



Alternative and canonical NF- κ B pathways DNA-binding hierarchies networks define Hodgkin lymphoma and Non-Hodgkin diffuse large B Cell lymphoma respectively

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Abstract

Purpose Despite considerable evidence that supports the NF- κ B role in the immune system and lymphomagenesis, it is unclear whether specific NF- κ B dimers control a particular set of genes that account for their biological functions. Our previous work showed that Hodgkin Lymphoma (HL) is unique, among germinal center (GC)-derived lymphomas, with respect to its dependency on Rel-B to survive. In contrast, diffuse large B-Cell lymphoma (DLBCL) including both Activated B-Cell-Like and Germinal Center B-Cell-Like, requires cREL and Rel-A to survive and it is not affected by Rel-B depletion. These findings highlighted the activity of specific NF- κ B subunits in different GC-derived lymphomas.

Methods Sequenced chromatin immunoprecipitated DNA fragments (ChIP-Seq) analysis revealed an extensive NF- κ B DNA-binding network in DLBCL and HL. The ChIP-Seq data was merged with microarray analysis following the Rel-A, Rel-B or cRel knockdown to determine effectively regulated genes.

Results Downstream target analysis showed enrichment for cell cycle control, among other signatures. Rel-B and cRel controlled different genes within the same signature in HL and DLBCL, respectively. BCL2 was exclusively controlled by Rel-B in HL. Both mRNA and protein levels decreased following Rel-B depletion meanwhile there was no change upon cRel knock-down. BCL2 exogenous expression partially rescued the death induced by decreased Rel-B in HL cells.

Conclusion The Rel-B hierarchical network defined HL and the cRel hierarchical network characterized DLBCL. Each Rel member performs specific functions in distinct GC-derived lymphomas. This result should be considered for the development of targeted therapies that are aimed to selectively inhibit individual NF- κ B dimers.

Keywords Hodgkin lymphoma · Non-Hodgkin lymphoma · DLBCL · Rel-B · NF- κ B alternative pathway · Germinal center-derived lymphomas

Introduction

B-cells have the well-documented property of requiring members of the nuclear factor- κ B (NF- κ B) family of transcription factors (TFs) at various developmental stages (Milanovic et al. 2017). A subset of these lymphomas arises

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from B-cells that undergo differentiation in the germinal center (GC) of lymph nodes in response to antigen stimulation including Hodgkin (HL) and Non-Hodgkin Lymphomas (NHL) (Carbone et al. 2009). Researchers have reported numerous mutations in the canonical pathway mediators that are responsible for the nuclear translocation of NF- κ B dimers and constitutive NF- κ B signaling in both HL and NHL (Gilmore et al. 2004; Ranuncolo et al. 2012). Nonetheless, these data are largely based on the signaling pathways themselves, and the role played by each Rel family member is unclear (Lenz et al. 2008; Davis et al. 2010).

Certain B-cell lymphomas such as the Germinal Center B-Cell (GCB) molecular subtype of Diffuse Large B Cell Lymphoma (DLBCL), HL and Primary Mediastinal B-Cell Lymphoma (PMBL) show amplification of the cREL locus (Feuerhake et al. 2005; Martin-Subero et al. 2002; Barth et al. 2003). It has been shown that this amplification correlates with cRel translocation to the nucleus. We have reported that HL is unique, among GC-derived lymphomas, owing to its constitutive activation dependency on the alternative NF- κ B pathway (Ranuncolo et al. 2012). These findings suggest unique functions for the canonical and the alternative NF- κ B signaling arms and potentially for individual subunits in the pathogenesis of different lymphoma subtypes (Klein and Heise 2015).

The NF- κ B family consists of two signaling arms, which are named as canonical (classical) and alternative (nonclassical or noncanonical). The five members of this family are Rel-A, Rel-B, cRel, p50 and p52 (the last two are NF κ B1/p105 and NF κ B2/p100 cleavage products, respectively). Only the Rel proteins contain a homology domain that is responsible for DNA binding and a transactivation domain (TADs) that is necessary for their transcriptional activation properties. These TADs are only 10% similar which suggests that their activation biochemistries are different, possibly requiring distinct coactivators. Furthermore, this diversity sustains the hypothesis that the three Rel proteins have different transactivation functions, likely targeting different genes (Oeckinghaus and Ghosh 2010).

We aimed to address the specificity of the Rel proteins in HL and DLBCL, the most common NHL in adults, which has not been investigated before.

HL has an incidence of 3/100,000 cases in Western countries. In accordance with the Revised European American Lymphoma (REAL) and the World Health Organization (WHO) lymphoma classification, 95% of cases belong to the classical type (cHL). The remaining 5% represents the nonclassical form, which is named as Nodular Lymphocyte Predominant HL (Harris et al. 1997; Kapatani and Murray 1999; Campo et al. 2011). Based on histological differences and the microenvironment features, cHL is further subdivided into nodular sclerosis, mixed cellularity, lymphocyte-depleted and lymphocyte-rich (Campo et al. 2011).

In cHL the tumor cell, which is designated as the Hodgkin and Reed–Sternberg cell (HRS), represents 0.1–10% of the tumor. It is now accepted that HRS cells are derived from late-stage GC B-cells that have often acquired nonproductive immunoglobulin gene rearrangements and that have been subsequently rescued from apoptosis via unknown mechanisms (Cabannes et al. 1999; Jungnickel et al. 2000).

HRS cells consistently show strong constitutive NF- κ B activity. Several genetic lesions have been identified that affect the classical NF- κ B pathway (Emmerich et al. 1999). Amplification or gains of the REL gene that encodes for cRel are present in almost 50% of cHL patients; mutations in different I κ Bs such as the NF- κ B1A encoding I κ B α are inactivated in 15–20% of cHL cases and I κ B ϵ which has been reported to be mutated in a similar percentage of cases (Cabannes et al. 1999; Jungnickel et al. 2000; Joos et al. 2002; Emmerich et al. 2003; Lake et al. 2009). More recently, the tumor suppressor gene TNFAIP3 that encodes A20, a negative regulator of the NF- κ B acting upstream of the kinases, has been reported as the target of inactivating mutations in 30–40% of cases (Kato et al. 2009; Schmitz et al. 2009a, b). We have previously shown that NIK (NF- κ B Inducing Kinase) accumulation, due to disruption of the molecular complex that targets NIK for degradation, leads to constitutive alternative NF κ B signaling (Ranuncolo et al. 2012).

DLBCL is the most common GC-derived NHL in adults. While 50% of patients are cured with standard therapy, a large fraction either relapses or exhibits primary refractory disease. Relapse is the major cause of treatment failure and death in these patients. DLBCL represents a very heterogeneous disease in multiple aspects including clinical outcome. Gene expression profiling has classified DLBCL into two subgroups: ABC and GCB (Alizadeh et al. 2000). ABC is characterized by canonical constitutive NF- κ B activation that sustains its viability (Davis et al. 2001). The molecular basis has been elucidated using functional and structural genomic studies. Upon B-Cell Receptor (BCR) engagement the signaling adapter CARD11 orchestrates the IKKs activation (Thome et al. 2010). In 10% of ABC lymphomas, CARD11 has activating oncogenic mutations that lead to spontaneous NF- κ B signaling (Lenz et al. 2008). In other ABC lymphomas, BCR engages wild-type CARD11 which leads to NF- κ B activation, a phenomenon known as chronic active BCR signaling (Davis et al. 2010). Despite more frequent NF- κ B signaling disruption in ABC, a smaller fraction of GCB also carries mutations associated with the NF- κ B pathway (Compagno et al. 2009). Despite the extensive literature, the real contribution of the canonical and alternative mediators in different B-cell lymphomas remains elusive.

In this current work, we have explored the genomic distribution of the NF- κ B family members using ChIP-Seq assay in representative human HL and DLBCL cell lines

and primary tonsillar centroblasts. Merging this set of data with gene expression array profiling, upon Rel factors knock-down, defined a Rel-B network in HL and a cRel network in DLBCL.

Materials and methods

Cell lines

BJAB, U-H01 and L1236 cell lines were cultured in RPMI, and HBL-1 and 293-T were grown in Dulbecco's Eagle modified medium, all were supplemented with 20% FBS (HyClone, Defined), 1% antibiotics and incubated at 37 °C with 5% CO₂.

Isolation of primary B-cells

Human tonsils were obtained (in accordance with the Helsinki Protocols) as otherwise discarded "leftovers" from routine tonsillectomies. After mincing, mononuclear cells were isolated via density centrifugation using Histopaque-1077. Naïve B-cells (NBC) [IgD+ (BD Biosciences 555778), CD27 – (BD Pharmingen 555439), CD38^{low} (BD Biosciences 347687)], centroblasts (CB) [CD38^{high} (Biosciences 347687), CD77+ (Immunotech 0175)] and centrocytes (CC) [CD38^{low} (Biosciences 347687), CD77- (Immunotech 0175)] were separated using the magnetic MidiMACS system according to published protocols (Klein et al. 2003). The purity of subpopulations was determined via flow cytometry. This protocol was approved by the Ethics Committee of Research Protocols of our Institution (CEPI) (E/110). Antibodies used are listed in Online Resource 1.

Retroviral production

Using Lipofectamine 2000, 293-T cells were transfected with the inducible pRSMX_PG-eGFP-Puro vector, which delivered the shRNA for Rel-B or cRel, along with the mutant ecotropic envelope-EA6×3* and pHIT60 Gag/Pol expressing plasmids. The virus supernatant was collected at 48 and 72 h post-transfection. Cells were spin-infected twice on consecutive days (Ngo et al. 2006). The small hairpin sequences used can be found in Online Resource 2.

Rescue experiment

L1236 and U-H01 cell lines were transduced with an inducible retrovirus expressing a HA-tag BCL2 cDNA cloned into the PGK-CMV (Open Reading Frame) (GeneCopoeia). After hygromycin selection for the cells that incorporated the cDNA BCL2 plasmid, each cell line was transduced with two different Rel-B shRNAs sequences and the shRNA

control. Doxycycline (20 ng/ml) was added to the culture medium two days after the second round of spin infection to induce the hairpin expression. The GFP+ cell population was measured by FACS (Becton–Dickinson) each two days during 10 days. For each time point and shRNA sequence, the GFP+ fraction was normalized to the GFP+ fraction in parallel cultures transduced with the corresponding shRNA-control, and compared to the initial GFP+ cell population measured two days after the spin-infection.

Real-time PCR

Total RNA was prepared using TRIzol. The Superscript II first-strand cDNA synthesis kit (Life Technologies, Carlsbad, California, USA) was used to obtain cDNA following the manufacturer's instructions. The mRNA expression of Rel-B and its target genes (BCL2, CDK6, CNND3) was analyzed using pretested assay-on-demand primer sets from Applied Biosystems (Hs00232399, Hs00153350, Ha00608037 and Hs00236949, respectively) (Foster City, California, USA) for 45 cycles at an annealing temperature of 60°C. Gene expression was normalized to B2MG (Assay-on-demand primer set from Applied Biosystems Hs00187842) and data are presented as fold change relative to the control according to the 2^{-ΔΔCT} method (Livak and Schmittgen 2001).

Immunoblot analysis

Infected cells (10 × 10⁶) were lysed in a solution containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 50 mM β-glycerophosphate, 1% Tween-20, 0.2% Nonidet P-40 and protease inhibitors. Proteins were resolved using 10% SDS-PAGE gels. The membranes were incubated with primary antibodies [Rel-A (sc-372), Rel-B (sc-226), cRel (sc-71), p50 (sc-114), p52 (sc-848), BCL2 (sc-7382) all used at 1:200 and Actin-HRP (sc-1615) used at 1:1000 from Santa Cruz Biotech] for 1 h at RT. Peroxidase-conjugated anti-rabbit (Jackson ImmunoResearch 111-035-003) and anti-mouse (Jackson ImmunoResearch 115-035-003) were used as secondary antibodies and incubated for 30 min at RT. The antibodies used are listed in Online Resource 3.

Expression arrays

U-H01 and HBL-1 cells transduced with retroviral Rel-B and cRel shRNAs, respectively, were puromycin-selected for GFP+ cells. Half of the GFP+ cells were treated with doxycycline to induce shRNA expression and the other half were not induced. Total RNA was isolated at days 1 and 2 after induction. Samples were hybridized on Human GeneChip Gene 1.0 ST array (Affymetrix Inc, Santa Clara, California, USA). The gene expression was analyzed by comparing the

signal from cells infected with noninduced shRNA to that from cells infected with induced shRNA. Statistical and clustering analysis was performed using the Partek Genomics Suite software and the RMA normalization algorithm. Differentially expressed genes were identified via ANOVA analysis. Genes that were downregulated more than twofold with a $p < 0.001$ were considered significant. Data have been deposited in the NCBI GEO database under the GSE109803 accession number.

Chromatin immunoprecipitation (ChIP)

ChIP was performed for Rel-A, Rel-B, cRel, p50, p52 (Antibodies used are the same described in immunoblot analysis) and normal rabbit serum (sc-2027, Santa Cruz Biotech). Thirty million U-H01, BJAB and HBL-1 cells and CB were treated with formaldehyde 1%. Crosslinking was stopped using glycine 0.125 M. Cells were resuspended in Lysis Buffer (50 mM Hepes pH 8.0, 150 mM NaCl, 1% Triton-X 100, 0.1% Na-deoxycholate, 1 mM EDTA, protease inhibitors). DNA was sheared by sonication in a Bioruptor (high energy for 30 s on and 30 s off over 30 min). Precipitation of DNA fragments were performed using Protein G-magnetic-beads (Dyna/Invitrogen); 10 μ g of antibody and normal rabbit serum were prebound to 50 μ l of washed beads in 100 μ l of 0.1 M NaOAc, 0.2% Tween-20 and 0.2% BSA. Immunoprecipitation was performed by incubating the lysate (5×10^6 cells) with antibody bounded beads. Unprocessed lysate (10%) was used as “input”. DNA was recovered using 100 μ l Chelex-100 (BioRad 142–1253) at boiling temperature over 10 min. Samples were cleaned up using the QIAquick Spin columns (QIAGEN, Hilden, Germany) according to manufacturer’s recommendation and eluted in 40 μ l of Elution Buffer (EB). The antibodies used are listed in Online resource 3.

Sample preparation for ChIP-Seq analysis

DNA ChIP fragments were repaired by generating phosphorylated blunt ends using T4 DNA Polymerase, Klenow DNA polymerase and T4 polynucleotide kinase following the Illumina protocols (San Diego, California, USA). Samples were run on a 2% agarose gel for the library size selection (200 \pm 25 bp range). DNA purification was performed using the QIAGEN Gel Extraction ki (Hilden, Germany). The DNA was amplified via PCR to enrich for the adapter-modified fragments. PCR products were cleaned on a QIAGEN MinElute purification column (Hilden, Germany) and DNA diluted to 10 nM in EB with 0.1% Tween-20. To prepare for cluster generation, 1–4 pM DNA was used, leading to a 120–150,000 clusters/tile density. DNA was denatured with NaOH to 0.5 nM.

Sequencing and analysis

Following the first base run, the process was paused to allow quality control. A small section of each lane was imaged. Cluster density and the average intensity value for the four bases were compared to the minimum thresholds. Subsequently, sequencing proceeded for 36 cycles. The images acquired were processed through the bundled Solexa image extraction pipeline, which identified colonies positions, performed base-calling and generated quality controls statistics. Sequences were aligned using the bundled ELAND software to the human genome NCBI build 37 as the reference. Only sequences that were perfectly and uniquely mapped to the genome were used. The ChIP-Seq data have been deposited in the NCBI GEO database under the GSE110348 accession number and can be found at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110348>.

Results

To address the NF- κ B subunits target genes in GC-derived B-cell HL and DLBCL, we analyzed the genome-wide distribution of Rel-A, Rel-B, cRel transcription factors and the p50 and p52 co-factors via ChIP-Seq.

Hodgkin Lymphoma

In U-H01 cells 29,414 Rel-B peaks were genome-wide distributed. To further identify genes with higher probabilities of being directly regulated by Rel-A, Rel-B, and/or cRel we concentrated on the peaks that localized within a ± 2 kb window, relative to the gene transcription start site (TSS). The ± 2 kb Rel-B peaks were distributed on 4509 genes; meanwhile the ± 2 kb cRel peaks were on 1994 genes and the Rel-A peaks were on 830 genes (Table 1). Out of the 4509 genes with the Rel-B peaks, only 6% overlapped with the Rel-A peaks and 11% with the cRel peaks. The Rel factors distribution showed a Rel-B DNA-binding hierarchy in HL.

The analysis of Rel-B-bound genes showed enrichment for genes related to B-cells TFs, BCR signaling, cell cycle

Table 1 Total number of ± 2 kb peaks from TSS in U-H01, BJAB, HBL-1 cell lines and centroblasts

| NF- κ B subunits | Cells | | | |
|-------------------------|-------|------|-------|------|
| | U-H01 | BJAB | HBL-1 | CB |
| Rel-A | 830 | 1553 | 1202 | 610 |
| Rel-B | 4509 | 1368 | 1904 | 5548 |
| cRel | 1994 | 3648 | 6690 | 1110 |
| p50 | 1924 | 4555 | 3352 | 4572 |
| p52 | 2029 | 33 | 1111 | 828 |

and cell death regulation, NF- κ B mediators and DNA damage and repair pathways. We used the DAVID v6.7 software package to extract biological meaning from the list of genes that were obtained after merging the ChIP-Seq with the cDNA array datasets.

Expression arrays compared U-H01 shRel-B transduced-induced vs transduced-uninduced cells read-out at days one and two after induction. Genes that were up- or downregulated more than twofold with a $p < 0.001$ were considered significant. Merging the genes that were downregulated \geq twofold and the ChIP-Seq data showed that 50% of genes were bound and transcriptionally regulated by Rel-B (Table 2).

Table 2 ChIP-Seq and expression array data merged in ABC-Like DLBCL and Hodgkin Lymphoma cell lines

| Sample | Genes \geq folds downregulated N (%) |
|---|--|
| Uninduced shRel-B ₁ /24 h induced shRel-B ₁ | 18.847 (57) |
| Uninduced shRel-B ₁ /48 h induced shRel-B ₁ | 19.371 (58) |
| Uninduced shRel-B ₂ /48 h induced shRel-B ₂ | 18.649 (56) |
| Uninduced shRel-B ₂ /48 h induced shRel-B ₂ | 14.642 (45) |
| Uninduced shRel ₁ /24 h induced shRel ₁ | 16.792 (50.4) |
| Uninduced shRel ₁ /48 h induced shRel ₁ | 15.749 (47.3) |
| Uninduced shRel ₂ /48 h induced shRel ₂ | 17.101 (51.4) |
| Uninduced shRel ₂ /48 h induced shRel ₂ | 17.267 (51.9) |

shRel-B₁ and shRel-B₂ indicate two different sequences used
shRel₁ and shRel₂ indicate two different sequences used

Table 3 Number of genes with \pm 2kB TSS overlap peaks among Rel factors and co-factors in U-H01, BJAB, HBL-1 cell lines and centroblasts

| U-H01 | Intersections (number of genes) | | | | | |
|-------|---------------------------------|-------|-------|------|-----|----------------|
| | cRel | Rel-A | Rel-B | p50 | p52 | cRel and Rel-B |
| Rel-A | 188 | – | 269 | 194 | 152 | 73 |
| cRel | – | 188 | 477 | 614 | 339 | |
| Rel-B | 477 | 269 | – | 677 | 555 | |
| BJAB | cRel | Rel-A | Rel-B | p50 | p52 | cRel and Rel-B |
| Rel-A | 650 | – | 198 | 726 | 21 | 41 |
| cRel | – | 650 | 439 | 1712 | 27 | |
| Rel-B | 439 | 198 | – | 596 | 22 | |
| HBL-1 | cRel | Rel-A | Rel-B | p50 | p52 | cRel and Rel-B |
| Rel-A | 897 | – | 358 | 604 | 93 | 328 |
| cRel | – | 897 | 1311 | 3310 | 455 | |
| Rel-B | 1311 | 358 | – | 1899 | 156 | |
| CB | cRel | Rel-A | Rel-B | p50 | p52 | cRel and Rel-B |
| Rel-A | 77 | – | 175 | 209 | 58 | 45 |
| cRel | – | 77 | 345 | 557 | 89 | |
| Rel-B | 342 | 175 | – | 1412 | 237 | |

Centroblasts

Centroblasts were isolated from human tonsils and subjected to ChIP-Seq. A predominant Rel-B DNA-binding was observed. There were \pm 2 kb Rel-B peaks on 5,548 genes. Considering equal window there were cRel peaks on 1110 genes and Rel-A peaks on 610 genes (Table 1).

DLBCL

Rel-B, an alternative NF- κ B pathway mediator, showed a dominant DNA-binding activity in HL meanwhile cRel, one of the canonical pathway mediators, showed the highest number of peaks in DLBCL. The same scenario was observed in ABC-Like and GCB-Like DLBCL. Regarding Rel-A, \pm 2 kb peaks from TSS were found on 1553 and 1202 genes in the BJAB and HBL-1 cell lines, respectively (Table 1).

The number of genes that showed p50 and p52 \pm 2kB peaks is indicated in Table 1 for all cell lines and CB. Additionally Table 3 depicts the number of genes with \pm 2kB overlapping Rel-A/cRel peaks, as well as the gene number with overlapped \pm 2 kb peaks for Rel-A, Rel-B or c-Rel with either p50 or p52 for the BJAB and HBL-1 cells. Finally, the number of Rel-A, Rel-B and cRel bound genes shared by different cell lines and CB, is summarized in Table 4.

Expression arrays compared the read-out from HBL-1 sh-cRel transduced-induced vs transduced-uninduced cells at days one and two after induction. Genes that were up- or downregulated more than twofold with a $p < 0.001$ were considered significant. Merging the genes that were downregulated \geq twofold and the ChIP-Seq data showed that 50%

Table 4 Rel-A, Rel-B and cRel bound genes shared by different cell lines

| Factor | Cell line 1 | Cell line 2 | N of common genes | % cell line 1 | % cell line 2 |
|--------|-------------|-------------|-------------------|---------------|---------------|
| Rel-A | U-H01 | HBL-1 | 105 | 12.7 | 8.8 |
| | U-H01 | BJAB | 119 | 14.4 | 7.7 |
| | U-H01 | CB | 44 | 5.3 | 7.2 |
| | HBL-1 | BJAB | 225 | 18.7 | 14.5 |
| | HBL-1 | CB | 58 | 4.8 | 9.5 |
| | BJAB | CB | 77 | 5 | 12.6 |
| Rel-B | U-H01 | HBL-1 | 538 | 13 | 30.6 |
| | U-H01 | BJAB | 348 | 7.8 | 25.4 |
| | U-H01 | CB | 1204 | 26.7 | 21.7 |
| | HBL-1 | BJAB | 240 | 12.6 | 17.5 |
| | HBL-1 | CB | 591 | 31 | 10.7 |
| | BJAB | CB | 375 | 27.4 | 6.7 |
| c-Rel | U-H01 | HBL-1 | 1076 | 54 | 16.1 |
| | U-H01 | BJAB | 604 | 30.3 | 16.6 |
| | U-H01 | CB | 205 | 10.3 | 18.5 |
| | HBL-1 | BJAB | 1949 | 29.1 | 53.4 |
| | HBL-1 | CB | 593 | 8.9 | 53.4 |
| | BJAB | CB | 403 | 11 | 36.3 |

of genes were bound and transcriptionally regulated by cRel (Table 2).

Rel-B and cRel bound different genes corresponding to a same signature, such as cell cycle-cell death and B-cell transcription factors, in HL and DLBCL cells, respectively (Figs. 1 and 2).

The DNA sequences from all the ± 2 kB peaks were evaluated for the presence of the most frequent cognate DNA-binding motif, 5'-GGGACTTTC-3', indicated as canonical kB-site, which accounted for a low percentage (Online Resource 4).

The data strongly highlighted two GC-derived lymphomas dissected by a different NF-KB pathway hierarchical arm at a DNA-binding level and transcriptionally controlled set of target genes. There is a dramatic difference between HL and DLBCL in terms of Rel-B and cRel genome-wide distribution.

Rel-B binds to and effectively regulates the transcription of the anti-apoptotic genes in HL

For a deeper investigation of the Rel-B transcriptional regulation activity in HL cells we chose the BCL2 gene. Single-locus ChIP showed enriched Rel-B binding compared to the control, which confirmed the result observed in the U-H01 ChIP-Seq study (Fig. 3a, a1). No Rel-A binding was observed meanwhile cRel enrichment on BCL2

was also found via single-locus ChIP (Fig. 3a, a1). Nevertheless, its protein level was not affected following cRel knockdown (Fig. 3b, b2). The BCL2 gene is not regulated by cRel, even though its promoter was bound by cRel, as shown by single-locus ChIP. It is effectively controlled by Rel-B in HL cells which was proven by the decrease in mRNA and protein levels after shRNA Rel-B depletion, as determined by western blot and Qt-PCR (Fig. 3b, b1, c, c2). These results were reproduced with a second HL cell line L1236, which confirmed BCL2, as bonafide Rel-B regulated gene in HL cells (Online Resource 5). Furthermore, BCL2 exogenous expression, that could not be targeted by the Rel-B shRNA sequences used, was able to partially rescue the death induced by Rel-B depletion in U-H01 and L1236 HL cell lines.

Furthermore, BCL2 exogenous expression, that could not be targeted by the Rel-B shRNA sequences used, was able to partially rescue the death induced by Rel-B depletion in U-H01 and L1236 HL cell lines.

Figure 1 depicts a set of cell cycle and cell death control genes bound by Rel-B in HL and by cRel in ABC-DLBCL cells.

NF-kB subunits in primary B-cells

NBC, CB and CC were isolated from human tonsil and total lysate was prepared to address the protein expression of NF-kB subunits (Fig. 4a).

The most significant differences among the three subsets of primary B-cells were observed regarding Rel-B and its co-factor p52 (Fig. 4a). Rel-B expression was increased in CC compared to NBC and CB. Along with the highest Rel-B expression in CC, western blot showed a significant decrease in p100 expression that positively correlated with an increase in the levels of its cleavage product p52. This result is expected since Rel-B/p52 is the major well-known heterodimer that mediates the alternative pathway signaling (Fig. 4a).

The centroblasts ChIP-Seq results showed a hierarchical Rel-B DNA-binding network as in HL. We assessed the BCL2, CDK6 and CCND3 mRNA levels in the naïve, centroblasts and centrocytes primary B-cell subsets.

Single-locus ChIP showed that Rel-B bound to CDK6 and CNND3 promoters in centroblasts (Fig. 4, b1–b2). CDK6 exhibited cRel-binding enrichment as well. On the other hand, no Rel-B enrichment was observed on BCL2 (Data not shown). The mRNA levels according to Qt-PCR showed the highest expression of CDK6 and CNND3 in CB with a significant difference compared to NBC and CC (Fig. 4c, c1–c2). The lowest BCL2 mRNA expression was observed in CB (Fig. 4c, c3).

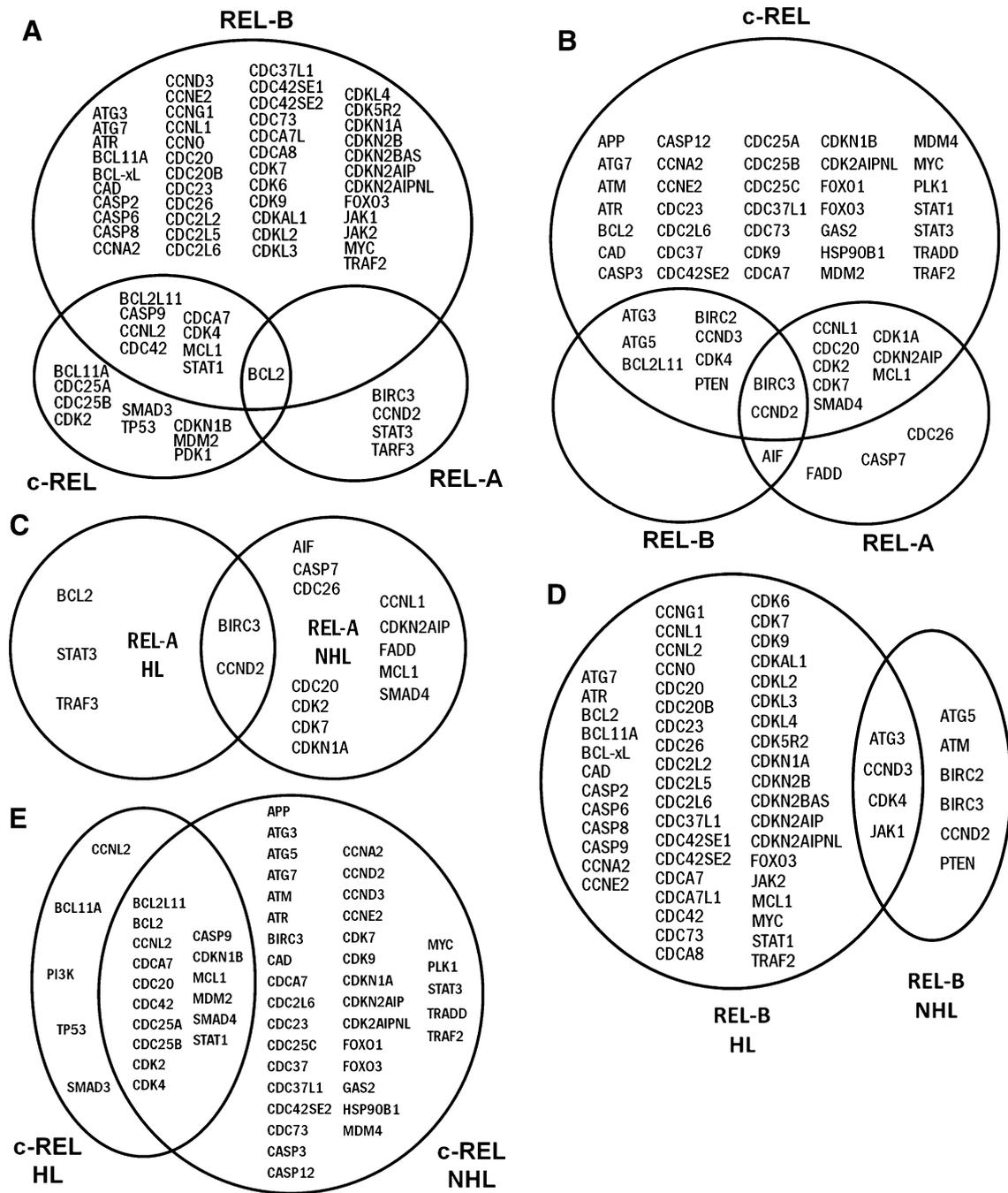


Fig. 1 Cell cycle and cell death genes signature. Venn diagrams depict genes with Rel-A, Rel-B and cRel binding in Hodgkin (a) and ABC-Like DLBCL (b) cells within a ±2 kb window from the gene transcription start site. The figure shows both Rel-A, Rel-B and cRel

uniquely bound genes, as well as genes that exhibited overlapping peaks. c–e Rel-A-, Rel-B- and cRel-bound genes that were shared by HL and DLBCL cells can be appreciated from the intersection of the corresponding Venn diagrams

Discussion

Despite extensive knowledge on the NF-κB biology, certain aspects are still not elucidated. It is well established that GC B-cell derived lymphomas frequently harbor genetic mutations which leads to the constitutive activation

of NF-κB signaling. Although insights into distinct roles for the NF-κB subunits during GC reaction have been reported (Klein and Heise et al. 2015), the role of each NF-κB family members in GC-originated lymphomas has not yet been determined.

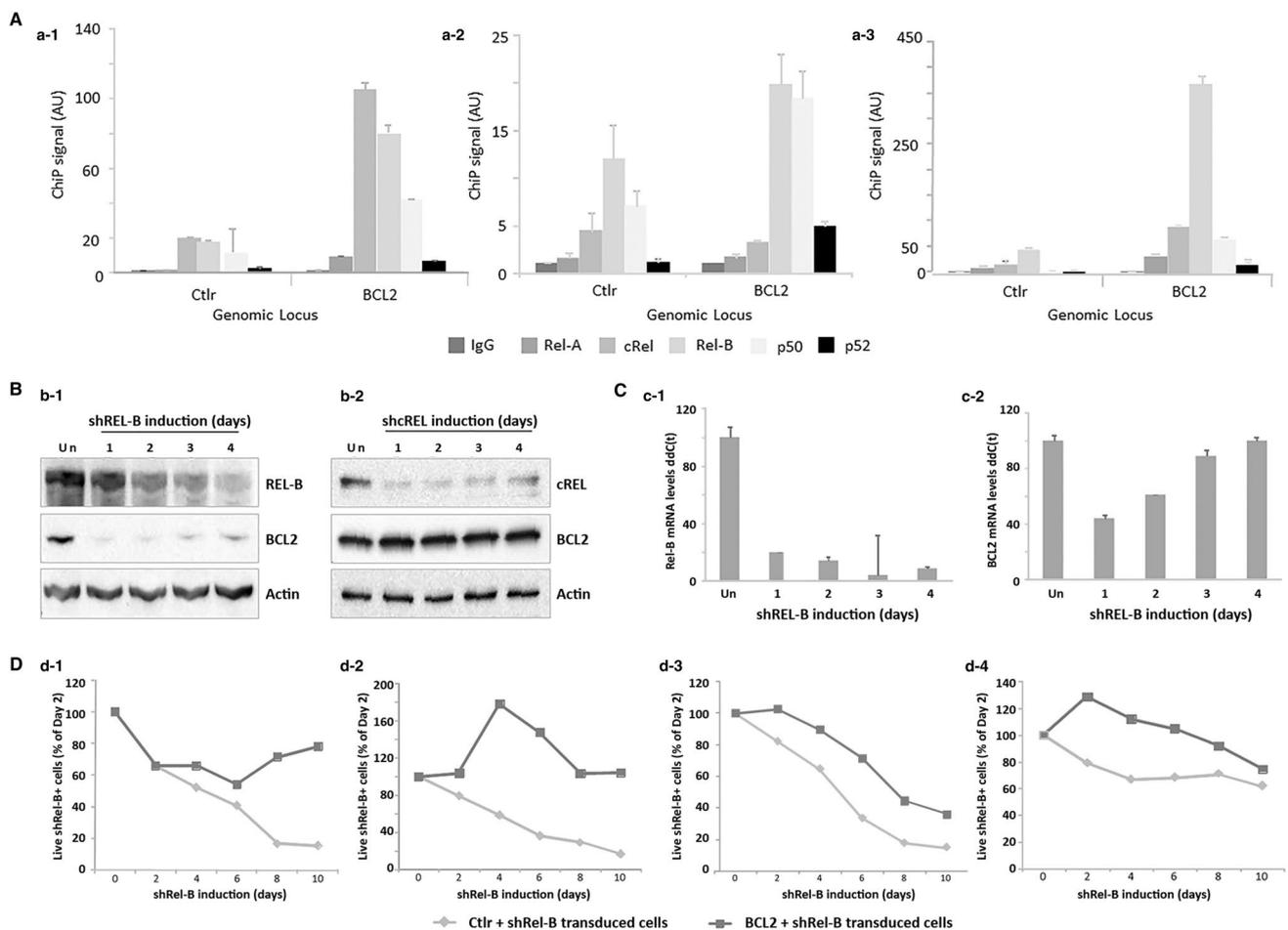


Fig. 3 Rel-B regulates CDK6, CCND3 and BCL2 expression in U-H01 HL cell line. **a** Single-locus ChIP confirmed the ChIP-Seq results and showed Rel-B binding enrichment on CDK6 (a-1), CCND3 (a-2) and BCL2 (a-3) gene promoters. Error bars represents the mean and SD. Three independent experiments were performed in triplicates. A representative assay is shown. **b** A significant decrease in CDK6, CCND3 and BCL2 protein level was observed in U-H01 cells following Rel-B depletion (b1) meanwhile their levels did not change upon cRel downregulation by a specific shRNA (b2). **c** The transcriptional control of the CDK6, CCND3 and BCL2 genes by

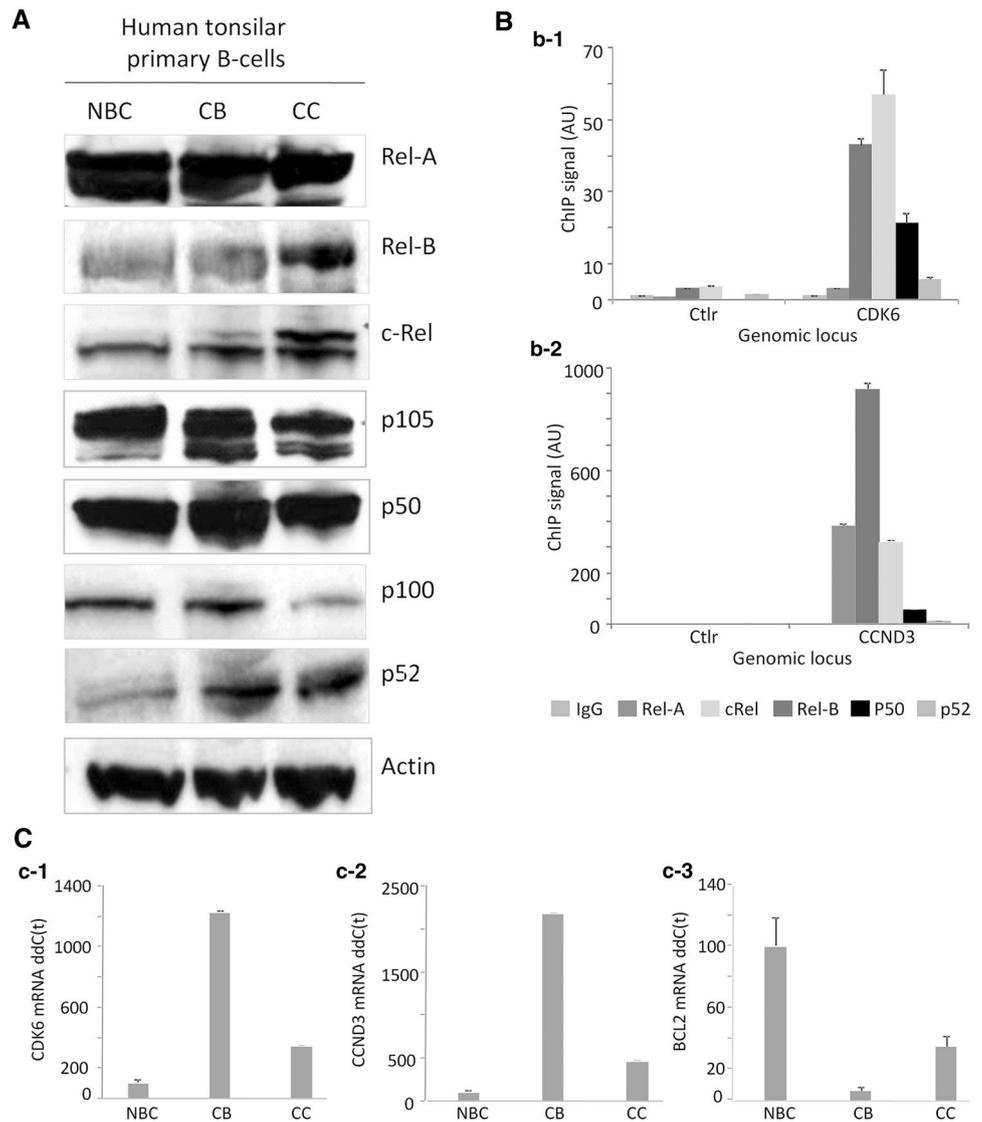
Rel-B as assessed by Qt-PCR, showed decreased mRNA levels in response to Rel-B depletion via a specific shRNA. Error bars mean SD. Three independent experiments were performed in triplicates. This is a representative assay (c1–c4). Single-locus ChIP shows cRel enrichment on these promoters in GCB-Like BJAB (**d**) and ABC-Like HBL-1 cell lines. Error bars mean SD. Three independent experiments were performed in triplicates. This is a representative assay (**e**). Results shown in A, B and C were performed in L1236 HL cell line in supplementary Fig. 1 (SF1)

2007). Western blot proved that Rel-B is responsible for the high expression of BCL2 in HL because Rel-B depletion significantly decreased its mRNA and protein levels (Fig. 3b, b2, c, c1–c2). BCL2 showed to be a key Rel-B downstream target gene since its exogenous expression was partially enough to rescue the cell death induced by Rel-B knockdown in HL cell lines. Although single locus ChIP showed cRel bound on BCL2 promoter in HL, its protein expression did not change in response to cRel depletion by a specific shRNA (Fig. 3b, b1). This shows that regardless of the overlap at the DNA-binding level, the classical and the alternative NF- κ B pathways, control different genes even within the same signature in HL and DLBCL (Figs. 1, 2).

Our findings reinforce the report that HRS cells originate from late-stage GC B-cells (Küppers and Rajewski 1998). The expression pattern of the Rel-B protein was accompanied by the acquisition of the p52 co-factor expression with a concomitant loss of its precursor NF- κ B2/p100 (Fig. 4a). ChIP-Seq analysis showed that Rel-B exhibited the highest number of peaks as compared to Rel-A and cRel in CB (Table 1). It has been reported that knockout mice for Rel-B or p52 fail to develop GCs (Weih et al. 2001).

Prior evidence for the alternative NF- κ B activity in HL can be inferred from EBV + HL tumors. It is thought that the expression of EBV proteins, LMP1 and LMP2, mimic the CD40 signaling that can activate both the canonical and

Fig. 4 Human Primary B cells isolated from human tonsil. **a** Expression of Rel-A, Rel-B, cRel, NFkB1/p105, p50, NFkB2/p100 and p52 proteins in naïve B cells (NBC), centroblasts (CB) and centrocytes (CC) was determined via western blotting. **b** Single-locus ChIP showed both Rel-B and cRel enrichment on CDK6 and CCND3 promoters. Error bars mean SD. Three independent experiments were performed. This is a representative assay. **c** Qrt-PCR showed enhanced CDK6 and CCND3 mRNA expression in centroblasts compared to that in naïve B cells and centrocytes. Regarding BCL2 mRNA the lowest level was found in centroblasts. Error bars mean SD. Three independent experiments were performed in triplicates. This is a representative assay



alternative pathways, with LMP1 specifically involved in the latter one (Weih et al. 1995). Another piece of data which suggests its potential involvement in HL, is the reported overexpression of NIK associated with tumorigenesis of Adult T-cell Leukemia and HRS cells (Kapatai and Murray 2007). We have previously shown the constitutive alternative NF- κ B signaling in HL cell lines due to NIK accumulation as a consequence of the TRAF2–TRAF3–cI κ A1–cI κ A2 complex disruption which causes NIK ubiquitination and consequently proteasome degradation failure (Ranuncolo et al. 2012).

Extensive literature supports that aberrant NF- κ B signaling plays an important role in B cell lymphomagenesis. It has been largely documented that structural alterations of the chr2p, which includes cRel and BCL11, is highly frequent in Hodgkin and HRS cells (Saitoh et al. 2008; Joos et al. 2002). The REL amplification leads to

nuclear accumulation of its protein (Martin-Subero et al. 2002). The HL dependence on both NF- κ B signaling arms might not be redundant since a crosstalk has been reported between the canonical and alternative pathways at different levels (Barth et al. 2003; Zarnegar et al. 2011). NIK is one of the molecules involved in this crosstalk. NIK was described as being primarily involved with the alternative pathway (Shih et al. 2011). Nevertheless, it has been reported that Rel-A-containing dimers can be activated through the NIK–IKK2 axis when NIK protein accumulates. The crosstalk is an aspect that might also affect therapeutic strategies for cancer treatments. The accumulation of NIK in the HRS cells could be the event that first determines the constitutive activation of the alternative pathway and that could further contribute to the miss-regulation of the canonical arm after exceeding an accumulation threshold.

The Rel-A/cRel, Rel-A/p50 or cRel/p50 (which represent the most frequent NFkB canonical dimers), overlap peaks, represented a low percentage in all cell lines and in CB. The same was observed when analyzing the Rel-B/p52 (which mediates the alternative pathway) overlap peaks. This result suggests that the NFkB factors might recruit other partners to induce gene expression which has been previously proposed by different studies (Kerr et al. 1993; de la Paz et al. 2007). In this regard, other DNA-binding proteins have been described as being able to specifically recognize kB-like sites motifs (Wong et al. 2011; Badis et al. 2009). Strikingly, the search for canonical kB DNA-binding sites GGG ACTTTC in the NFkB subunits peaks, indicated that a low percentage showed a canonical binding site. When allowing up to three mismatches in the decameric sequence, a larger number of peaks showed potential binding sites. The NF-kB family members might be part of molecular complexes larger than the expected homo- and heterodimers. This finding implies that novel protein–protein interactions and NF-kB factors are recruited to promoters by other factors. Binding to a “nonperfect” site leads to the possibility of being prompt release; which may be important for the rapid control of gene expression as it might be the case during GC B-cell transit.

The data reported herein contributes to the knowledge that different B-cell lymphomas are characterized by a preferential activity of the canonical, the alternative or both NF-kB signaling arms simultaneously. This specificity should be taken into account for the development of therapies that are aimed at selectively inhibit the NF-kB signaling pathway or even interfere particular subunits of this TFs family that are involved in the pathogenesis of a GC-derived B-cell lymphoma subtype.

Author contributions AMGC and MC performed experiments, WX analyzed the ChIP-Seq data and developed the algorithm for this purpose. TAW discussed results. SMR performed experiments, analyzed data, discussed results and wrote the manuscript.

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Data availability Availability of data and materials: Expression arrays data have been deposited in the NCBI GEO database under the GSE109803 accession number. ChIP-Seq data have been deposited in the NCBI GEO database under the GSE110348 accession number and can be found at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110348>. All gene lists that are derived from our analysis of the ChIP-Seq and expression arrays experiments, as well as more detailed protocols, are available upon request.

Compliance with ethical standards

Conflict of interests The authors declare no competing interests.

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