-κB activation was analyzed by electrophoretic mobility shift assay (EMSA) using the human immunodeficiency virus long terminal repeat tandem

κB oligonucleotide as κB probe as previously described (24). The oligonucleotidic probe used (5'-ACAAGGGACTTTCCGTCGGGGACTTTCCAAGG-3') is marked with [gamma-32P]-ATP (100μCi) by a Poly-nucleotide Kinase (Roche) for 1 hour at 37°C. After 3-minute centrifugation at 7000 rpm with the Quick Spin Column (Roche), eliminating non-specific radioactivity, the probe is purified. Incubation of 10 μg of total protein extract takes place in room temperature with DTT 0,058 mol/L, PolyIdC 1,08 μg/μL (Roche 5 μg/μL), TB 10X EMSA buffer and the radioactive probe 14,5 ng/μL, for 40 minutes. For the supershifts, an additional step of 30 min incubation with 2 μl of antibody (Santa Cruz – RelA C-20X; RelB C-19X; c-Rel (C)X; p50 H-119X; p52 K-27X) at 4°C previous to incubation with the mix is required. Water is added to make a total volume of 17 μL for each sample. Non-denaturizing deposit blue (2μL) is added and the extracts migrate in a polyacrylamide 5% gel for 1 hour and 50 minutes at 170V in TBE 0,25X buffer. Afterwards it is dried over a Whatman paper at 80° C for 45 minutes and exposed to an autoradiographic film overnight, conserved at -80°C.

Cases were analyzed and classified independently by 3 different researchers. A consensus was achieved for the discordant cases.

M.5 - Immunoblotting

Immunoblotting were performed as previously described (24). Protein extracts (20 μg) are denaturized at 95°C for 4 minutes together with the migration blue. They migrate through a 5% concentration gel, followed by a 7,5 to 12% separation gel (depending on size of proteins analyzed), in a TG-SDS 1X buffer at 70V for 1h30. The proteins are transferred to a nitrocellulose membrane for 1

hour at 100V in cold transfer buffer (1X alcohol 100%, 1X transfer buffer). After saturated for 1 hour in TBS 1X-Tween®20 1% plus 5% milk the membrane is incubated overnight with primary antibodies diluted in TBS 1X-Tween®20 1% plus 5% bovine serum albumin (BSA) (see table M3 for details). Following, it is washed in TBS-T and incubated during 1 hour with the secondary antibodies and revealed using Pierce™ ECL (Thermo Scientific), Pierce™ ECL plus (Thermo Scientific) or ECL select™ (GE healthcare), with aid of Amersham Imager™ 600 (GE Healthcare).

Antibody	Company	Reference	species	Dilution
cIAP2	Cell Signaling	58C7	Rabbit	1/1000
RelB	Santa Cruz	D4	Mouse	1/1000
γH2AX	Cell Signaling	20E 3	Rabbit	1/2000-2500
Cleaved caspase 3	Cell Signaling	5A1E	Rabbit	1/1000
Bcl-2	Santa Cruz	N19	Mouse	1/1000
Bcl-xL	Cell Signaling	54H6	Rabbit	1/1000
β-actin	Sigma	A5316	Mouse	1/1000
ATM	Santa Cruz	G12	Mouse	1/1000
p-ATM (s1981)	Cell Signaling	D25E5	Rabbit	1/1000
RelA	Santa Cruz	C20	Goat	1/1000

Table M3: Antibodies used for immunoblotting. Antibodies, as well as their references, and dilutions used for performing immunoblots are listed in the table.

M.6 - Lentiviral production and transduction

Production of infectious recombinant lentiviruses was performed by transient transfection of 293T cells as previously described (121). Two lentiviral vector constructions were used: one expressing the shRNA directed against RelB and other against Luciferase, used as a control vector (pTRIPDU3-EF1a-GFP and pTRIPDU3-MND-GFP). The vectors contain:

- HIV 5' LTR of HIV and deleted (DU3) HIV 3' LTR
- SV40 origin of replication
- RNA polymerase III promoter H1 controls the expression of the cloned shRNAs
- Reporter gene Green Fluorescent Protein (GFP) under control of promoter EF1a or MND

Integration of vectors occurs in low copy numbers (1-10 copies) with no replication.

For infections, cells were incubated overnight with recombinant lentiviruses (3×10^5 cells/500 μ l culture medium in each condition). An equal amount of fresh culture medium was added 24 hours later (500 μ l). Cells were washed and seeded in fresh culture medium (usual culture density) after 48h. GFP positive cells were sorted with FACS Aria™ sorter (Becton Dickinson). Only cells with a high expression of GFP, above 40000 of mean fluorescence intensity (MFI), were selected and then amplified. Cells were regularly counted in trypan blue for evaluation of viability.

M.7 - Annexin V binding assay

Cells were harvested and washed twice with cold PBS. Cells were resuspended in 1X binding buffer containing Annexin V-APC (BD Biosciences Pharmingen) and 4',6-diaminidino-2-phenylindole (DAPI, Molecular Probes) following the manufacturer's instructions. The samples were subjected to cytometric analysis with a MACSQuant cytometer (Miltenyi Biotec) and the data was statistically evaluated using the Flowjo v10.2 software.

M.8 - γ H2AX foci

Cells were incubated with or without doxorubicin (doses and times described further) then fixated in formalin 4%, permeabilized in SDS 1%, and then stained with an anti- γ H2AX antibody. Samples were incubated with secondary anti-body Alexa Fluor 647® and then analyzed using an ImageStream X Mark II Imaging Flow Cytometer. Data were acquired at a 60× magnification with EDF using the 642 nm laser at 150 mW and INSPIRE software. At least 8000 events of cells per sample were analyzed. Acquired data were analyzed using the IDEAS analysis software (v6.1; Merck-Millipore). Cells were gated for focused cells using the Gradient RMS feature. Cells were gated for single cells using the aspect ratio and area features. The spot counting analysis wizard was used.

M.9 – RNA extraction and RT-qPCR

Total RNA extraction and reverse transcription were performed as previously described (24). Real-time PCR analysis was carried out with LightCycler FastStart DNA Master plus SYBR Green I on a Light Cycler 1.5 (Roche Applied Science). All values were normalized to the level of HPRT mRNA. Primer sequences are listed in table M4.

Gene	Sequence
<i>BCL2</i>	5'-GTCTGGGAATCGATCTGG AA-3' 5'-GCAACGATCCCATCAATCTT-3'
<i>BCLXL</i>	5'-CCCGACCTGTGATACAA-3' 5'-ATCCAAAGCCAAGATAAGATT-3'
<i>CIAP2</i>	5'-GACTGGGCTTGTCTTGCT-3' 5'-AAGAAGTCGTTTTCTCCTTTGT-3'
<i>RELA</i>	5'-TTGAGCCCACAA AGCCTTATCAAGT-3' 5'-GGACAATGCCAGTGCCATACAG-3'
<i>TRAF2</i>	5'-GCATACCCGCCATCTTCTC-3' 5'-CGTTCAGGTAGATACGCAGACA3'
<i>XIAP</i>	5'-GCAAGAGCTCAAGGAGACCA-3' 5'-AAGGGTATTAGGATGGGAGTTCA-3'

Table M4: Primer sequences used for RT-qPCR.

M.10 - p53 functional status

p53 gene functional status is determined by the functional analysis of separated alleles in yeast (FASAY) method, as described before (122). Total RNA is extracted from samples (cell lines or tumor samples) and reverse transcribed using the Superscript II reverse transcriptase (Thermo Fisher Scientific). Then, p53 transcripts are amplified by polymerase chain reaction and co-transfected, with the GAP repair plasmid into yeast. The yeast growth is adenine independent if p53 is functional and colonies are white, but impaired on a adenine low medium if p53 is non functional, leading to small and red colonies. p53 status was considered mutated when: (a) >10% of the yeast colonies are red, (b) analysis using the split versions of the test could identify the defect in the 5' or 3' part of the gene, and (c) sequence analysis from mutant yeast colonies (Sanger) could identify an unambiguous genetic defect.

M.11 - Immunohistochemistry

Immunoperoxidase staining was centrally performed on an Ultra automated system (Roche Ventana, Tucson, AZ) using UltraVIEW detection Original kits and optimized protocols for BCL-2, and MYC staining as previously described (77) In the absence of an internal positive control, immunostains were considered non-evaluable. The tissue core with the highest percentage of tumor cell staining was considered for analysis. The thresholds employed were 40% for MYC and 50% or 70% for BCL2 as previously reported (123,124).

M.12 - Statistical analysis

M.12.1 – Cell death

Statistical significance was assessed using unpaired t tests (Prism 5.0c, GraphPad Software). A value of $p=0.05$ was considered as statistically significant with the following degrees: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

M.12.2 – Patients

Clinical analysis

Statistical analyses were performed using the software Statistical Package for the Social Sciences (SPSS, version 24), including descriptive statistics and statistical tests (chi-square for categorical variables or Fisher's exact test when Chi-square was not appropriate; T test for comparison of means; Kaplan-Meier method for evaluating prognostic impact of predictors; Cox proportional hazard model for survival-time outcomes on one or more predictors). Survival curves were created for overall survival based on Kaplan-Meier method, according to clinical data obtained from GHEDI cohort, previously described elsewhere (119) Cox proportional hazard regression analyses for univariate and multivariate were

based on forward stepwise regression, with a cutoff for p-values ≤ 0.2 . Two-side *P*-values < 0.05 were considered statistically significant ($\alpha = 0.05$).

Transcriptomic analysis

Microarray experiments were performed on Affymetrix Human Genome HGU133plus2.0 GeneChips (a genome wide array with 54674 probe sets targeting 19418 transcripts). Gene expression levels were normalized using the GC-RMA algorithm and flags were computed using MAS5. Quality assessment of the chips has been performed with affyQCReport R package. MAS5 algorithm produces a flag “P” for “Present”, “M” for “Marginal” or “A” for “Absent” associated to each intensity measure. This flag is an estimation of the statistical difference between PM (Perfect Match) and MM (Mismatch). Three probe lists have been used for each comparison according to flagged measurement in the relevant chips. The “PP” list is made of probes only flagged as “Present” for all chips involved in the comparison. The “P50” list has been created filtering probes flagged as “Present” for at least half of the chips. The “All” list is made of all probes without any filter. Three groups of two biologically independent samples were compared. The group comparisons were done using Student’s t test. To estimate the false discovery rate we filtered the resulting p values at 5% and used the Benjamini and Hochberg (BH), Bonferroni (B) or without correction (SC). Cluster analysis was performed by hierarchical clustering using the Spearman correlation similarity measure and average linkage algorithm. Data were subsequently submitted to Ingenuity Pathway Analysis (IPA) to model relationships among genes and proteins and to construct putative pathways and relevant biological processes. The R project for Statistical Computing [<http://www.r-project.org/>] Ingenuity Pathways: [<http://www.ingenuity.com>]

RESULTS

Section I - Cell lines

1. RelB is frequently activated in DLBCL derived cell lines

We have tested 29 human DLBCL cell lines for their NF- κ B DNA binding status by EMSA combined with supershift. Results are summarized on table R1. Amongst the 25 cell lines that presented constitutive NF- κ B activity, ten (40%) presented a constitutive RelB DNA binding activity. RelA was almost always present (22/25; 88%), being the 2 remaining cell lines positive exclusively for cRel activity. Interestingly, RelB activity coexisted with cRel activity only when both were weaker than RelA. A strong RelB activity (as strong as or stronger than RelA) was never associated with cRel binding activity. Inversely, when cRel had strong activity (as strong as or stronger than RelA), RelB activity was never detected.

Cell line	COO classification	NF- κ B	
		Predominant subunit	RelB activity
380	GCB	RelB	Positive
DB	GCB	cRel	Negative
K231	GCB	RelB	Positive
K422	GCB	RelA	Negative
OCILY7	GCB	cRel	Negative
OCILY8	GCB	RelA	Positive
OCILY19	GCB	RelA	Positive
OZ	GCB	cRel	Positive
PFEIFFER	GCB	cRel	Negative
RL	GCB	RelA	Negative
SC-1	GCB	RelA	Negative
SUDHL6	GCB	RelA	Positive
VAL	GCB	cRel	Negative
WSUDLCL2	GCB	RelA	Negative
WSUNHL	GCB	RelA	Negative
HBL1	ABC	RelA	Negative
HLY1	ABC	RelA	Positive
MD901	ABC	RelB	Positive
RIVA	ABC	cRel	Negative
U2932	ABC	cRel	Negative
FARAGE	Unclassified	cRel	Negative
HT	Unclassified	RelA	Negative
NUDUL1	Unclassified	RelA	Positive
SUDHL7	Unclassified	RelA	Negative
TOLEDO	Unclassified	RelA	Positive

Table R1: Characterization of human derived DLBCL cell lines. EMSA with supershifts were performed using total protein extracts. Cell lines are distributed by the COO classification and were classified according to presence or absence of DNA binding activity of RelB, regardless of intensity, and according to the predominant active subunit. RelB and cRel activity were considered predominant when intensity was equal or superior to RelA. RelA activity was considered predominant when superior to the others in intensity. Cell lines presenting RelB activation are highlighted in grey.

2. RelB protects ABC and GCB DLBCL derived cell lines against doxorubicin-induced DNA damage and apoptosis

We chose four of the RelB-positive DLBCL cell lines (1 ABC: MD-901, and 3 GCB: Oci-Ly19, Oci-Ly8 and K231), which presented constitutive RelB activity equal or superior to RelA in intensity, and little or no cRel activity (Figure R1).

We developed a stable RelB knockdown approach by stable RNA interference in these four cell lines using either a lentivirus carrying an shRNA targeting RelB or a scrambled control. As shown in figure R2, RelB protein levels were efficiently and significantly decreased in the ABC MD901 and the three GCB K231, Ocily8, and Ocily19 cells. Importantly, RelB knockdown also resulted in a marked decrease in constitutive RelB binding activity without affecting RelA binding activity.

Unlike the team previously reported in multiple myeloma cells (25), we did not observe significant spontaneous cell death following RelB expression inhibition. A similar phenotype, with no spontaneous death upon inhibition of RelB expression has been observed before in a colon cancer model (125). This finding probably reflects more complex cell death regulatory mechanisms.

To go one step further, we have then evaluated the impact of RelB in DLBCL cell survival upon doxorubicin treatment, the main genotoxic drug for DLBCL first line therapy (75).

Since RelB activation classically requires longer times (102), we have established a time of doxorubicin exposure superior to 6 hours but no longer than 24 hours to avoid triggering of compensatory mechanisms or other pathways that might have added up to complexity of the interpretation of our results. We have

chosen the dose of 2 μ M for an overnight experiment of 16h for MD901 and we have adapted time and dose for the other cell lines. The more resistant Ocily8 had a longer time of exposure (24 hours) and more sensitive Ocily19 had a shorter time (8 hours) and smaller dose (1 μ M).

Doxorubicin promotes RelB DNA binding after 6 hours in all four cell lines tested, even though in different intensities (Figure R3). Strong RelA activation is also observed in all four cell lines, with high intensity since early times. Importantly, we did not observe any differences in RelA DNA binding activity between controls and knockdowns.

Doxorubicin-induced apoptosis, evaluated by annexin V/DAPI staining, was markedly increased upon knockdown of RelB expression in all 4 DLBCL cell lines (Figure R4A). This effect was associated with greater cleavage of caspase 3 (Figure R4B). These findings indicate that RelB protects DLBCL cells from genotoxic stress-induced caspase-dependent apoptosis.

Focal phosphorylation of histone H2AX on serine 139 (γ H2AX) is one of the earliest events found on either endogenous or exogenous DNA double strand breaks. The foci have a 1:1 relation with double strand breaks, making γ H2AX an accurate and sensitive method to estimate DNA damage (126). Remarkably, we have observed by immunoblotting that knocking down RelB led to increased γ H2AX expression in response to doxorubicin (Figure R4C). Similarly, γ H2AX immunofluorescence staining revealed a greater number of foci upon RelB expression inhibition, after doxorubicin exposure (Figure R4D).

Considering p53 major role in apoptosis (127) and its important crosstalk with NF- κ B (128), it seemed critical to evaluate the presence of *TP53* mutations

in the 4 DLBCL cell lines (Table R2). Three out of the four cell lines tested present mutations, with FASAY assays showing >90% of function loss. Interestingly, two of them presented the same rare mutation. The mutation found in Ocily8 cell line was concordant with previous reports (129). Not surprisingly, the one cell line bearing a wild type (WT) *TP53*, Ocily19, was the one overall more sensitive to doxorubicin-induced apoptosis. In spite of it, the RelB-dependent effect on doxorubicin-induced apoptosis and DNA damage accumulation was comparable to the other cell lines.

Collectively, our data indicate that RelB protects DLBCL cells from doxorubicin-induced apoptosis and DNA damage accumulation. It is likely that it occurs in a p53-independent manner.

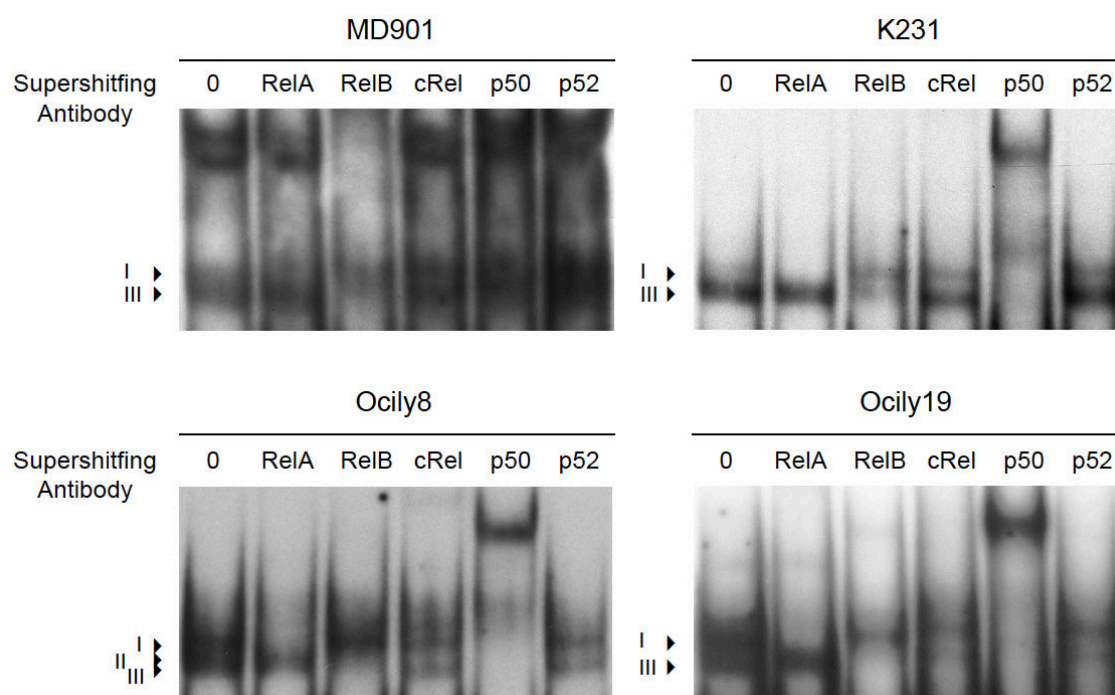


Figure R1: NF-κB subunits activation status in chosen DLBCL cell lines.

Four cell lines with strong RelB DNA binding activity were chosen after

characterization of NF- κ B activation by EMSA with supershifts. MD901, an ABC, and the 3 GCB cell lines K231, Ocily8 and Ocily19. Dash heads I, II and II indicate RelA- a cRel- and a RelB-containing complexes, respectively.

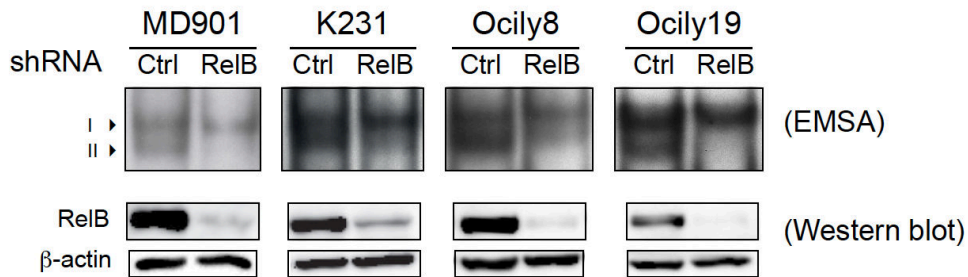


Figure R2: Verification of efficient knockdown of RelB in DLBCL cell lines.

Efficient inhibition of RelB DNA binding activity (upper) and protein expression (lower) by RNAi upon stable lentiviral transduction of a shRNA directed against RelB vs a shRNA control in 4 DLBCL cell lines. Total protein extracts were analyzed by Western blot (lower) and EMSA (upper), as indicated on the right. The shRNA is indicated above. Ctrl, shRNA control. Dash heads I and II indicate RelA- and RelB-containing complexes, respectively.

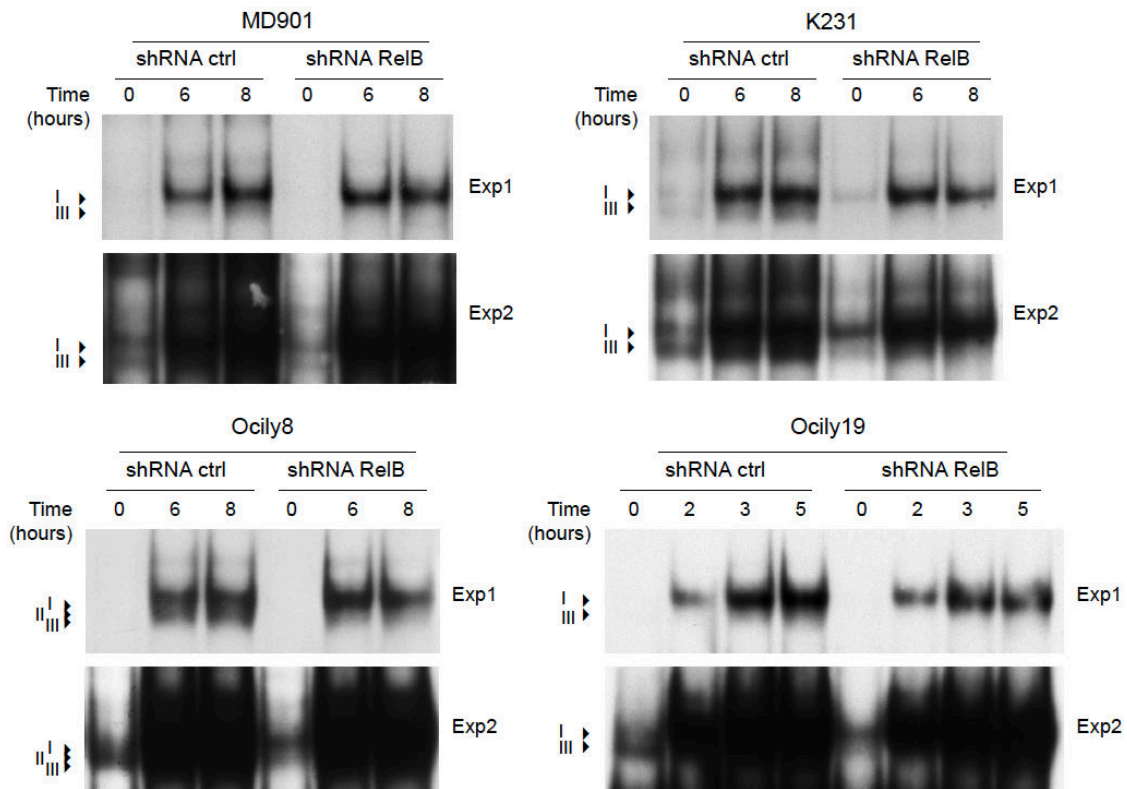


Figure R3: Effect of doxorubicin on NF-κB DNA binding in DLBCL cell lines.

EMSAs were performed with total protein extracts obtained from cell lines incubated with doxorubicin at times indicated above, at the concentration of 1μM for Ocily19 and 2μM for the remaining cell lines. Due to the intensity of NF-κB DNA binding induced by doxorubicin, two different times of exposition of the same gel are presented for each cell line. Dash heads I, II and III indicate RelA- a cRel- and a RelB-containing complexes, respectively.

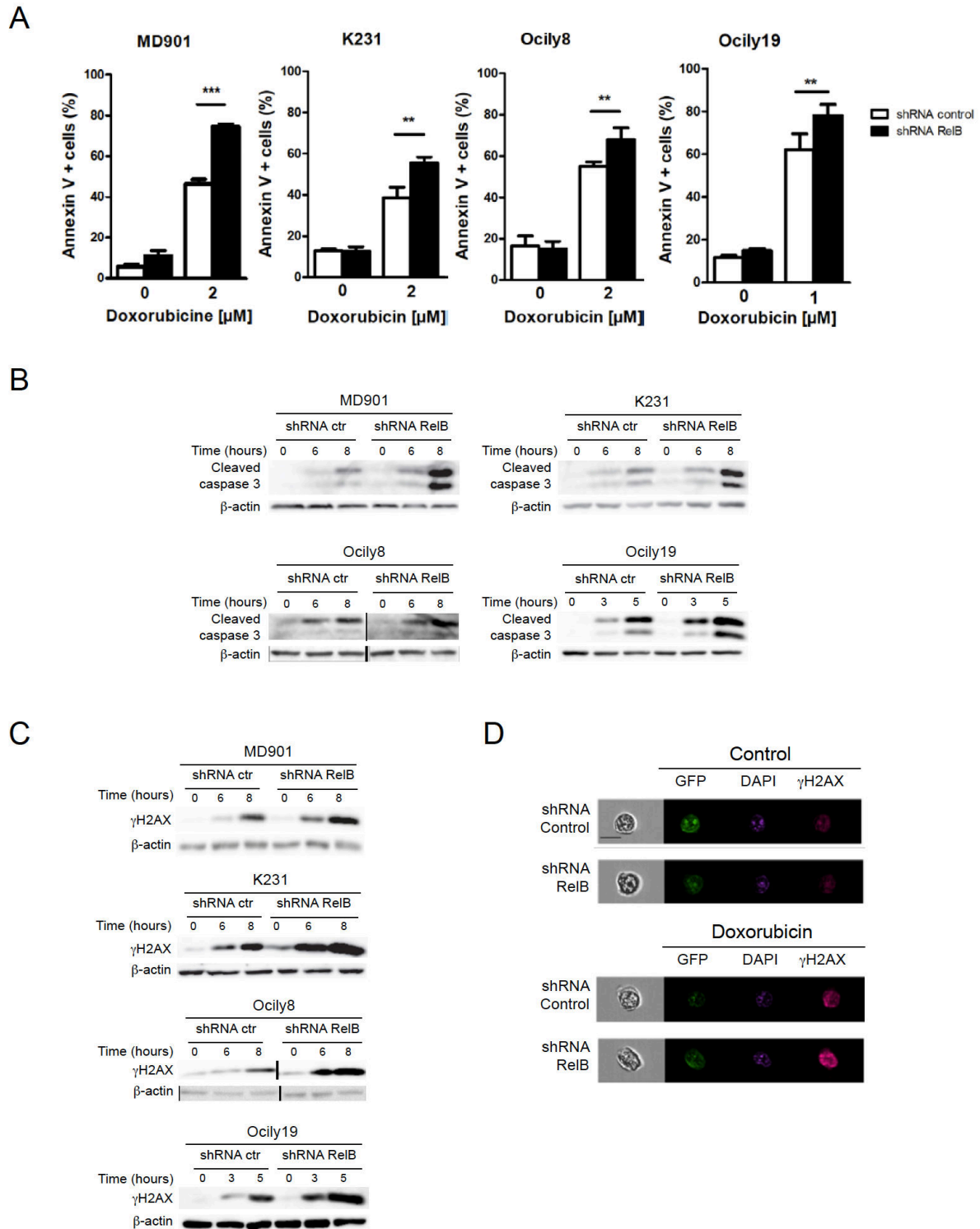


Figure R4: RelB protects DLBCL cell lines against caspase 3 dependent apoptosis and DNA damage accumulation induced by doxorubicin. (A) RelB protected cell lines against doxorubicin-induced apoptosis in the GCB and ABC cell lines. Cells were incubated with doxorubicin in the indicated doses for 8

hours (Ocily19), 16 hours (MD901 and K231) or 24h (Ocily8). They were then stained and analyzed by flow cytometry (see “Materials and Methods”) (MD901 n=3; K231 n=4; Ocily8 n=5; Ocily19 n=5). RelB activity was associated with reduced caspase 3 cleavage **(B)** and increased γ H2AX expression **(C)**, representing DNA damage accumulation in response to doxorubicin. Total protein extracts obtained from cells incubated with doxorubicin 2 μ M for MD901, K231 and Ocily8, and 1 μ M for Ocily19, for indicated times were analyzed by western blot. Antibodies are indicated on the left. **(D)** Inhibition of RelB expression resulted in greater accumulation of γ H2AX foci in MD901 cells. Cells were incubated with or without doxorubicin 2 μ M for 6 hours then fixated, stained and analyzed as described in “Materials and Methods”.

Cell line	p53 status	FASAY assay	Mutation
MD901	mutated	non functional	c.527G>t, p.C176F
K231	mutated	non functional	c.527G>t, p.C176F
Ocily8	mutated	non functional	c.845G>C, p.Arg282Pro
Ocily19	WT	inaltered	x

Table R2: Characterization of TP53 in DLBCL cell lines. FASAY assays were performed with RNA extracted from four DLBCL cell lines. Results and the mutations found are listed in the table.

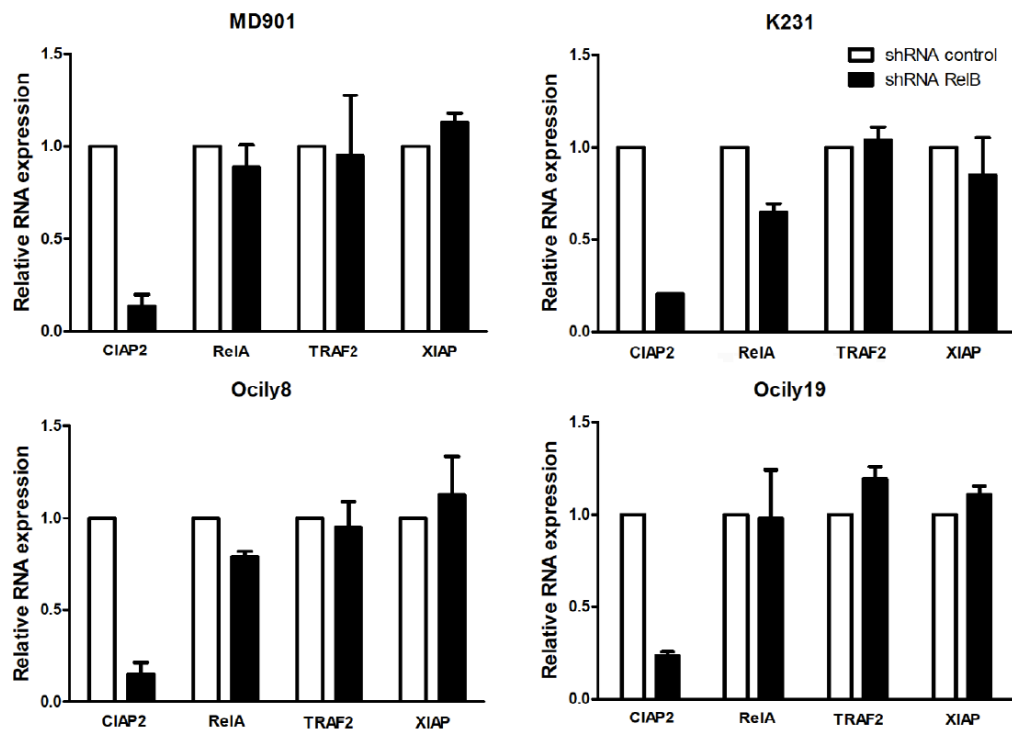
3. RelB constitutive activation is associated with up-regulation of cIAP2 in DLBCL cells

To better understand the protective effect of RelB, we have determined the mRNA expression levels of major anti-apoptotic genes such as Bcl-2, Bcl-xL, XIAP, TRAF2 and cIAP2 (Figure R5A). Cellular inhibitor of apoptosis protein 2

(cIAP2) mRNA expression levels are remarkably decreased upon inhibition of RelB expression while expression of other anti-apoptotics remained unaffected. cIAP2 protein expression was also confirmed to be under the control of RelB activity by immunoblotting (Figure R5B).

Protein expression of other main anti-apoptotics Bcl-2 and Bcl-xL was not affected neither by inhibition of RelB, nor by doxorubicin (Figure R5B).

A



B

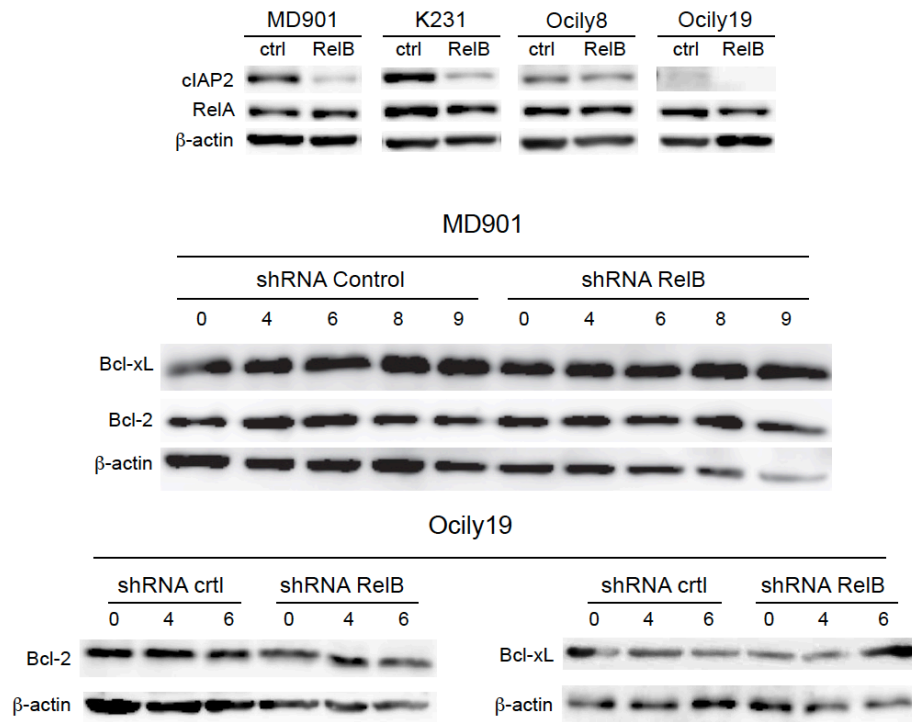


Figure R5: Effect of RelB on anti-apoptotic agents. (A) Knockdown of RelB results in decreased cIAP2 mRNA expression in all 4 DLBCL cell lines tested without affecting other classic anti-apoptotic agents. RNA was extracted from cells bearing either shRNA control or shRNA RelB and analyzed by RT-qPCR. Results are shown in expression relative to controls. **(B)** RelB-dependent effect was also confirmed in cIAP2 protein expression for all cell lines. Neither inhibition of RelB protein expression, nor doxorubicin affected Bcl-2 and Bcl-xL expression in MD901 and K231. Total protein extracts obtained from cells incubated without and with doxorubicin 2 μ M for indicated times were analyzed by western blot. Antibodies are indicated on the left.

Section II – Patients

1. RelB is frequently activated in both GCB and ABC DLBCL subtypes.

We have directly assessed by EMSA combined with supershift the activation status of classical (RelA and cRel) and alternative (RelB) NF- κ B subunits in 70 *de novo* DLBCL patients from the GHEDI cohort from the Lymphoma Study Association (LYSA) national cooperator group (see “Material and Methods”). Four cases were excluded in reason of technical issues related to protein extraction. The characteristics of the patients included in the study are summarized in table R3.

All cases were analyzed by 3 researchers independently, and then reanalyzed by the same 3 as a group until a consensus was achieved. We used two different approaches to classify the cases: the first was based on the relative intensity of each transcriptionally active NF- κ B subunit (RelA, RelB and cRel). Cases were considered RelA, RelB or cRel “strong” or “dominant” accordingly to the subunit that presented the strongest DNA-binding activity relative to the others. Cases were considered strong RelA when DNA binding activity of this subunit was stronger than observed for cRel and RelB. When RelB or cRel presented equal or stronger to RelA DNA binding activity they were considered as strong RelB or strong cRel. There were no cases presenting equal RelB and cRel intensity, stronger than RelA (discussed later). Each case can only present one “dominant” subunit.

The second classification used concerned RelB. Cases were considered “RelB positive” when they presented detectable RelB DNA binding activity,

regardless of intensity. They were “RelB negative” when this activity was not detected. The other subunits were not taken into account in this classification.

Constitutive NF- κ B activation was detected in all cases. RelB subunit presented a DNA binding activity in 44 cases (66.6%) (Figure R6) with no preference for ABC or GCB subtype (Table R3). RelA binding activity, regardless of the intensity was observed in all cases in combination with either cRel or RelB, and less often RelB together with cRel (19 cases – 43.1% of all RelB positive and 47.5% of all cRel positive cases). cRel was activated in 40 cases (60.6%). Interestingly, when RelB DNA binding activity was as strong or superior in comparison to RelA (henceforth called strong RelB), there was only very weak or no cRel activity (2/16). Inversely, a cRel strong DNA binding activity very rarely coexisted with significant RelB activity (3/13). This mutually exclusive effect was statistically significant ($p < 0.01$).

Stronger RelA activity compared to the other subunits tended to be preferentially present in ABC cases (30/37; 81%). Inversely, those with cRel presenting the strongest DNA binding activity, tended to be of GCB type (10/13; 76.9%). This association was statistically significant ($p = 0.001$ and $p = 0.002$, respectively).

Finally, antibody against p50 supershifted RelA, cRel and RelB complexes almost completely, thus indicating that the great majority of dimers contains p50. Antibody against p52 had very little effect on NF- κ B complexes.

Our findings indicate that RelB is frequently activated in DLBCL patients. Additionally, we have demonstrated NF- κ B frequent engagement in GCB DLBCL.

Characteristics	RelB EMSA - Total cases (n=66)			RelB Signature (n=202*)		
	Positive n(%)	Negative n(%)	p-value	Positive n(%)	Negative n(%)	p-value
Age > 60 years	23 (52.3)	9 (40.9)	0.392	51 (44.3)	55 (63.2)	0.008
Sex (F)	25 (56.8)	12 (54.5)	0.861	58 (50.4)	40 (46)	0.53
ECOG						
0	18 (40.9)	8 (36.4)	-	55 (47.8)	37 (42.5)	-
1	12 (27.3)	10 (45.5)	-	43 (37.4)	33 (37.9)	-
2	13 (29.5)	3 (13.6)	-	14 (12.2)	16 (18.4)	-
3	1 (2.3)	1 (4.5)	-	3 (2.6)	1 (1.1)	-
ECOG > 1 ¹	14 (31.8)	4 (18.2)	0.241	17 (14.8)	17 (19.5)	0.371
Stage						
I	3 (6.8)	2 (9.1)	-	14 (12.2)	9 (10.3)	-
II	7 (15.9)	6 (27.3)	-	22 (19.1)	13 (14.9)	-
III	4 (9.1)	5 (22.7)	-	22 (19.1)	12 (13.8)	-
IV	30 (68.2)	9 (40.9)	-	57 (49.6)	53 (60.9)	-
Stages III/IV ²	34 (77.3)	14 (63.6)	0.241	79 (68.7)	65 (74.7)	0.349
IPI score						
0	3 (6.8)	3 (13.6)	-	18 (15.7)	10 (11.5)	-
1	10 (22.7)	5 (22.7)	-	24 (20.9)	10 (11.5)	-
2	5 (11.4)	4 (18.2)	-	24 (20.9)	17 (19.5)	-
3	10 (22.7)	6 (27.3)	-	22 (19.1)	28 (28)	-
4	8 (18.2)	4 (18.2)	-	19 (16.5)	16 (18.4)	-
5	8 (18.2)	0 (0)	-	8 (7)	6 (6.9)	-
IPI scores 3-5 ³	26 (59.1)	10 (45.5)	0.294	49 (42.6)	50 (57.5)	0.036
Extranodal site > 1	20 (45.5)	5 (22.7)	0.073	40 (34.8)	27 (31)	0.575
LDH > ULN	29 (67.4)	15 (68.2)	0.952	67 (58.3)	57 (65.5)	0.294
COO classification						
GCB	15 (34.1)	9 (40.9)	-	40 (34.8)	43 (49.4)	-
ABC	28 (63.6)	13 (59.1)	-	49 (42.6)	36 (41.4)	-
Unclassified	1 (2.3)	0 (0)	-	26 (22.6)	8 (9.2)	-
COO Classification ABC ⁴	28 (63.6)	13 (59.1)	0.634	40 (44.9)	43 (54.4)	0.22
Treatment						
R-CHOP 14	9 (20.5)	4 (18.2)	-	9 (7.8)	5 (5.7)	-
R-CHOP 21	15 (34.1)	7 (31.8)	-	4 (3.5)	4 (4.6)	-
Mini-R-CHOP 21	3 (6.8)	2 (9.1)	-	42 (36.5)	22 (25.3)	-
R-ACVBP + Conso	14 (31.8)	5 (22.7)	-	18 (15.7)	15 (17.2)	-
R-ACVBP + ASCT	1 (2.3)	2 (9.1)	-	37 (32.2)	33 (37.9)	-
ACVBP	2 (4.5)	2 (9.1)	-	5 (4.3)	8 (9.2)	-
Treatment R-CHOP ⁵	27 (61.4)	13 (59.1)	0.859	60 (52.2)	56 (64.4)	0.083
Total	44 (100)	22 (100)	-	115 (100)	87 (100)	-

Table R3: Characterization of the patients from the GHEDI cohort. For analysis, patients who received R-CHOP 14, R-CHOP 21 and mini-R-CHOP were grouped in “R-CHOP”. Patients who received R-ACVBP+conso, ACVBP+ASCT and ACVBP were grouped in “R-ACVBP”. ECOG, Eastern Cooperative Oncology Group performance status; IPI, international prognostic index; LDH, lactate dehydrogenase; ULN, upper limit of normal; COO, cell of origin; GCB, germinal center B-cell; ABC, activated B-cell; R, rituximab; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; ACVBP,

doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone; Conso, consolidation; ASCT, autologous stem cell transplantation.

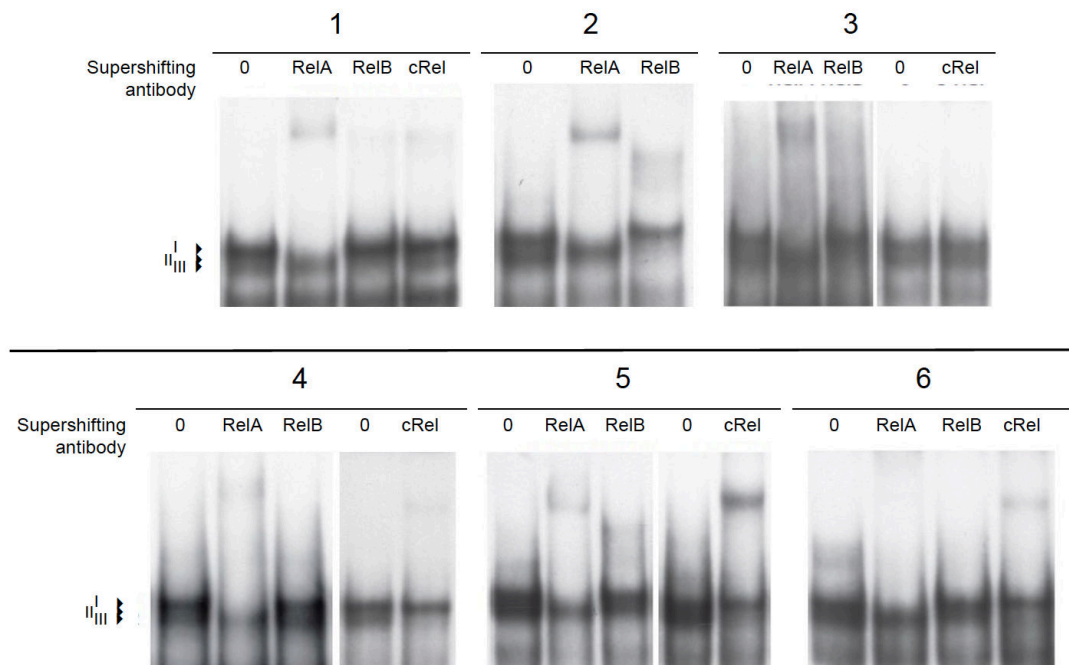


Figure R6: Analysis of the DNA-binding activity of NF- κ B in a cohort of newly diagnosed DLBCL patients. Examples of typical EMSA profiles. Upper cases (1,2 and 3) present RelB constitutive activation. Lower cases (4,5 and 6), do not show RelB DNA binding activity. “Dominant” RelB activity can be seen in 2 and 3; “dominant” cRel, in cases 5 and 6; cases 1 and 4 are examples of RelA “dominant” activity (see text). Total protein extracts from 70 *de novo* DLBCL frozen samples were analyzed by EMSA combined with supershift. Dash heads I, II and II indicate RelA- a cRel- and a RelB-containing complexes, respectively.

2. RelB constitutive activation is not associated with NF- κ B-related mutations or current gene expression signature in DLBCL

RelB activation did not correlate with any of the mutations commonly associated with NF- κ B in DLBCL (e.g. *MYD88*, *CARD11*, *CD79A/B*, *TNFAIP3*) (92,130), or any other commonly DLBCL-associated mutations which were evaluated (Table R4).

Next, we have evaluated in our training cohort of 66 DLBCL patients whether RelB activation is linked to the published NF- κ B transcriptional signature composed of six genes (54). Remarkably, RelB activation status was not associated to the expression of any of these genes, indicating that this NF- κ B signature does not reflect the status of RelB activation. As described, this so-called NF- κ B signature marked preferentially the ABC DLBCL with an overexpression of four out of six genes ($p < 0.05$). When comparing patients with strong RelA vs cRel activity, four genes showed significant overexpression in the RelA group ($p < 0.05$). This finding is in line with RelA activation correlating with the ABC subtype (Table R5).

Altogether, these observations indicate that the current tools to evaluate NF- κ B activity only apply to the classical NF- κ B pathway and do not provide valuable information on RelB activation status.

Gene	RelB (EMSA)		p-value
	Positive (43)(%)	Negative (19)(%)	
<i>TNFAIP3</i>	7 (16.3)	0 (0)	0.089
<i>MYD88</i>	7 (16.3)	4 (21.1%)	0.724
<i>CARD11</i>	6 (14)	4 (21.1)	0.479
<i>CD79A</i>	0 (0)	0 (0)	–
<i>CD79B</i>	5 (11.6)	5 (26.3)	0.259
<i>BCL2</i>	4 (9.3)	3 (15.8)	0.665
<i>PIM1</i>	9.7 (16.3)	7 (4.3)	0.102
<i>TNFRSF14</i>	2 (4.7)	2 (10.5)	0.58
<i>KMT2D</i>	19 (44.2)	9 (47.4)	0.816
<i>B2M</i>	8 (18.6)	2 (10.5)	0.71
<i>TP53</i>	7 (16.3)	2 (10.5)	0.709
<i>PRDM1</i>	6 (14)	0 (0)	0.067
<i>EP300</i>	6 (14)	1 (5.3)	0.422
<i>IRF4</i>	5 (11.6)	1 (5.3)	0.657
<i>MEF2B</i>	5 (11.6)	4 (21.1)	0.437
<i>CREBBP</i>	5 (11.6)	5 (26.3)	0.259
<i>ITPKB</i>	4 (9.3)	2 (10.5)	1
<i>ID3</i>	4 (9.3)	1 (5.3)	1
<i>NOTCH1</i>	3 (7)	1 (5.3)	1
<i>CIITA</i>	3 (7)	3 (15.8)	0.359
<i>FOXO1</i>	3 (7)	1 (5.3)	1
<i>GNA13</i>	3 (7)	4 (21.1)	0.187
<i>MYC-A</i>	3 (7)	3 (15.8)	0.359
<i>NOTCH2</i>	2 (4.7)	0 (0)	1
<i>STAT6</i>	2 (4.7)	2 (10.5)	0.58
<i>SOC3</i>	2 (4.7)	2 (10.5)	0.58
<i>CD58</i>	2 (4.7)	2 (10.5)	1
<i>EZH2</i>	2 (4.7)	3 (15.8)	0.163
<i>TCF3</i>	1 (2.3)	0 (0)	1
<i>CDKN2B</i>	1 (2.3)	0 (0)	1
<i>XPO1</i>	1 (2.3)	0 (0)	1
<i>BRAF</i>	0 (0)	0 (0)	–
<i>CDKN2A</i>	0 (0)	0 (0)	–
<i>MFHAS1</i>	0 (0)	2 (10.5)	0.09

Table R4: DLBCL frequent mutations according to RelB status. EMSA assessed patients were tested for DLBCL frequent mutations and no association with RelB constitutive activation was found. p-values are shown in the right column.

Gene expression comparison			
	ABC vs GCB	sRelA vs scRel	RelB pos vs RelB neg
Bcl-2	p<0.001	p=0.76	p=0.27
Cyclin D2	p<0.001	p<0.001	p=0.93
CCR7	p=0.63	p=0.21	p=0.85
c-FLIP	p=0.027	p=0.01	p=0.50
IRF4	p=0.001	p=0.002	p=0.91
I κ B α	p=0.76	p=0.03	p=0.43

Table R5: NF- κ B DLBCL gene expression signature (Davis et al.) comparison by groups. Expression of the 6 genes whose overexpression was published to define a NF- κ B signature was compared in groups (ABC vs GCB; strong RelA vs strong cRel; RelB positive vs RelB negative). The table shows the p-values for statistical significance of differences encountered (significant p values are in bold). Four out of 6 showed significant overexpression in ABC compared to GCB; Four out of 6 showed significant overexpression in strong RelA compared to strong cRel; there was no association of the signature and RelB expression. sRelA, strong RelA; scRel, strong cRel; RelB pos, RelB positive; RelB neg, RelB negative.

3. Establishment of RelB-associated gene expression signature

Using 61 cases directly assessed for RelB DNA binding status, we established a gene expression signature of 140 genes associated with RelB activation (Figure R7). In order to use this RelB gene expression signature to predict RelB activation status, we have revalidated it in the cohort of patients tested by EMSA (n=61). The RelB-associated gene expression signature was able to predict the directly determined RelB activation status with 85% of

sensitivity, 100% of specificity and a positive predictive value of 100% (Table R6).



Figure R7: RelB-associated gene expression signature. Heat map resulting from hierarchical clustering of the 140 genes that exhibited an average 1.5-fold or higher change in RelB positive cases defined by EMSA.

		RelB activation by EMSA		
		Positive	Negative	Total
RelB activation by GEP	Positive	34	0	34
	Negative	6	21	27
	Total	40	21	61

* Sensitivity: 85%; Specificity: 100%; PPV: 100%; NPV: 77,8%; Accuracy: 90,2%

Table R6: Definition of RelB activation status by EMSA vs gene expression profile. The RelB gene expression signature was projected on 61 EMSA evaluated cases for prediction of RelB activation status. The gene expression

signature was highly concordant with EMSA presenting a specificity of 100% and sensitivity of 85%.

4. RelB activation has a negative impact on patient overall survival

In this series of 66 EMSA-tested cases of our test cohort, patients with high risk IPI (IPI=3-5) have a worse overall survival (OS) ($p<0.001$) (Figure R8), in line with classical DLBCL series. Interestingly, although GCB/ABC does not predict a different OS ($p=0.503$) (Figure R8), RelB activation was associated to a worse outcome ($p=0.037$) (Figure R8).

This observation was confirmed among patients treated with R-CHOP ($n=40$) ($p=0.034$) (Figure R9).

When we evaluated the impact of RelB signature among patients treated with R-CHOP ($n=98$), RelB did not predict worse OS ($p=0.129$), whereas GCB/ABC and IPI demonstrated a prognostic impact ($p=0.011$ and $p<0.001$, respectively) (Figure R10).

However, in multivariate analysis, RelB gene expression signature predicts a worse OS ($p=0.016$) in patients treated with R-CHOP, independently from the IPI ($p=0.003$) and GCB/ABC classification ($p=0.036$) (Table R6).

Considering the small size of the EMSA evaluated cohort, RelB activation defined by EMSA was close to predict a worse outcome, independently from IPI ($p=0.069$ and $p=0.031$, respectively) (Table R6).

We have performed the same evaluation for patients who received R-ACVBP. In univariate and multivariate analysis, COO classification and grouped IPI, but not RelB activation, had an impact on the overall survival (Table R6). Due

to small number of cases (n=26) and lack of sufficient events, we did not perform the analysis of R-ACVBP-treated patients in the cohort evaluated by EMSA.

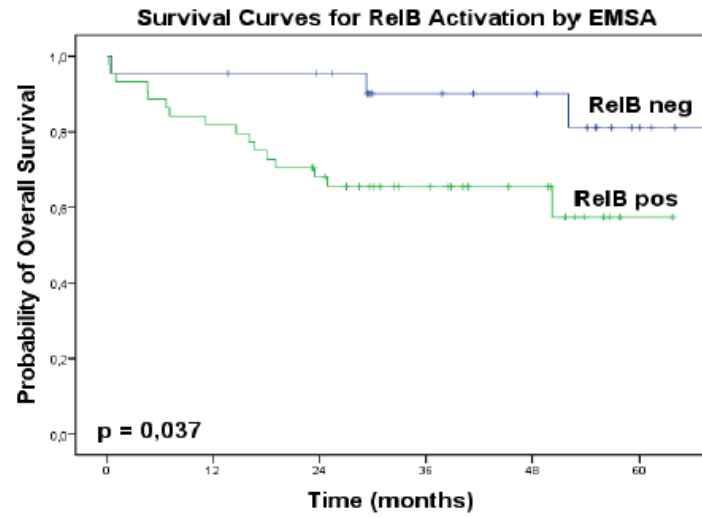
We did not observe any significant impact of RelB activation in the progression free survival (PFS) (data not shown).

Additionally, expression of prognostic markers BCL2 and MYC was assessed by IHC in 45 cases. There was no association between RelB activation and BCL2, MYC or double expression BCL2/MYC (Table R7). Of note, double expression of MYC and BCL2 by IHC did not affect the overall survival of patients (p=0.652) (data not shown).

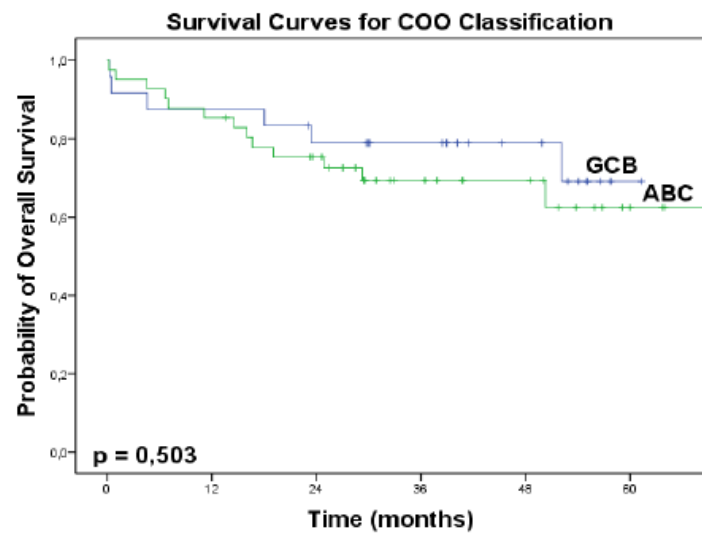
FISH assessment for translocation of *MYC* and *BCL2* was performed in 53 cases. RelB activation showed no association with *MYC*, *BCL2* translocations (p=1 and p=0.145, respectively). Only four cases were double hit *BCL2/MYC*, being statistically insufficient for analysis.

In conclusion, we have developed a gene expression signature that can predict accurately RelB activation status in DLBCL patients. Our study brings up RelB as a potential new independent marker of worse prognosis among patients with DLBCL.

A



B



C

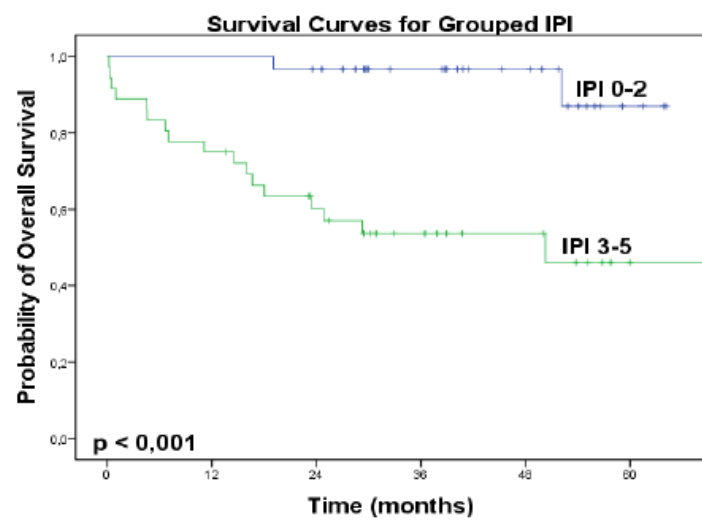
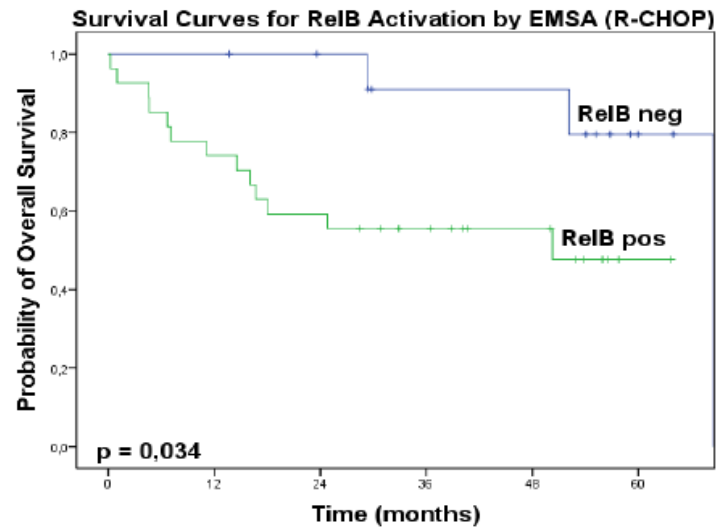
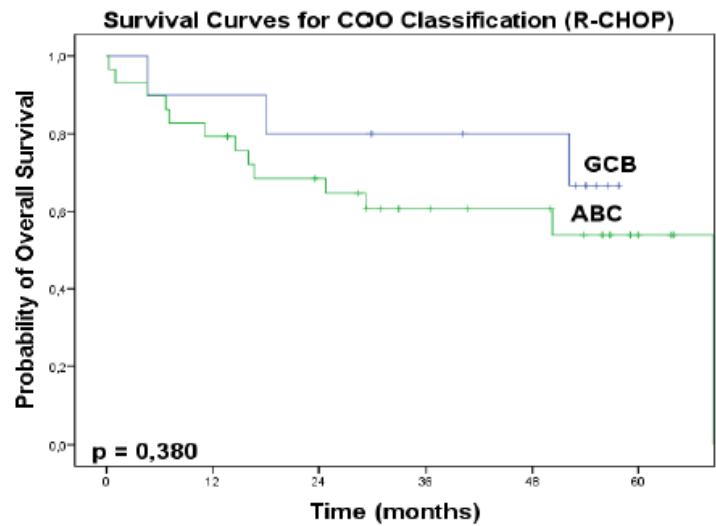


Figure R8: Prognostic impact of RelB activation defined by EMSA on the GHEDI cohort of DLBCL patients. (A) Kaplan-Meier overall survival curves for patients with RelB activation status defined by EMSA (n=66). The estimated probability of overall survival in 2 years is 75.4% (SE 0.054), and in 5 years 65.1% (SE 0.074). The estimated probability of overall survival for RelB negative and positive patients in 2 years is of 95.5% (SE 0.044) and 65.5% (SE 0.072), respectively; and for 5 years, 81.1% (SE 0.104) and 57.3% (SE 0.099), respectively. The mean survival for RelB negative and positive patients is 61.9 (CI 95%: 53.4-70.3) and 44.6 (CI 95%: 37-52.1) months, respectively. **(B)** Univariate analysis of overall survival according to COO classification in the same group (n=66). **(C)** Univariate analysis of overall survival according to grouped IPI in the same group (n=66).

A



B



C

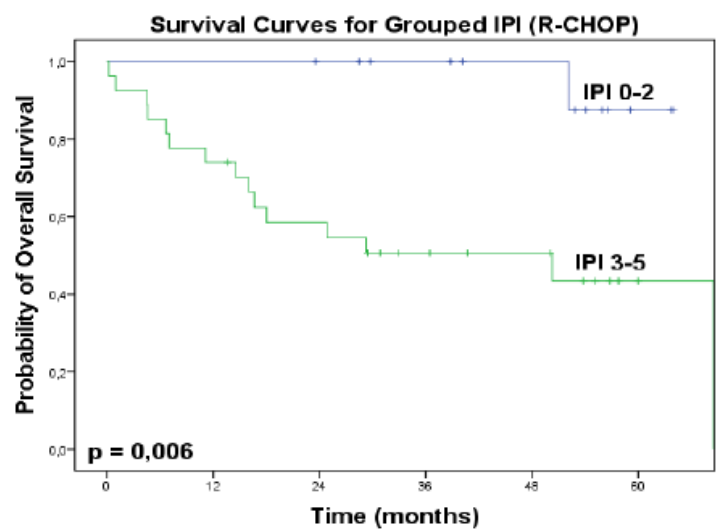
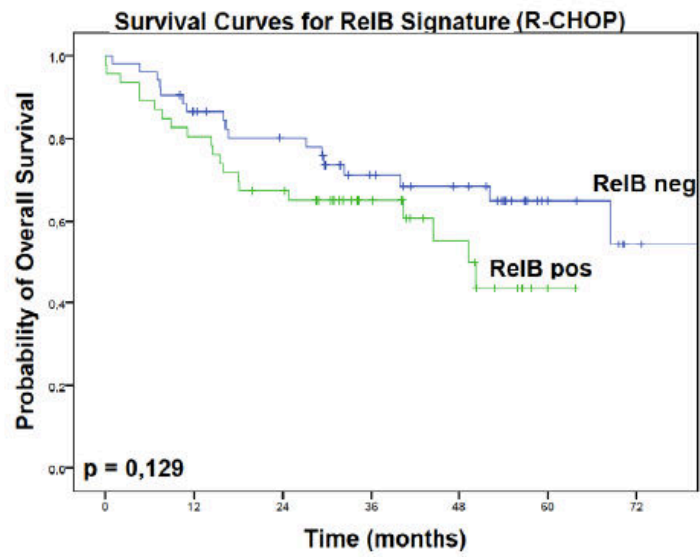
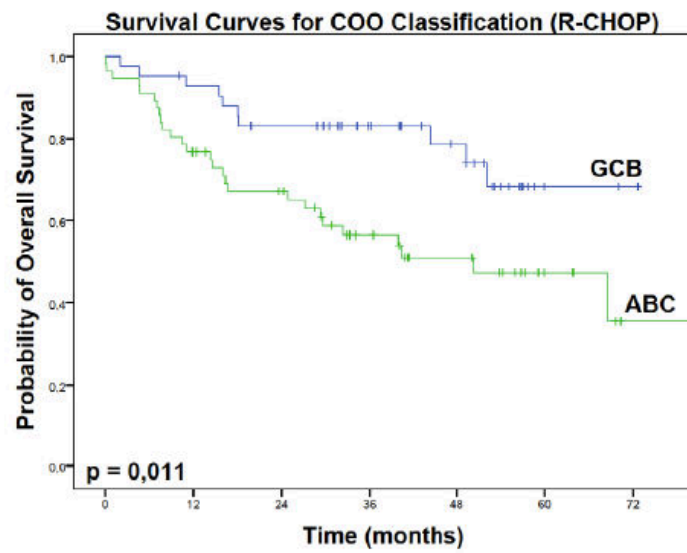


Figure R9: Prognostic impact of RelB activation defined by EMSA on R-CHOP treated DLBCL patients of the GHEDI cohort. (A) Kaplan-Meier overall survival curves for patients with RelB status defined by EMSA, including only R-CHOP treated patients (n=40). The estimated probability of overall survival in 2 years is 72.2% (SE 0.071), and in 5 years 57.8% (SE 0.088). The estimated probability of overall survival for RelB negative and positive patients in 2 years is of 100% and 59.3% (SE 0.095), respectively; and for 5 years, 79.5% (SE 0.131) and 47.6% (SE 0.110), respectively. The mean survival for RelB negative and positive patients is 63.1 (CI 95%: 54.3-71.9) and 39 (CI 95%: 29-49) months, respectively. **(B)** Univariate analysis of overall survival according to COO classification in the same group (n=40). **(C)** Univariate analysis of overall survival according to grouped IPI in the same group (n=40).

A



B



C

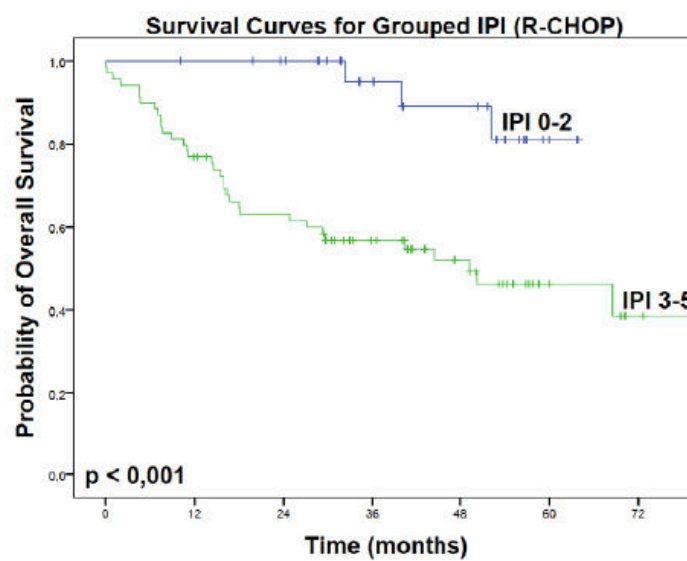


Figure R10: Prognostic impact of RelB activation defined by RelB gene expression signature on the GHEDI cohort of DLBCL patients. (A) Kaplan-Meier overall survival curves for patients with RelB status defined by RelB signature, including only R-CHOP treated patients (n=98). The estimated probability of overall survival in 2 years is 74% (SE 0.045), and in 5 years 56.6% (SE 0.059). The estimated probability of overall survival for RelB negative and positive patients in 2 years is of 80.1% (SE 0.056) and 67.4% (SE 0.069), respectively; and for 5 years, 65% (SE 0.073) and 43.5% (SE 0.102), respectively. The mean survival for RelB negative and positive patients is 59 (CI 95%: 50.4-67.6) and 41.4 (CI 95%: 34.1-48.7) months, respectively. **(B)** Univariate analysis of overall survival according to COO classification in the same group (n=98). **(C)** Univariate analysis of overall survival according to grouped IPI in the same group (n=98).

Tested Cohorts	Features	Prognostic variables		Multivariate Cox HR Regression		
				HR	95% CI (LL-UL)	p-value
Cohorts evaluated by EMSA	Total (n=66)	RelB EMSA	neg x pos	0.315	0.09-1.105	0.071
		Grouped IPI	0-2 x 3-5	0.105	0.024-0.459	0.003
	R-CHOP (n=40)	RelB EMSA	neg x pos	0.246	0.054-1.117	0.069
		Grouped IPI	0-2 x 3-5	0.106	0.014-0.814	0.031
Cohorts evaluated by GEP	R-CHOP/ GCB/ABC (n=98)	RelB GEP	neg x pos	0.435	0.221-0.858	0.016
		Grouped IPI	0-2 x 3-5	0.455	0.218-0.951	0.003
		COO classification	GCB x ABC	0.163	0.0490.541	0.036
	R-ACVBP/ GCB/ABC (n=70)	RelB GEP	neg x pos	0.848	0.194-3.703	0.826
		Grouped IPI	0-2 x 3-5	0.1	0.02-0.5	0.005
		COO classification	GCB x ABC	1.262	0.289-5.509	0.757

Table R6: Cox proportional hazard regression for multivariate analyses in cohorts with RelB status evaluated by EMSA and gene expression signature.

IHC	RelB (EMSA)		p-value
	Positive (30)(%)	Negative (15)(%)	
MYC ($\geq 40\%$)*	15 (50)	5 (35.7)	0.375
BCL2 ($\geq 50\%$)	25 (83.3)	12 (80)	0.542
BCL2 ($\geq 70\%$)	22 (73.3)	12 (80)	0.46
MYC/BCL2 ($\geq 50\%$)	14 (46.7)	3 (20)	0.082
MYC/BCL2 ($\geq 70\%$)	13 (43.3)	3 (20)	0.123

* One RelB negative case by EMSA was not interpretable by IHC for MYC

Table R7: Bcl-2 and c-Myc protein expression according to RelB activation status by EMSA. IHC evaluation was performed in tissue micro arrays, revealing no association between RelB activation verified by EMSA and expression of BCL2, MYC or double expression MYC/BCL2. Cut-off values used follow the literature (66).

DISCUSSION

We have analyzed for the first time a large panel of human DLBCL-derived cell lines for DNA-binding activity status of all NF- κ B subunits, establishing a frequent activation of RelB.

Previous studies involving DLBCL cell lines have failed to present convincing evidence of the importance of RelB in DLBCL, in part for difficulties in the detection of RelB constitutive activation. A portion of the studies uses p52 as a surrogate to establish the activation status of the alternative pathway by IHC (70,96,99,101). Nonetheless, this approach has two main setbacks: the first and more obvious one is that p52 is a product of partial processing of p100. As such, all antibodies directed to p52 will also detect p100, the main inhibitor of RelB. Thus, detection of “activation of the alternative pathway of NF- κ B” might actually be the detection of an inhibited complex. The second point to consider is that in our study, EMSA with supershifts using p52 antibody revealed that a very small proportion of the DNA-bound NF- κ B complexes is composed by this subunit. Restraining the evaluation to p52 exclusively might result in important underestimation of RelB activity.

Another issue is that authors frequently neglect verification of the actual constitutive RelB activation status prior to choosing cell lines (104,131). The significance of works concerning RelB in which cell lines have little, if any, RelB constitutive activation is debatable.

RelB controlled cIAP2 expression in DLBCL cell lines. Curiously, we have previously reported that cIAP2 is a direct target of RelB (25). Even though we have not demonstrated it in our cell lines, it is tempting to imagine it may be the

case. In spite of not being further explored, relative upregulation of BIRC3 (cIAP2) has been identified in DLBCL cell lines which presented greater doxorubicin resistance (129). Interestingly, in the same study, levels of BCL2 and BCLXL did not correlate with cell line sensitivity to doxorubicin either. In a different study, DLBCL cell lines that showed NF- κ B activation have been shown to be more resistant to doxorubicin (129).

IAPs inhibit apoptosis by preventing the cytochrome c induced cleavage of caspase 3 by caspase 9 (132). Nevertheless, apoptosis due to reduced IAP surveillance requires loss of more than one IAP (133,134), which may explain why our cell lines did not die spontaneously. It is possible that under stable basal conditions DLBCL cells can compensate for the lack of cIAP2 through other mechanisms. Yet, RelB-dependent regulation of cIAP2 might have been responsible at least in part for the protective effect of RelB against doxorubicin-induced apoptosis.

Of note, even though the knockdown through shRNA reduces RelB expression dramatically, the minimal residual activity might be sufficient to maintain a possible RelB dependent cell survival. True knockouts by CRISPR/Cas9 technique may help answer this question. However, off-target effects and triggering of compensatory mechanisms may present as setbacks of this method.

Genotoxic stress has been reported to cause cytokine-induced depletion of IAP proteins (135) and doxorubicin has been described to downregulate cIAP2 with correlated apoptosis (136). In this context, a previous downregulation of

clAP2 by the inhibition of RelB expression may enhance cell line susceptibility to this drug.

Inhibition of RelB expression resulted in increased doxorubicin-induced DNA damage accumulation. As evoked earlier, doxorubicin is responsible for single- and double-strain DNA breaks through several different mechanisms, initiating at least 5 different types of DNA repair response (137). Due to this complexity we were not able to further explore the influence of RelB activation on each specific response. Analyzing different types of stimuli that trigger single DNA-repair responses would be perhaps a more reasonable method for investigating whether RelB plays a role in DNA-repairing. However intriguing, this investigation seemed out of reach for us. Whether the increased DNA damage accumulation is a consequence of increased DNA aggression, impaired DNA repair or even both remains to be investigated. All the same, protection against this DNA damage accumulation is very likely a crucial contributor to RelB-dependent reduced sensitivity to doxorubicin.

Chromosomal instability has been pointed out as a bad prognosis marker in DLBCL (138). Ramachandiran et al. have found an inverse correlation between RelB nuclear localization and DNA damage in DLBCL cells from patient samples, regardless of their GCB/ABC status (104). Importantly, they have demonstrated that the effect observed was p53-independent, which is also in consonance with our own observations. Interestingly, Chapuy et al. have described a group of DLBCL with characteristics of cell cycle markers and chromosomal instability that is composed by both GCB and ABC patients (58). Further analysis of common traits shared by this group and our group of RelB activated patients might bring useful insight.

ROS production in response to doxorubicin has been studied as one of the responsible for its cardiotoxicity (139). More recently ROS homeostasis has gained more importance in anti-cancer therapy (140). Efficient methods for cellular ROS assessment involve the use of fluorochromes that are based on the structure of 2'-7' dichlorfluorescein (DCF) (141). Unfortunately, cell lines bearing the lentiviral vector are sorted based on GFP expression. In addition to it, doxorubicin has a very large spectrum of orange/red excitability/emission. Those two factors prevent the use of DCF fluorochromes, which fall in the same spectrum. On account of these technical difficulties, we were not able to investigate the effects of RelB on ROS production in response to doxorubicin.

We have demonstrated for the first time by direct DNA binding that RelB is frequently activated in DLBCL patients and that its constitutive activation is associated with worse outcome.

RelB gene expression signature predicted accurately RelB activation. Even though further validation in larger cohorts is needed, it appears as a promising more dependable alternative to IHC in the detection of RelB activation.

RelB was a stronger outcome predictor among patients treated with R-CHOP. However, as the population in the GHEDI study, although comprising patients included in trials, included patients with very different therapy regimens and IPI, it might be interesting to confirm our results in an independent cohort of patients with DLBCL and treated with R-CHOP. In our series, RelB activation status did not predict the OS in R-ACVBP-treated patients. It is interesting however to underline that R-ACVBP regimen contains a higher dose of doxorubicin (75mg/m^2) than R-CHOP (50mg/m^2) in the induction phase and includes a consolidation phase with different genotoxic agents. To better evaluate

the potential interaction between RelB activation and treatment arm (R-CHOP vs R-ACVBP), it might be interesting to design a FFPE RelB-related signature to evaluate in the patients included in the LNH03-2B, a randomized trial comparing R-ACVBP and R-CHOP, the prognostic value of RelB in both treatment arms, as we have too few patients of this trial with frozen tissue in our GHEDI series.

COO remains the main classification to stratify patients and “unclassified” patients are also a very heterogeneous group. The cohort assessed by EMSA, based on which RelB gene expression signature was established, had only one case unclassifiable as either GCB or ABC. Also, we did not analyze unclassified cell lines in our *in vitro* studies, for it would add in complexity in a poorly understood subject. For these reasons, we did not evaluate the prognostic impact of RelB gene expression signature in unclassified cases. Enrichment in “unclassified” samples in future studies by direct evaluation may help improve the signature’s prediction of RelB activation status also in these cases. RelB may become a useful tool for better risk stratification of this group of patients. Direct evaluation of NF- κ B subunits DNA-binding status in “unclassified” cases might also add in knowledge of the biology of the group.

Curiously, the COO classification did not predict prognosis in our EMSA evaluated cohort. A relatively small total number of cases, heterogeneity of patient populations and treatment arms, relatively smaller number of GCB cases and the fact that the cases had generally short survivals might have had an influence. Nevertheless, RelB activation predicted worse OS in this same group. This finding suggests RelB may be a good predictor even when the COO fails to stratify prognostic groups.

RelB constitutive activation did not show a preference for any of the COO groups. However, strong cRel activity almost excluded any RelB activity and was strongly associated to GCB status. Thus, RelB status is not influenced by the COO classification but RelB's relation with other NF- κ B subunits may indirectly interfere with its relation with the COO. In spite of it, RelB was still an independent prognosis predictor.

We have confirmed that constitutive NF- κ B activation is present in both GCB and ABC subtypes of DLBCL. NF- κ B activation in GCB cell lines has been reported (129), and it has been suggested in DLBCL patients by IHC and ELISA evaluation in previous studies (65,70,93–96). Most recently, genetic studies by new methodology have also been broadening the concept of NF- κ B activation to certain GCB cases (58,61). Nevertheless, we showed that the classical pathway seems to activate preferentially different NF- κ B subunits in ABC and GCB cases. This probably results from the genetic differences between the two subtypes and is possibly a reflex of the biological functions of the different NF- κ B subunits in B-cells. Interestingly, a tendency to RelA strong activity preferentially in ABC and cRel in GCB has been previously observed in a smaller set of patients, using ELISA technique (96).

The previously published NF- κ B gene expression signature (54) was as strongly associated with ABC subtype as with RelA strong DNA binding activity. This signature failed to predict the activation of the other NF- κ B subunits. This finding may also help explain why NF- κ B has been thought to be an exclusive trait of the ABC subtype, and why RelB has been for so long neglected.

None of the recurrent DLBCL mutations reflected RelB activation. Of note, mutations on *TRAF3* have been described to cause activation of the alternative pathway of NF- κ B (65). However, they account for only 10% to 15% of DLBCL patients, being insufficient to explain the frequency of RelB activation observed by us. Unfortunately, *TRAF3* mutational status was not available for our cohort and it might be interesting to test it. The genetic alterations that contribute to the constitutive activation of the alternative NF- κ B pathway and RelB are a field to be explored.

In conclusion, we have demonstrated that RelB is frequently activated in DLBCL patients and cell lines, and predicted poorer prognosis in patients, independently from known prognosis predictors.

We have shed light on possible mechanisms through which RelB acts in order to promote tumor cell survival. Further investigation will allow a better understanding of these mechanisms and on how to target RelB in patient treatment.

Additionally, by directly assessing the DNA binding of each NF- κ B subunit individually, we were able to demonstrate that not simply NF- κ B activation status, but each subunit activation status and the interaction between them are determinant in DLBCL tumor biology. In such way, a more detailed approach must be considered in dealing with NF- κ B in the context of DLBCL.

Our findings emphasize the importance of addressing both classical and alternative pathways when targeting NF- κ B in DLBCL. They also underline that targeting NF- κ B should concern GCB DLBCL as well.

Finally, engagement of RelB is a potential new prognostic marker for DLBCL patients and might open the road for improvement in patient stratification and new targeted therapy.

CONCLUSIONS

Section I: cell lines

1. RelB is frequently activated in human DLBCL-derived cell lines
2. RelB protects DLBCL cell lines against caspase 3-dependent apoptosis induced by doxorubicin
3. RelB protects DLBCL cell lines against DNA damage accumulation induced by doxorubicin
4. RelB controls the transcription of anti-apoptotic cIAP2 in DLBCL cell lines.

Section II: patients

1. NF- κ B activation is a feature of both GCB and ABC DLBCL patients
2. NF- κ B classical pathway activates preferentially different subunits in GCB and ABC DLBCL patients
3. RelB constitutive activation is frequent in DLBCL patients and is independent from the COO classification
4. RelB activation defined by gene expression is an independent predictor worse outcome for R-CHOP treated DLBCL patients
5. RelB gene expression signature obtained is able to predict RelB activation status in DLBCL patients
5. RelB activation is not associated with current NF- κ B gene expression signature or frequent mutations known in DLBCL patients.
6. Current methods of evaluation of NF- κ B activation in DLBCL are only able to evaluate RelA activation, not NF- κ B activation as a whole.

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ANNEXES

List of publications

1 - Frequent activation of RelB NF- κ B alternative pathway subunit and its associated gene expression signature predict worse prognosis in diffuse large B-cell lymphoma. **Collares, D**, Nuan-Aliman, S, Faumont, N, Lordello, L, Eluard, B, Bordereaux, D, Martins, I, Cagnard, N, Taoui, O, Lehmann-Che, J, Martinez-Climent, JA, Jardin, F, Copie-Bergman, C, Mauiri, C, Jais, JP, Leroy, K, Kroemer, G, Molina, T, Feuillard, J, Baud, V. *In preparation*, 2019.

2 - Tumor necrosis factor receptor family co-stimulation increases regulatory T cell activity via NF- κ B. Lubrano di Ricco, M, Ronin, E, **Collares, D**, Divoux, J, Wajant, H, Gomes, T, Grinberg-Bleyer, Y, Baud, V, Marodon, G, Salomon, BL. *J Exp Med*, *submitted*.

3 - AXL targeting overcomes human lung cancer cell resistance to NK and CTL-mediated cytotoxicity. Terry, S, Abdou, A, Engelsen, AS, Buart, S, Dessen, P, Corgnac, S, **Collares, D**, Meurice, G, Gausdal, G, Baud, V, Saintigny, P, Lorens, JB, Thiery, JP, Mami-Chouaib, F. *Cancer Immunol Res*, *accepted*.

4 - NF- κ B RelA transcription factor is critical in regulatory T cell activation and stability. Ronin, E, Lubrano di Ricco, M, Vallion, R, Divoux, J, Kwon, HK, Grégoire, S, **Collares, D**, Fradin, D, Rouers, A, Baud, V, Benoist, C, Salomon, BL. *In preparation*.

5 - Post-translational modifications of RelB NF- κ B subunit and associated functions. Baud V and **Collares D**. *Cells*, 4;5(2), 2016. pii: E22.

Review

Post-Translational Modifications of RelB NF- κ B Subunit and Associated Functions

Véronique Baud * and Davi Collares

NF- κ B, Differentiation and Cancer, Université Paris Descartes, Sorbonne Paris Cité, 75014 Paris, France

* Correspondence: veronique.baud@inserm.fr; Tel.: +33-1-7064-9458

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Abstract: The family of NF- κ B transcription factors plays a key role in diverse biological processes, such as inflammatory and immune responses, cell survival and tumor development. Beyond the classical NF- κ B activation pathway, a second NF- κ B pathway has more recently been uncovered, the so-called alternative NF- κ B activation pathway. It has been shown that this pathway mainly controls the activity of RelB, a member of the NF- κ B family. Post-translational modifications, such as phosphorylation, acetylation, methylation, ubiquitination and SUMOylation, have recently emerged as a strategy for the fine-tuned regulation of NF- κ B. Our review discusses recent progress in the understanding of RelB regulation by post-translational modifications and the associated functions in normal and pathological conditions.

Keywords: NF-kappaB; RelB; post-translational modifications; cell motility; phosphorylation; ubiquitination; SUMOylation; NF- κ B alternative pathway

1. Introduction

Nuclear factor κ B (NF- κ B) was first described in 1986 as nuclear factor binding the kappa light chain enhancer in B cells [1]. Since then, it has been demonstrated to play a central role in the inflammatory and immune responses, but it also controls cell proliferation and protects the cell from apoptosis [2–4]. The relevance of NF- κ B in tumor maintenance, tumor development and possibly even in tumor initiation is becoming more evident [5–8] and, recently, activation of NF- κ B has been implicated in tumor resistance to chemotherapy and radiotherapy [9].

In mammals, the NF- κ B family is composed of five members, RelA (p65), RelB, cRel (Rel), NF- κ B1 (p50 and its precursor p105) and NF- κ B2 (p52 and its precursor p100) [10]. These proteins form a variety of homo- and hetero-dimers that, in a resting cell, are retained in a latent cytoplasmic form through binding to a member of the inhibitor of NF- κ B (I κ B) protein family. Upon cell stimulation, NF- κ B is activated by two main pathways (Figure 1). The first one is called the classical NF- κ B pathway. It involves activation of the I κ B kinase (IKK) complex, leading to phosphorylation of I κ B proteins and their subsequent ubiquitinylation and degradation by the proteasome [11] (Figure 1, *left*). This releases active complexes to translocate to the nucleus and execute their transcription functions. The classical pathway usually regulates the activity of RelA and cRel containing dimers. It is typically responsible for a strong and rapid NF- κ B activating signal in response to stress situations and plays a crucial role in the regulation of inflammation and innate immunity. Inflammatory cytokine tumor necrosis factor α (TNF α), toll-like receptors (TLR), interleukine-1 (IL-1) and lipopolysaccharide (LPS) are some of the stimuli involved in its activation. The second one, the more recently described alternative NF- κ B pathway, leads to the activation of RelB-containing dimers (Figure 1, *right*) [7,12,13]. This pathway involves the NF- κ B inducing kinase (NIK) that activates IKK α , thereby leading to the phosphorylation and proteasome-dependent processing of p100, resulting in the release of RelB/p50 and RelB/p52 dimers (Figure 1, *right*). It is known to be involved in diverse processes such as lymphoid organogenesis

and B cell survival, as well as in the regulation of adaptive immunity. It is activated by a more restricted subset of TNF family members (e.g., lymphotoxin β (LT β), B-cell activating factor (BAFF) and CD40 ligand).

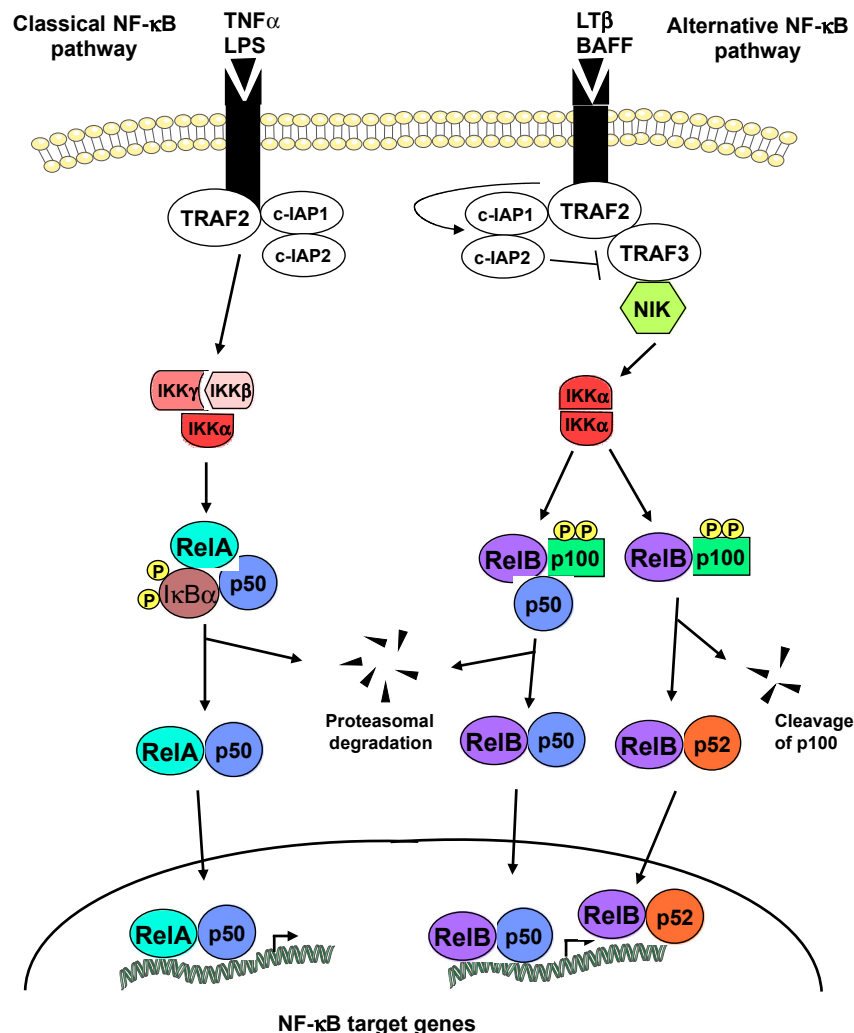


Figure 1. The classical and alternative NF- κ B activation pathways. The classical NF- κ B pathway (*left*): Activation of various receptors, such as TNFRs, causes phosphorylation of the inhibitory I κ B proteins by the IKK complex, leading to their phosphorylation at two specific serine residues, and thereby their degradation by the proteasome 26S. Freed from their inhibitory interaction with the I κ Bs, RelA- and cRel-containing dimers translocate to the nucleus where they activate the transcription of specific NF- κ B target genes. The alternative NF- κ B pathway (*right*): Activation of a more restricted set of receptors (e.g., BAFF, lymphotoxin β), causes the degradation of TRAF3 by the cIAP1/2 E3 ligases, leading to the activation of the MAP3K NIK that activates IKK α , subsequently leading to the phosphorylation and proteasome-dependent processing of p100 and ultimately resulting in the release of either RelB/p50 or RelB/p52 dimers.

RelB is the only NF- κ B member that cannot homodimerize, and it only triggers potent transcriptional activation when coupled to p50 or p52 [14–17]. Beyond the alternative NF- κ B signaling cascade, RelB-dependent DNA-binding activity is negatively regulated at the nuclear level by several mechanisms, such as trapping in RelA/RelB or p100/RelB complexes [18–20], and post-translational modifications (see above). RelB-containing dimers also display DNA-binding specificity [21–23]. RelB recruitment to some genes correlates with transcriptional downregulation (IL12-p40), whereas in

other cases (EBV-induced molecule 1 ligand chemokine (ELC) and macrophage-derived chemokine (MDC)), it increases transcriptional activity well over the level achieved by RelA or cRel [24]. Altogether, this emphasizes the importance and unique role of RelB.

Analyses of RelB-deficient mice have shown that RelB is essential to the development of medullary epithelium, mature dendritic cell function, and secondary lymphoid tissue organization [25–28], indicating that RelB exerts a crucial positive effect for these developmental processes that cannot be compensated for by the presence of other NF- κ B proteins. RelB-deficient mice also spontaneously develop a generalized persistent non-infectious multi-organ inflammatory syndrome that contributes significantly to their premature mortality [29]. RelB is a critical element involved in dendritic cell maturation and immune tolerance to inflammation [30,31]. RelB also represses expression of immediate-response proinflammatory genes during endotoxin tolerance in monocytes, [32–34]. The participation of RelB in non-hematopoietic related function has also emerged. RelB has been shown to play an essential role in limiting the expression of proinflammatory mediators in lipopolysaccharide-induced fibroblasts [35,36], thereby playing an important role in the resolution of acute inflammation. RelB promotes mitochondrial biogenesis in muscle cells [37–39], participates in the regulation of the circadian rhythm in murine fibroblasts [40] and supports the xenobiotic-detoxifying pathway in lung fibroblasts [41,42]. RelB also plays an important role in RANKL-induced osteoclastogenesis that cannot be compensated for by RelA [43–45].

Furthermore, accumulating evidence strongly suggest that an abnormal activity of RelB is involved in the development of both hematopoietic malignancies and solid cancers [13]. Constitutive activation of RelB/p50 dimers participates in the inhibition of DNA-damage-induced apoptosis in certain types of MALT lymphoma [46]. A frequent constitutive RelB DNA-binding activity was reported in a cohort of newly diagnosed multiple myeloma patients [47]. It was demonstrated that RelB plays a crucial role in promoting multiple myeloma cell survival *via* the increased expression of a subset of anti-apoptotic NF- κ B target genes (e.g., cIAP2) by a direct transcriptional control [47]. Inhibition of Notch-induced RelB/p52 activity in Hodgkin lymphoma cell lines is associated with apoptosis and decreased expression of cIAP2 [48]. Moreover, bone marrow stem cells (BMSCs) prevent apoptosis of primary B lymphoma cells, at least in part, through RelB-dependent increased expression of NF- κ B-dependent anti-apoptotic genes (including cIAP1/2 and XIAP) [49]. Thus, it is likely that the prosurvival effects of RelB observed in multiple myeloma might be generalized to other B-cell neoplasms, especially those addicted to NF- κ B. RelB also assisted TEL-JAK2-induced T-cell leukemogenesis [50]. Interestingly, in non-hematopoietic stromal cells, RelB has a role favoring leukemia onset and increasing disease severity.

Abnormal high level of RelB expression has been reported in various solid cancers (e.g., glioblastoma, prostate, breast, bladder and non-small cell lung cancers) and appears to correlate with tumor aggressiveness [51–55]. RelB is the most frequently detected NF- κ B subunit in the nucleus of prostate cancer tissue [51]. The level of nuclear RelB correlates with a patient's Gleason score, suggesting that RelB expression levels are associated with prostate cancer progression. Moreover, RelB exerts a radioprotective role in aggressive prostate cancer cells, at least partially via the induction of the MnSOD gene [56,57]. RelB promotes glioma cell survival and proliferation, and controls invasion independently from RelA [53,58]. In addition, inhibition of RelB in human breast cancer cells reduced cyclin D1 and c-myc expression, slower proliferation, and repressed transformed phenotype [59]. These data suggest that RelB promotes mammary gland carcinogenesis. Higher RelB expression was demonstrated in estrogen receptor α (ER α)-negative breast cancer *versus* ER α -positive one. Moreover, it has been shown that RelB promotes a more invasive phenotype in ER α -negative cancer via induction of the anti-apoptotic BCL2 gene [52]. RelB also favors resistance of these cells to γ -irradiation and the chemotherapeutic agent doxorubicin [60]. RelB mRNA levels were also associated with bladder cancer tumor grade, clinical stage and lymph node metastasis profile [54].

Post-translational modifications are changes or alterations in a protein occurring after the completion of the translational process, either when a functional group is covalently added to the protein, or during the proteolytic and folding processes. These structural changes act

as a mechanism for the specification of proteins and increase their variety. Post-translational modifications have emerged as one of the diverse strategies known for to the fine-tuned regulation of NF- κ B. Reported modifications targeting NF- κ B activity include phosphorylation, acetylation, methylation, ubiquitinylation, SUMOylation, and isomerization of specific amino acid residues, and target either the IKKs, the I κ Bs, the NF- κ B subunits, or critical adaptor proteins that feed into NF- κ B [61–67]. Such modifications influence initiation and duration of NF- κ B response, its specificity for a determined signaling cascade, cell-specific response to a certain stimulus and specific gene transcription. Depending on the cell type and stimulus, such modifications activate or repress NF- κ B activity [61,62,68]. Among those involving NF- κ B transcription factors, site-specific modifications of RelA is by far the most well known [62–64,69,70]. Our review discusses recent progress in the understanding on RelB regulation by post-translational modifications (Table 1) and its associated functions.

Table 1. Post-translational modifications of RelB. The modification, the site(s) involved, the functional effect and reference are indicated in chronological order.

Modification	Site(s)	Enzyme(s)	Effect	Reference
Phosphorylation	Threonine 84, Serine 552	Unknown	Degradation	Marienfeld <i>et al.</i> 2001 [71]
Phosphorylation	Serine 368	Unknown	Dimerization	Maier <i>et al.</i> 2003 [72]
Polyubiquitination	Lysine 273, 274, 305 and 308	Unknown	Transcriptional activity	Leidner <i>et al.</i> 2008 [73]
Phosphorylation	Serine 472	IKK α /IKK β	Cell migration	Authier <i>et al.</i> 2014 [74]
SUMOylation	Lysine 387, 388, 390, 411, 414, 415, and 416	Unknown	Transcriptional activity	Leidner <i>et al.</i> 2014 [75]

2. Phosphorylation of RelB

2.1. Serine 552 and Threonine 84

Marienfeld *et al.* were the first to describe by *in vitro* kinase assays that RelB can be phosphorylated on threonine 84 and serine 552 [71]. Furthermore, TPA-ionomycin-induced RelB phosphorylation was shown to depend on these two specific sites as evaluated by *in vivo* labeling in murine EL-4 T cells. The authors report a marked decrease in RelB protein expression upon TPA-ionomycin stimulation in human peripheral blood T cells and Jurkat cells. In contrast, TNF α has no effect on RelB expression levels. Interestingly, a phosphorylation-defective RelB mutant serine 552 to cysteine and threonine 84 to alanine (S552C/T84A) leads to the stabilization of RelB. Thus, it indicates that TPA-ionomycin-induced S552 and T84 phosphorylation of RelB leads to its degradation. Remarkably, a cleaved form of RelB was best observed upon pretreatment of T-cells by proteasome inhibitors, suggesting that RelB cleavage can precede its degradation by the proteasome. Notably, cleavage of RelB near its N-terminus (after arginine 85) by the paracaspase MALT1 has been reported [76]. However, mutation of serine 552 and threonine 84 did not prevent RelB cleavage by MALT1 in 293T cells, thereby indicating that these two sites do not appear to be involved in MALT1-dependent RelB cleavage [76].

2.2. Serine 368

Maier *et al.* identified RelB serine 368 in the C-terminal part of the Rel Homology domain (RHD) as a conserved residue in human and drosophila NF- κ B subunits. [72] As evaluated by luciferase reporter assays, both S368A inactivating and S368E phosphomimetic RelB point mutants exhibited a markedly reduced transcriptional activity in RelB-defective murine S107 plasmacytoma cells compared to that seen in wild-type (WT) RelB. It thus suggests that serine 368 alone rather than its phosphorylation

is critical for the control of RelB activity. Mutation of serine 368 severely affects RelB dimerization with its interacting partners p50, p52, RelA and p100. Remarkably, absence of serine 368 correlates with a strong decrease in p100 half-life along with an increase in p100 proteolysis into p52. No similar effect was seen with p105. Whether the phosphorylation of serine 368 can occur on endogenous RelB is still unknown.

2.3. Serine 472

Although $\text{TNF}\alpha$ is known to induce a massive nuclear accumulation of RelB, it is generally accepted that RelB global DNA-binding activity is not induced upon $\text{TNF}\alpha$ treatment in fibroblasts [18]. Our laboratory has recently uncovered that RelB plays a crucial role in promoting fibroblast migration upon prolonged $\text{TNF}\alpha$ stimulation. Remarkably, RelB pro-migratory function is driven by its induced phosphorylation on serine 472 [74] (Figure 2). We have identified the two kinases $\text{IKK}\alpha$ and $\text{IKK}\beta$ as novel RelB-interacting partners whose activation by $\text{TNF}\alpha$ promotes RelB phosphorylation on serine 472. Moreover, using a custom antiphospho-serine 472-specific RelB monoclonal antibody, we have shown that RelB phosphorylation on serine 472 is induced in fibroblasts in response to both $\text{TNF}\alpha$ and $\text{PDGF}\beta$ [74]. We have demonstrated that nuclear RelB phosphorylated on serine 472 dissociates from its interaction with the inhibitory protein $\text{I}\kappa\text{B}\alpha$ and binds to the promoter of critical migration-associated genes, such as the metalloproteinase matrix metalloproteinase 3 (MMP3) (Figure 2). Finally, we have shown that RelB serine 472 phosphorylation status controls MMP3 expression and pro-migration activity downstream of TNF receptors (TNFRs) [74] (Figure 2). Interestingly, phosphorylation of RelA on threonine 505, induced by Chk1 kinase, has been reported to inhibit constitutive fibroblast migration [69]. Such observation reinforces the idea of non-redundant functions for RelA and RelB in the control of cell motility.

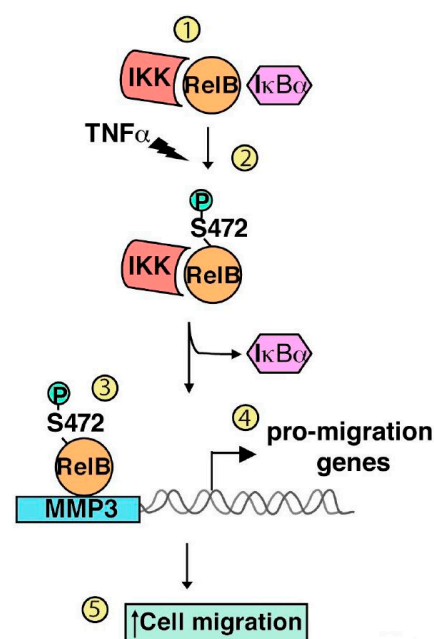


Figure 2. Model for RelB serine-472 phosphorylation acting as an activator of inflammation-mediated cell migration. The $\text{I}\kappa\text{B}$ kinase (IKK) complex constitutively interacts with the RelB subunit of NF- κB [1]. Activation of IKK upon prolonged $\text{TNF}\alpha$ treatment (at least 6 hours) causes phosphorylation of RelB on serine 472 [2]. It allows nuclear ReB to dissociate from its interaction with the inhibitory protein $\text{I}\kappa\text{B}\alpha$ and to bind to the promoter of pro-migration genes such as MMP3 [3], thereby resulting in selective NF- κB target gene expression involved in the control of $\text{TNF}\alpha$ -induced cell migration [4]. $\text{TNF}\alpha$ -induced IKK-driven ReB serine-472 phosphorylation is subsequently required for efficient cell migration in an MMP3-dependent manner [5].

2.4. Other Putative Phosphorylation Sites

Mass spectrometry approaches have highlighted several other putative sites that can be modified by phosphorylation throughout RelB, such as serine 20, serine 37, serine 116, serine 139, serine 217, tyrosine 293, serine 425, and threonine 579 [77]. Whether phosphorylation of these residues exists *in vivo* and their functional consequences are currently unknown. Nevertheless, it presumes that functional regulation of RelB by phosphorylation is highly complex.

3. Polyubiquitination of RelB

In 2008, Leidner *et al.* pinpointed for an ubiquitination-dependent enhancement of RelB transcriptional activity that is not linked to an increase in RelB nuclear localization or DNA binding [73]. Of note, RelB serine 368, serine 552 or threonine 84 (see above) do not seem to be involved in RelB polyubiquitination [73]. RelB ubiquitinylation assays using HA-ubiquitin mutants defective for either Lys⁴⁸ degradative-conjugated polyubiquitin chain or Lys⁶³ non-degradative-conjugated polyubiquitin chain, or defective for both, still showed an efficient RelB polyubiquitination. Thus, it indicates that polyubiquitination of RelB might involve other types of polyubiquitin conjugation [78]. Mapping of the ubiquitination target sites revealed the existence of various lysine residues which serve as ubiquitination acceptors throughout the RelB protein. Nonetheless, Lys273/274 and Lys305/308 appeared to be critical for the ubiquitination-dependent increase in RelB transcriptional activity. The nature of polyubiquitin-chain conjugation involved in this process remains unclear. The molecular mechanisms controlling the increase in RelB activity, especially the identity of the recruited co-activators or released co-repressors (e.g., Daxx, EZH2 or G9a) [34,79,80] still need to be explored.

4. SUMOylation of RelB

Another post-translational modification reported to modulate the functionality of NF- κ B is the conjugation of SUMO peptides at lysine residues, a process that is termed SUMOylation [67]. SUMOylation of a target protein involves the enzymes SUMO-activating protein (E1), the SUMO conjugating protein UBC 9 (E2) and a panel of SUMO ligases (E3), a panel of enzymes quite similar to the ubiquitination machinery. SUMOylation and ubiquitination frequently have antagonistic effects when affecting the function of a particular protein [81].

Seeking a mechanistic explanation for the dual behavior of RelB either as an activator or a repressor of NF- κ B target gene expression, Leidner *et al.* have shed light on a SUMOylation-dependent weakening of RelB transcriptional activity. This effect does not rely on changes in RelB nuclear localization or its DNA-binding ability [75]. Mutational analysis of lysine residues throughout RelB revealed that SUMOylation of RelB can occur at numerous sites, and inactivation of seven lysine residues—positions 387, 388, 390, 411, 414, 415, and 416—is required to affect RelB SUMOylation. The mechanism that connects SUMOylation of RelB to a decrease in RelB transcriptional activity is currently unknown.

5. Conclusions

Considering the presence of 22 lysine, 46 serine, 24 threonine and 10 tyrosine residues in human RelB, it is clear that we have just scratched the surface concerning RelB post-translational modification possibilities.

As reviewed here, phosphorylation, ubiquitinylation and SUMOylation have been reported to have an effect on RelB activity, either enhancing or weakening it. Knowing that RelB has been previously shown to behave either as a transcriptional activator or a transcriptional repressor, we can hypothesize that post-translational modifications can be a key determinant to whether RelB will exert an inhibitory or activation function. Such post-translational-modifications can change the cofactor that interacts with RelB, leading to a different outcome in the specificity of RelB-dependent nuclear factor κ B (NF- κ B) response. In the same way, the same cofactor recruited by different post-translational

modifications could lead to different target pools of genes, thus conveying on RelB different functions. Furthermore, a modification-dependent RelB degradation could be implicated in determining the duration of the response to a certain stimulus, as its degradation would stop the RelB-dependent response. In support of this hypothesis, Marielfeld *et al.* showed a site-specific phosphorylation on threonine 84 and serine 552 that determines the cleavage and subsequent degradation of RelB [71]. In another study, RelB protein expression levels were shown to control the magnitude of classical NF- κ B pathway activation through induced RelB cleavage by the paracaspase MALT1 in B and T cells [76]. However, whether or not in this context RelB post-translational modifications are involved in the control of RelB cleavage and subsequent relief of the classical NF- κ B activation pathway is currently unknown. All these possibilities, considered together with all those of other NF- κ B family members that interact with and regulate RelB, could explain the versatility of this factor.

We have recently revealed a novel activating molecular mechanism leading to RelB transcriptional activation downstream of TNF receptors. It relies on RelB-serine 472 phosphorylation and is critical for the control of inflammation-induced cell migration [74]. We thus have shed light on a specific RelB post-translational modification that drives RelB to exert a specific biological function. It has been recently reported that RelB can promote the more invasive phenotype of ER α -negative breast cancer cell lines [52], and RelB increases the incidence of metastatic tumors in a mice xenograft model of prostate cancer [51]. Furthermore, RelB knockdown strongly reduces glioma cell migration and invasion [53]. However, whether RelB serine 472 phosphorylation can participate in the invasiveness of cancer cells is currently unknown but is nevertheless worth further investigation.

Unveiling RelB post-translational modifications will provide us not only with a better understanding of the normal regulation of RelB (and the alternative NF- κ B pathway), but also with the understanding of its deregulated activity and the pathological consequences that follow. Since this area of research is moving at a rapid pace, there is hope that the processes behind RelB post-translational modifications influencing global NF- κ B activity and its involvement in pathological processes will soon be uncovered.

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