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Overexpression of an activated REL mutant enhances the transformed state of the human B-lymphoma BJAB cell line and alters its gene expression profile

M Chin[1], **M Herscovitch**[1], **N Zhang**, **DJ Waxman**, and **TD Gilmore**[*]

Department of Biology, Boston University, Boston, MA 02215, USA

Abstract

The human *REL* proto-oncogene encodes a transcription factor in the NF-κB family. Overexpression of REL is acutely transforming in chicken lymphoid cells, but has not been shown to transform any mammalian lymphoid cell type. In this report, we show that overexpression of a highly transforming mutant of REL (RELΔTAD1) increases the oncogenic properties of the human B-cell lymphoma BJAB cell line, as demonstrated by increased colony formation in soft agar, tumor formation in SCID mice, and adhesion. BJAB-RELΔTAD1 cells also show decreased activation of caspase in response to doxorubicin. BJAB-RELΔTAD1 cells have increased levels of active nuclear REL protein as determined by immunofluorescence, subcellular fractionation, and electrophoretic mobility shift assay. Overexpression of RELΔTAD1 in BJAB cells has transformed the gene expression profile of BJAB cells from that of a germinal center B-cell subtype of diffuse large B-cell lymphoma (GCB-DLBCL) to that of an activated B-cell subtype (ABC-DLBCL), as evidenced by increased expression of many ABC-defining mRNAs. Up-regulated genes in BJAB-RELΔTAD1 cells include several NF-κB targets that encode proteins previously implicated in B-cell development or oncogenesis, including *BCL2*, *IRF4*, *CD40* and *VCAM1*. The cell system we describe here may be valuable for further characterizing the molecular details of REL-induced lymphoma in humans.

Keywords

c-Rel; NF-kappaB; malignant transformation; BJAB; lymphoma; microarray

Introduction

The human c-*rel* proto-oncogene (*REL*) encodes an NF-κB family transcription factor. Misregulated *REL* is associated with B-cell malignancies in several ways (Gilmore *et al.*, 2004). Overexpression of REL protein can transform chicken lymphoid cells *in vitro*. Additionally, the *REL* locus is amplified in several types of human B-cell lymphoma, including diffuse large B-cell lymphoma (DLBCL), follicular lymphoma and primary

[*]Correspondence: TD Gilmore, Biology Department, Boston University, 5 Cummington Street, Boston, MA 02215, USA; 617-353-5444 (phone); 617-353-6340 (fax); gilmore@bu.edu.

[1]These authors contributed equally to this work

mediastinal lymphoma. Moreover, *REL* mRNA is highly expressed in *de novo* DLBCLs, and this elevated expression correlates with increased expression of many putative REL target genes (Rhodes *et al.*, 2005). Nevertheless, REL has not been shown to be oncogenic in any mammalian B-cell system, either *in vitro* or *in vivo*.

REL contains an N-terminal Rel Homology Domain, which mediates DNA binding, dimerization, nuclear localization, and binding to its inhibitor IκB. The C-terminal half of REL contains a transactivation domain, which can be divided into two subdomains (Martin *et al*., 2001; Starczynowski *et al.*, 2003). Deletion of either C-terminal transactivation subdomain enhances the *in vitro* transforming activity of REL in chicken spleen cells (Starczynowski *et al.*, 2003). Similarly, v-Rel lacks a transactivation subdomain found in avian c-Rel, and this deletion contributes to v-Rel's increased transforming activity as compared to c-Rel (Gilmore, 1999). In addition, deletions and mutations that alter the REL transactivation domain have been identified in a small percentage of human B-cell lymphomas, and one such mutation can enhance the transforming activity of REL in chicken lymphoid cells (Kalaitzidis and Gilmore, 2002; Barth *et al.*, 2003; Starczynowski *et al.*, 2007). Nevertheless, REL's role in mediating oncogenesis in mammalian cells is not clear.

Herein, we show that overexpression of a REL mutant lacking transactivation subdomain 1 (RELΔTAD1) enhances certain "transformed" properties of the human B-lymphoma cell line BJAB. Furthermore, RELΔTAD1-transformed BJAB cells have an altered gene expression profile that is consistent with them having been converted to a more aggressive form of DLBCL. As such, these results are the first direct demonstration that REL can contribute to human B-cell oncogenesis and describe an *in vitro* system for studying oncogenic conversion of B-cell lymphoma.

Results

Overexpression of RELΔTAD1 increases oncogenic properties of BJAB cells

A REL mutant (RELΔ424-490, or RELΔTAD1 herein) that is missing the first C-terminal transactivation subdomain has an enhanced ability to transform primary chicken spleen cells *in vitro* as compared to wild-type REL (Starczynowski *et al.*, 2003). In an effort to establish a human cell assay for REL-induced oncogenesis, we first created an MSCV-based retroviral vector for expression of RELΔTAD1; as a control for our experiments, we used the MSCV vector backbone that contains only the puromycin resistance gene (Figure 1a).

Retroviral stocks of MSCV and MSCV-RELΔTAD1 were used to infect human B-lymphoma BJAB cells, and these cells were then selected for puromycin resistance to establish stable pools of retrovirally transduced cells. By Western blotting, we identified a pool of MSCV-RELΔTAD1-transduced cells that expresses high levels of RELΔTAD1, which migrates faster than full-length endogenous REL (Figure 1b). The expression of RELΔTAD1 is approximately 2.4-fold greater than endogenous REL, which is expressed at approximately equal levels in both MSCV-RELΔTAD1-transduced cells and control MSCV-transduced cells. The expression of RELΔTAD1 was stable during more than six months of continued passage of MSCV-RELΔTAD1-transduced cells (not shown).

To determine whether overexpression of RELΔTAD1 affects oncogenic properties of the BJAB cell line, we first compared the soft agar colony-forming abilities of MSCV-RELΔTAD1 cells and MSCV-transduced cells. As shown in Figure 1c, BJAB-RELΔTAD1 cells had an approximately 2.3-fold increased ability to form colonies in soft agar as compared to BJAB-MSCV cells. Moreover, colonies formed by BJAB-RELΔTAD1 cells were generally larger than those formed by BJAB-MSCV cells (not shown). Similarly, BJAB-RELΔTAD1 cells had increased tumor-forming ability in SCID mice (Table 1). Nevertheless, the growth rates of BJAB-MSCV and BJAB-RELΔTAD1 cells in liquid media were similar (Figure 1d). Doxorubin-induced activation of caspase-3 and cleavage of the caspase substrate PARP are delayed in BJAB-RELΔTAD1 cells as compared to BJAB-MSCV cells; however, there is no difference in the ability of doxorubicin to decrease viability in these two cell types (Figure 1e).

RELΔTAD1-expressing BJAB cells have increased nuclear REL protein activity

As a first step towards determining the basis for the enhanced transformed properties of BJAB-RELΔTAD1 cells, we characterized RELΔTAD1 protein in these cells. By biochemical subcellular fractionation, BJAB-RELΔTAD1 cells showed increased nuclear REL protein—for both RELΔTAD1 and endogenous REL—as compared to BJAB-MSCV cells, in which the low level of endogenous REL is almost exclusively cytoplasmic (Figure 2a). As controls for these fractionation experiments, we show that two cytoplasmic proteins (CD40 and 14-3-3) and a nuclear protein (RNA polymerase) are exclusively present in their respective fractions in both cell types. Indirect immunofluorescence showed that BJAB-RELΔTAD1 cells have increased overall REL staining as compared to BJAB-MSCV cells and also have detectable nuclear REL staining (Figure 2b), which is not seen in BJAB-MSCV cells. Nuclear extracts from BJAB-RELΔTAD1 cells also have increased levels of NF-κB p50, but not of RelA (Figure 2c).

BJAB-RELΔTAD1 cells show increased nuclear κB-site DNA-binding activity as compared to BJAB-MSCV cells (Figure 2d). The κB site-binding activity in BJAB-RELΔTAD1 cells was competed by the relevant unlabelled probe and was almost completely supershifted by anti-REL antiserum. Therefore, by three criteria, nuclear REL protein is increased in BJAB-RELΔTAD1 cells as compared to control BJAB-MSCV cells.

In coimmunoprecipitations from BJAB cells, REL and RELΔTAD1 interact equally well with IκBα, suggesting that the changes in DNA binding and nuclear localization seen in BJAB-RELΔTAD1 cells are not due to changes in association with IκB (Supplementary Figure S1).

The expression of many known REL/NF-κB target genes is increased in RELΔTAD1-expressing BJAB cells

We next compared the overall gene expression profiles of BJAB-RELΔTAD1 cells and BJAB-MSCV cells by using an extensive human microarray, which contains over 41,000 probes, representing unique gene products. Using a two-fold change and *P*-value of less than 0.005 (Holloway *et al*., 2008), we found that 538 mRNAs were decreased and 663 mRNAs were increased in BJAB-RELΔTAD1 cells (Supplementary Table S1). The levels of 67

transcripts were increased at least ten-fold in BJAB-RELΔTAD1 cells (Table 2). Serving as an internal control, *REL* mRNA showed approximately 25-fold increased expression in BJAB-RELΔTAD1 cells, presumably because the *REL* probe on the microarray can detect both endogenous *REL* and exogenous RELΔTAD1 mRNA/cDNA.

Several mRNAs that show greatly elevated expression in BJAB-RELΔTAD1 cells are known REL/NF-κB targets, including *CXCR7* (77-fold increase), *IRF4* (32-fold), *CD44* (26-fold), *VCAM1* (24-fold), chemokine *CCL22* (21-fold) and the anti-apoptotic protein *BCL2* (13.5-fold). However, out of approximately 400 reported REL/NF-κB targets (see www.nf-kb.org), only approximately 4% were at least two-fold elevated in BJAB-RELΔTAD1 cells, 94% were unchanged, and 2% were decreased by at least two-fold.

Based on cDNA profiling, DLBCLs have been divided into two main subtypes: GCB (germinal center B-cell type) and ABC (activated B-cell type) (Alizadeh *et al*., 2000; Rosenwald *et al*.,2002; Shipp *et al*., 2002; Wright *et al*., 2002; Ngo *et al*., 2006). This division is based on the observation that one subset of DLBCLs has a gene expression profile similar to B lymphocytes in the germinal center while another subset has a gene expression profile similar to activated peripheral B cells (Alizadeh *et al*., 2000). Furthermore, the ABC subtype has increased expression of several NF-κB target genes as compared to the GCB subtype, and survival of ABC cell lines depends on expression of these NF-κB target genes (Davis *et al*., 2001, Lam *et al*., 2008). BJAB cells have a gene expression profile that is consistent with the GCB subtype (Kalaitzidis *et al*., 2002; Ngo *et al*., 2006). Using the literature, we assembled a comprehensive set of genes that have been used to define these two subsets of DLBCL: 102 for ABC and 62 for GCB (see Tables S2 and S3 for details). We then compared the levels of these ABC- and GCB-defining targets between BJAB-MSCV cells and BJAB-RELΔTAD1 cells, using a *P*-value < 0.005 as a cut-off. Overall, 30% of the 102 ABC-profile genes were upregulated in the BJAB-RELΔTAD1 cells (Table 3). We also found that BJAB cells overexpressing RELΔTAD1 showed increased expression of many of the ABC-defining genes that are NF-κB targets (Figure 3a): 17/29 (59%) ABC-specific NF-κB target genes were upregulated in BJAB-RELΔTAD1 cells (Table 3). Using the same filter criteria ($P<0.005$), only 6% of total transcripts showed increased expression in BJAB-RELΔTAD1 cells as compared to BJAB-MSCV cells.

We also found that 24% of the GCB-defining genes were downregulated in BJAB-RELΔTAD1 cells as compared to BJAB-MSCV cells (Table 3). In contrast, only 9% of the total transcripts were downregulated in BJAB-RELΔTAD1 cells.

A statistical comparison of the percent change in ABC-subtype genes (30% upregulated, 12% downregulated) versus GCB-subtype genes (10% upregulated, 24% downregulated) in BJAB-RELΔTAD1 cells (as compared to BJAB-MSCV cells) indicates that these two gene sets are affected in a significantly different manner (*P*-value, 0.0009; see Table 3).

To further analyze our gene expression data, we used Gene Ontology (http://david.abcc.ncifcrf.gov/) to categorize genes upregulated in BJAB-RELΔTAD1 cells. We focused on upregulated genes because REL is primarily a transcriptional activator. Using this analysis, we were able to classify 563 of the 663 upregulated genes (>2-fold, $P<0.005$)

in BJAB-RELΔTAD1 cells; many of these upregulated genes encode proteins associated with cell surface processes/regions, including ones involved in cell-cell communication, the plasma membrane, the extracellular matrix, biological adhesion and signal transduction in general (Table 4).

In addition, we classified this same set of upregulated genes in BJAB-RELΔTAD1 cells by their biological function (www.ingenuity.com); by this analysis we were able to classify 421 of 663 significantly upregulated genes. This analysis was consistent with our Gene Ontology annotation. Namely, over-represented molecular and cellular functions included those involved in cell-to-cell communication and cell growth and proliferation (Table 4). Furthermore, many genes (75 out of 421 annotated) that are statistically over-represented have been associated with immunological diseases (Table 4).

We next used RT-PCR to validate a subset of genes showing increased expression in BJAB-RELΔTAD1 cells. As controls, we used a primer set that could amplify both endogenous REL and RELΔTAD1 to show that *REL* mRNA expression is increased in BJAB-RELΔTAD1 cells as compared to BJAB-MSCV cells, whereas *GAPDH* expression is similar in both cell types (Figure 3b). Consistent with the microarray results, there was increased expression of *BCL2*, *CCR7*, *IRF4* and *VCAM1* mRNA in BJAB-RELΔTAD1 cells. In contrast, *CD10*, a marker for GCB-type DLCBL (van Imhoff *et al*., 2006), showed reduced mRNA expression in BJAB-RELΔTAD1 cells. Western blotting showed that protein levels of BCL2, VCAM1, CD40 and REL are all elevated in BJAB-RELΔTAD1 cells (Figure 3c), whereas CD10 protein is reduced in BJAB-RELΔTAD1 cells (Figure 3c). For CD40, the small (1.4-fold), but significant ($P \leq 8.97 \times 10^{-11}$), increase in *CD40* mRNA in BJAB-RELΔTAD1 cells seen on the microarray was mirrored by an approximately 1.4-fold increase in CD40 protein.

BJAB-RELΔTAD1 cells show increased adherence to culture dishes

During passage, we noticed that BJAB-RELΔTAD1 cells appeared to adhere more readily to culture plates than BJAB-MSCV cells. To compare the abilities of BJAB-MSCV and BJAB-RELΔTAD1 cells to adhere, we plated both cell types on petri dishes, and cultured the cells for 36 h. We then visualized these cells before and after washing with PBS. As shown in Figure 4a, many BJAB-RELΔTAD1 cells remained attached to the culture dish after washing, whereas the BJAB-MSCV cells were removed by washing. We quantified this difference in adherence by comparing the numbers of floating versus adhering cells for each cell type: approximately five-fold more BJAB-RELΔTAD1 cells were attached to the dish as compared to the BJAB-MSCV cells (Figure 4b).

BJAB cells have low levels of endogenous REL protein expression

BJAB cells have previously been shown to have a low level of REL mRNA as compared to a number of other lymphoma cell lines (Leeman *et al.*, 2008). To determine whether REL protein expression was also low in BJAB cells, we compared the expression of endogenous REL protein in BJAB cells to five other human B-cell lymphoma cell lines (SUDHL-4, RC-K8, IB4, BL41 and Daudi). SUDHL-4 cells have been characterized as having a GCB profile, whereas RC-K8 cells have an ABC cDNA expression profile (Kalaitzidis *et al.*,

2002). Among these six lymphoma cell lines, the expression of REL was lowest in BJAB cells (Figure 5a). As such, in BJAB cells, retrovirally transduced expression of RELΔTAD1 is higher than endogenous REL, whereas in Daudi cells, RELΔTAD1 expression is lower than endogenous REL (Figure 5b). Moreover, expression of RELΔTAD1 did not enhance the soft agar colony ability of Daudi cells (Figure 5c), at least when expressed at the level in the cell line we analyzed here.

Discussion

This paper represents the first direct demonstration of an oncogenic effect of REL protein expression in a human B-lymphoid cell system. That is, we show that overexpression of an activated REL mutant, RELΔTAD1, increases the oncogenic properties of the human B-cell lymphoma BJAB cell line, as measured by increased soft agar colony-forming ability, tumor formation in immunocompromised mice, and adhesion. Moreover, the mRNA expression profile of BJAB cells overexpressing RELΔTAD1 is substantially altered; in particular, there is increased expression of many NF-κB target genes whose expression is associated with the more aggressive ABC subtype of diffuse large B-cell lymphoma. Furthermore, many of the up-regulated genes in BJAB-RELΔTAD1 cells can be classified as genes implicated in immunological diseases (Table 4), suggesting that BJAB-RELΔTAD1 cells have a phenotype that is more similar to aggressive DLBCL than is the GCB-like phenotype of control BJAB cells. As such, the cell system we describe here may provide an *in vitro* model system for understanding DLBCL transition from a low-grade (GCB-like) to a high-grade (ABC-like) oncogenic state.

Although v-Rel, c-Rel and their derivatives have been shown to be oncogenic in avian and mouse systems (Gilmore, 1999; Gilmore *et al.*, 2004), there has been controversy about whether REL is a true oncoprotein for human B-lymphoid cells (Shaffer *et al*., 2002; Houldsworth *et al.*, 2004). For example, the *REL* gene is amplified in a high percentage of GCB-type DLBCLs, but these cells do not have particularly high levels of nuclear κB site-binding activity (Davis *et al*., 2001). Moreover, the lack of oncogenic activity by overexpressed REL in mouse B-lymphoid cells *in vitro* or *in vivo* has cast doubt on whether REL acts as an oncoprotein in human B-cell malignancies, which are the sole human cancer cell type wherein the *REL* gene has been found to undergo amplification and mutation (Gilmore *et al*., 2004). The results we present herein strongly suggest that REL can exert an oncogenic effect in human B-lymphoma cells, and indicate that REL or certain REL target genes may be suitable therapeutic targets for some human B-cell lymphomas.

There are several likely explanations for the susceptibility of BJAB cells to the transforming activity of RELΔTAD1. First, BJAB cells express relatively low levels of endogenous REL protein (Figure 5a) as compared to several other human B-lymphoma cell lines. Thus, in BJAB cells it is possible to achieve a higher ratio of RELΔTAD1 protein to endogenous REL, and this relatively high level of RELΔTAD1 may be required for its transforming effect in human B cells. Second, BJAB cells have a GCB mRNA profile (Ngo *et al*., 2006), which is correlated with a better clinical outcome in DLBCL patients (Rosenwald *et al*., 2002; Shipp *et al*., 2002), suggesting that BJAB cells are not as “transformed” as some other human B-cell lines. Third, in soft agar and tumor-forming assays similar to those we have

conducted here, BJAB cells have been shown to be susceptible to oncogenic effects of other factors, including the Epstein-Barr virus (EBV) LMP1 protein (Enberg *et al*., 1983; Wennborg *et al*., 1987), EBV small RNAs (Yamamoto *et al*., 2000) and the AP12-MALT1 fusion protein from MALT lymphomas (Ho *et al.*, 2005). Interestingly, LMP1 and AP12-MALT1 are both inducers of NF-κB (Hammarskjold *et al*., 1992; Lucas *et al*., 2007) and both can increase the resistance of BJAB cells to inducers of apoptosis (Stoffel *et al*., 2004; Ho *et al*., 2005; Lucas *et al*., 2007). In addition, LMP1 can induce expression of BCL2 and IRF4, which are required for apoptosis resistance (Henderson *et al.*, 1991; Finke *et al*., 1992; Snow *et al*., 2006), enhanced adhesion (Mainou and Raab-Traub, 2006), and cell motility (Mainou and Raab-Traub, 2006). Moreover, *MALT1* chromosomal gains are also associated with ABC-subtype gene expression, including high levels of BCL2 expression and poorer prognosis (Dierlamm *et al*., 2008).

Many of the upregulated genes in BJAB-RELΔTAD1 cells are connected with processes that involve the plasma membrane, i.e., cell-to-cell communication, the extracellular matrix, adhesion, and membrane binding (see Table 4). These genes include *VCAM1, CD44, CD40, ITGAX*, and many chemokines and chemokine receptors including *CCL22*, *CCR7, CXCR4* and *CXCL10*. Additionally, BJAB-RELΔTAD1 cells are more adherent to a culture dish than control BJAB-MSCV cells (Figure 4). This is consistent with the large cohort of increased cDNAs in RELΔTAD1 cells that are classified as related to adhesion (Table 4). NF-κB signaling is also known to be downstream of many adhesion-related signaling pathways (Perez *et al*., 1994; Lee *et al*., 1999; Zarnegar *et al*., 2004). Furthermore, CD40 and VCAM1 mRNA and protein expression are upregulated in the BJAB-RELΔTAD1 cells. While *CD40* mRNA was only modestly increased (1.4-fold) in BJAB-RELΔTAD1 cells, this did translate into similarly increased CD40 protein levels (Figure 3c). CD40 has been shown to be important in B-cell aggregation (Lee *et al*., 1999), and both VCAM1 and CD40 play roles in adhesion (Springer and Vonderheide, 1992; Lee *et al*., 1999). Taken together, these results suggest that overexpression of RELΔTAD1 in BJAB cells causes upregulation of many adhesion-associated genes, which results in a phenotype of the cells being more adherent, which may contribute to their enhanced ability to form colonies in soft agar and tumors in SCID mice.

BCL2 and *IRF4*, genes whose expression is up-regulated in BJAB-RELΔTAD1 cells, are markers for ABC DLBCL, whereas *CD10* is down-regulated in both ABC DLBCLs and BJAB-RELΔTAD1 cells (Alizadeh *et al*., 2000; Wright *et al*., 2003). The increased expression of *BCL2* in ABC DLBCLs correlates with a poorer clinical prognosis (Iqbal *et al.*, 2006). The transcription factor IRF4 can synergize with v-Rel in the transformation of chicken fibroblasts and knockdown of IRF4 expression reduces the soft agar colony-forming ability of v-Rel-transformed cells (Hrdlicková *et al*., 2001). Of note, multiple myelomas are dependent on IRF4 for growth, whereas the growth of GCB-DLCBL does not require IRF4 (Shaffer *et al*., 2008). Taken together, these results are consistent with BCL2 and IRF4 playing a role in the enhanced transformed phenotype that we describe for BJAB-RELΔTAD1 cells.

We also found that many other ABC-defining genes (including several not known to be NF-κB targets) are significantly upregulated in BJAB-RELΔTAD1 cells. These ABC genes

include *MARCKS*, *BATF*, *BMI1*, *LITAF* and others (see Tables 2 and S2). Some of these ABC-type upregulated genes may reflect an overall shift in gene expression, induced indirectly by NF-κB/REL. In addition, some GCB-subtype genes are significantly down-regulated in BJAB-RELΔTAD1 cells (Tables 3 and S3). These genes are, for the most part, non-NF-κB targets, suggesting that these decreases in GCB-type gene expression are also indirectly affected by RELΔTAD1.

Approximately 4% of total NF-κB targets (www.nf-kb.org) were up-regulated in BJAB-RELΔTAD1 cells as compared to 59% of ABC-specific NF-κB targets (Table 2). The selective increase in expression of only a small number of NF-κB target genes in BJAB-RELΔTAD1 cells suggests that the BJAB cells have been transformed to a more aggressive form of DLBCL by RELΔTAD1 through activation of a minor subset of NF-κB/REL targets. These ABC-specific NF-κB target genes may be poised for activation by RELΔTAD1 in B-lymphoma cells, possibly due to their chromosomal state or to cooperation of RELΔTAD1 with other B cell-specific transcription factors.

There are 40 genes whose expression is reduced by at least ten-fold in BJAB-RELΔTAD1 cells (Table S1). The reduced expression of CD10 mRNA and protein in BJAB-RELΔTAD1 cells (Figure 3) is consistent with the enhanced transformed properties of these cells, given that reduced CD10 expression correlates with a poorer prognosis in the clinic (van Imhoff *et al*., 2006). Gupta *et al.* (2008) have shown that expression of two B-cell proteins BLNK and BCAP are down-regulated directly by Rel in v-Rel-transformed avian cells. In our study, the level of only BLNK was significantly reduced in BJAB-RELΔTAD1 cells. Such results raise the possibility that down-regulation of gene expression is important for REL-induced effects on B-cell oncogenesis, and that some of these genes are specifically repressed by RELΔTAD1.

BJAB-RELΔTAD1 cells show a reduced induction of caspase-3 activity following treatment with 1 μg/ml doxorubicin, although the ability of doxorubicin to decrease viability is unchanged in BJAB-RELΔTAD1 cells (Figure 1e). These data are consistent with previous results showing that CD40 ligand, an inducer of NF-κB, can reduce the ability of this concentration of doxorubicin to induce caspase activity in BJAB cells without affecting its ability to induce apoptosis (Voorzanger-Rousselot *et al.*, 1998). These results indicate that doxorubicin induces apoptosis in BJAB cells through a caspase-independent mechanism, which is not blocked by increased Rel/NF-κB activity.

The majority of the nuclear κB site-binding activity in RELΔTAD1-BJAB cells contains REL protein, whereas in control BJAB-MSCV cells, only a small fraction of the binding activity is supershifted by REL antiserum (Figure 2d). In addition, there are increased nuclear levels of NF-κB p50 in BJAB-RELΔTAD1 cells, presumably because RELΔTAD1 and p50 readily interact (Figure S1). Taken together, these data suggest that a shift in the composition of nuclear NF-κB/REL dimers occurs upon overexpression of RELΔTAD1.

Only a small number of the genes upregulated by more than ten-fold in BJAB-RELΔTAD1 cells are ABC-defining (5 genes) or known NF-κB targets (8 genes) (Table 2). As such, some of these genes may be novel ABC DLBCL markers or NF-κB/REL targets. In

addition, there are 14 ABC-defining genes that are significantly up-regulated in BJAB-RELΔTAD1 cells that have yet to be classified as NF-κB/REL targets (Tables 3 and S2). Future work will be directed at determining which genes are direct RELΔTAD1 targets and which contribute to the phenotypic changes that occur in RELΔTAD1 "transformed" BJAB cells.

Material and methods

Plasmids, cell culture and infections

pMSCV has been described previously (Gilmore *et al*., 2003). pMSCV-RELΔTAD1 was created by subcloning a *Bgl*II to *Xho*I fragment containing the RELΔTAD1 cDNA into pMSCV.

Human A293T cells and BJAB or Daudi lymphoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 or 20% fetal bovine serum (Biologos, Montgomery, IL), respectively, as described (Starczynowski *et al.*, 2005). Virus stocks were generated by transfecting A293T cells with pMSCV or pMSCV-RELΔTAD1 plus helper plasmid pcL10a1, essentially as described previously (Gilmore *et al*., 2003). Approximately two days later, virus was harvested. One ml of virus (in the presence of 4 μg/ml polybrene) was used to infect 10^6 BJAB or Daudi cells using the spin infection method (Gilmore *et al*., 2003). Two days later, cells were selected with 2.5 μg/ml puromycin (Sigma) for 2-4 weeks.

Soft agar colony assays and tumor formation assays

For soft agar assays, equal numbers of the indicated BJAB or Daudi cells (250, 500, 1000 or 2000 cells) were placed in soft agar containing DMEM, 20% FBS and 0.3% bacto agar (Difco, Franklin Lakes, NJ), and plates were placed at 37°C in a humid incubator with 5% CO_2. To confirm cell counts, total cell protein assays (Bio-Rad) were performed on the cell dilutions used for plating. Macroscopic soft agar colonies were counted 14 days after plating.

Tumor studies were performed essentially as described previously (Yamamoto *et al*., 2000; Gapuzan *et al*., 2002). 5×10^6 cells were injected subcutaneously into SCID mice (Taconic Farms, Germantown, NY). Once tumors appeared, mice were monitored 3× weekly and animals were sacrificed when tumors reached 2.25 mm^2. All animal studies were performed in accordance with NIH guidelines and with approval of the Boston University Institutional Animal Care and Use Committee.

Caspase-3 and cell viability assays

Caspase-3 activity and cell viability following doxorubicin treatment were performed as described in Supplementary Material.

Western blotting, indirect immunofluorescence, biochemical fractionation and electrophoretic mobility shift assays

Western blotting and indirect immunofluorescence were performed as described previously (Starczynowski *et al.*, 2003, 2005). Details of antisera are in Supplementary Material. Indirect immunofluorescence was visualized using a confocal microscope (Olympus FLUOVIEW Laser Scanner Microscope BX 50, Center Valley, PA) (Starczynowski *et al.*, 2003).

Cytoplasmic and nuclear extracts were prepared as described previously (Liang *et al.*, 2003), and were used either for Western blotting of equalized fractions or in electrophoretic mobility shift assays (nuclear extracts). EMSAs for κB-site binding were performed using 5 μg of nuclear extracts as described previously (Kalaitzidis *et al.*, 2002). For supershift assays, 1 μl of REL antiserum (#1507, gift of Nancy Rice) was added after protein/DNA complex formation, and samples were then incubated for an additional hour on ice (Kalaitzidis *et al.*, 2002).

mRNA analysis: microarrays, data analysis and reverse transcriptase-PCR

The Agilent Whole Human Genome Microarray platform (product number G4112, Agilent Technology, Santa Clara, CA). This array contains 43,376 human oliognucleotide probes and also 1,468 positive controls and 153 negative controls. Within the array there are approximately 41,000 unique probes, which represent a smaller number of genes, reflecting the redundancy of the array platform. RNA was isolated from ~ 5×10^6 BJAB-MSCV and BJAB-RELΔTAD1 cells from four separate dishes for each on four separate days using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). RNA integrity was measured using an Agilent 2100 Bioanalyzer (Agilent Technology, Santa Clara, CA), and all samples had integrity values over 8.0. Samples from two RNA aliquots for each cell type were pooled, creating four pooled RNA samples: two of BJAB-MSCV and two of BJAB-RELΔTAD1 cells. Sample labeling, hybridization to microarrays, scanning and calculation of normalized expression ratios were performed as previously described (Holloway *et al*., 2008) at the Wayne State University Institute of Environmental Health Sciences microarray facility. As part of the platform, a dye swapping experiment was performed, where Alexa 555-labeled cDNA from one of the BJAB-MSCV pools was mixed with Alexa 647-labeled cDNA from one of the BJAB-RELΔTAD1 pools. In a reciprocal dye swap, Alexa 647-labeled cDNA from BJAB-MSCV cells was mixed with Alexa 555-labeled cDNA from BJAB-RELΔTAD1 cells.

The false discovery rate (FDR) was calculated as described previously (Clodfelter *et al*., 2007). Briefly, a filter of $P<0.005$ was applied for statistical significance. Of the total probes on the array, 1592 met the two-fold expression difference cut-off criterion between the two cell types. The number of genes predicted to meet the combined threshold ($P<0.005$ and a greater than two-fold change in expression) by type I errors is 0.005×1592, or 8 genes. In our array, the actual number of genes having a two-fold expression change and a $P<0.005$ is 1274. This corresponds to an FDR of 0.63% (8/1274). To eliminate duplicates in this analysis, we removed those genes with identical sequence names.

RT-PCR was performed as described (Leeman *et al.*, 2008). See Supplementary Material for details of primers and PCR conditions.

Adhesion assay

BJAB-MSCV and BJAB-RELΔTAD1 cells (1×10^6) were plated on petri dishes and were cultured for 36 h at 37°C and imaged. Cells were then washed once with PBS and the same field was imaged again using the same magnification (200 ×). To quantify the number of attached and floating cells, cells from triplicate dishes of each cell type were also isolated directly from the media and cells that remained adhered were collected separately. Both pools of cells were then lysed, and total protein was quantified from these lysates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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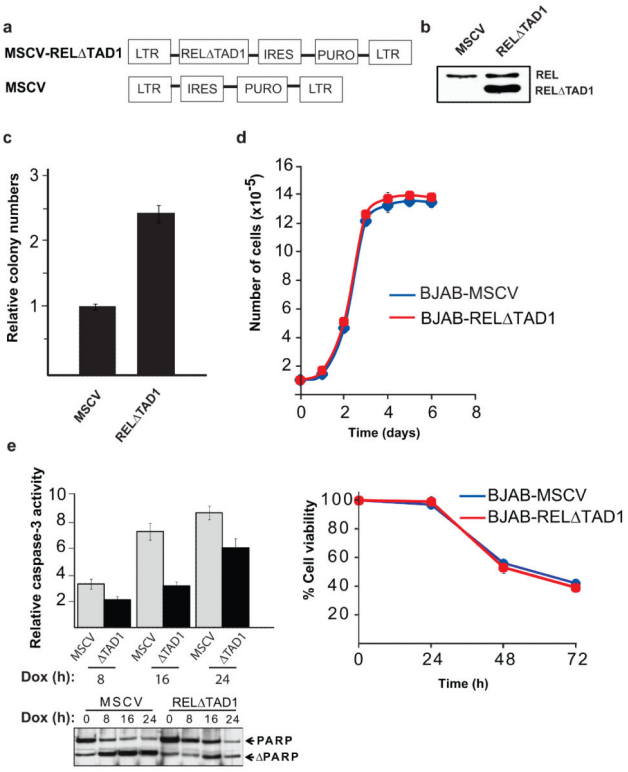

Figure 1.

Overexpression of RELΔTAD1 increases the soft agar colony-forming ability of BJAB cells. (**a**) Structure of MSCV retroviral vectors used in these studies. (**b**) Anti-REL Western blotting of cells stably transduced with MSCV or MSCV-RELΔTAD1 (RELΔTAD1). Endogenous REL and introduced RELΔTAD1 are indicated. (**c**) Relative soft agar colony formation of BJAB-MSCV cells (1.0) and BJAB-RELΔTAD1 cells. Values are the averages of four assays performed in triplicate; error bars indicate standard error. (**d**) Comparison of the proliferation of BJAB-MSCV cells and BJAB-RELΔTAD1 cells. Cells were plated at 10^5 cells/well and were counted each day following plating. (**e**) BJAB-MSCV cells (MSCV) and BJAB-RELΔTAD1 cells (ΔTAD1) were treated with 1 μg/ml doxorubicin (DOX) for the indicated times and caspase-3 activity was measured or PARP cleavage was monitored by Western blotting (bottom panel). For each cell type, caspase activity is relative to the activity seen with untreated cells at the same time point (1.0). Cell viability was measured after treatment with 1.0 μg/ml of doxorubicin at the indicated time points (right panel). Values are the averages of four (caspase-3 activity) or three experiments (cell viability), each performed with triplicate samples.

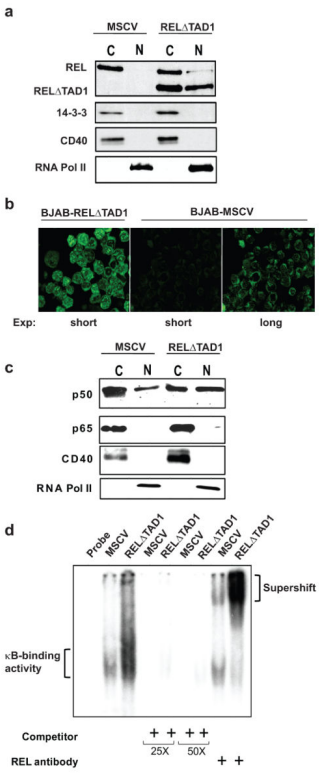

Figure 2.

BJAB-RELΔTAD1 cells have increased nuclear REL protein activity as compared to BJAB-MSCV cells. BJAB-RELΔTAD1 cells and BJAB-MSCV cells were compared by subcellular fractionation (**a, c**), indirect immunofluorescence using an anti-REL primary antiserum (**b**), and by EMSA analysis of nuclear extracts (**d**). In (**a)** and (**c**), nuclear (N) and cytoplasmic fractions (C) were subjected to Western blotting using equal proportions of each fraction for analysis of REL, p50, RelA, and 14-3-3 and CD40 proteins (as cytoplasmic controls) or RNA polymerase II (as a nuclear control). In (**b**), the indicated BJAB cells were stained with an anti-REL antibody and viewed by confocal microscopy. The left panel contains BJAB-RELΔTAD1 cells; the middle and right panels show BJAB-MSCV cells. The left and middle panels were imaged using the same exposure time (Exp), while the right panel was imaged using a longer exposure time to detect the low level of endogenous REL in BJAB-MSCV cells. In (**d**), an EMSA was performed on equalized amounts (5 μg) of nuclear extracts using a κB-site probe from the human *MHC1* enhancer. Where indicated, competitions were performed using an excess of cold probe or samples were supershifted (SS) using anti-REL antiserum. The relevant complexes are indicated.

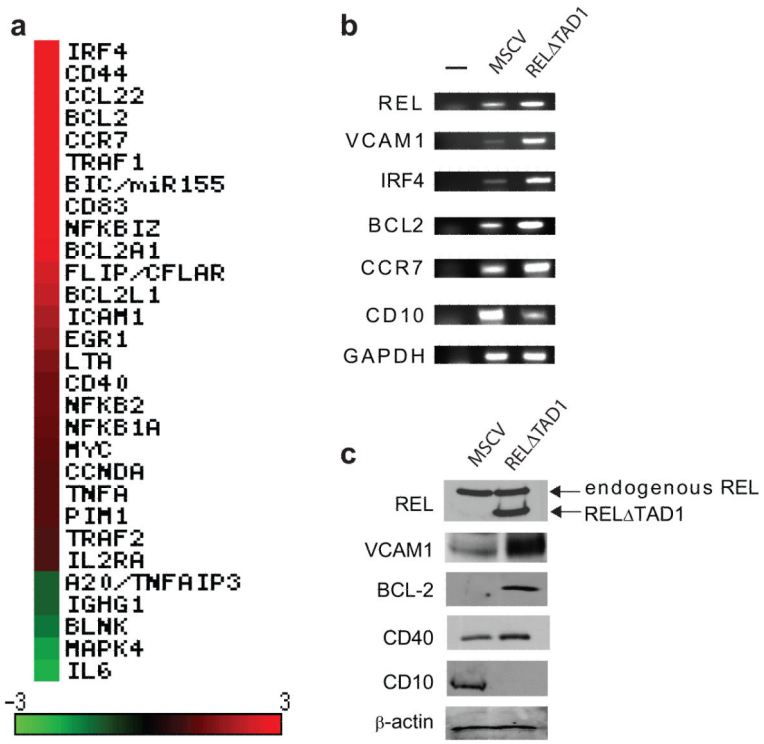

Figure 3.

Analysis of mRNA and protein from select genes in BJAB-MSCV and BJAB-RELΔTAD1 cells. **(a)** Heat map of NF-κB-specific ABC-target gene expression in BJAB-RELΔTAD1 cells as compared to BJAB-MSCV cells. The map was created using the matrix2png program (Pavlidis and Noble, 2003). The expression scale is shown below the map. (**b**) RT-PCR of the indicated mRNAs: *BCL2*, *IRF4*, *CCR7*, *CD10*, *VCAM1*, *REL* (as a positive control), and *GAPDH* (as a normalization control). Water control (-); BJAB-MSCV (MSCV); BJAB-RELΔTAD1 (RELΔTAD1). (**c**) Western blotting for REL, VCAM1, BCL2, CD40, CD10 and β-actin (as a normalization control) of extracts from BJAB-MSCV cells (MSCV) and BJAB-RELΔTAD1 cells (RELΔTAD1).

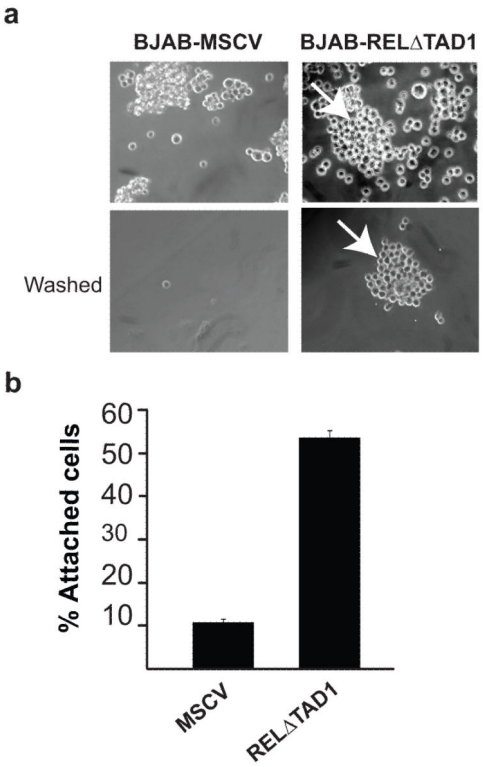

Figure 4.

BJAB-RELΔTAD1 cells show increased adherence to culture dishes. (**a**) BJAB-MSCV and BJAB-RELΔTAD1 cells (1×10^6) were grown in petri dishes for 36 h and imaged at 200 × magnification (top panel); dishes were then washed with PBS and cells in the same field were imaged again (“Washed” panels). The arrows point to a clump of BJAB-RELΔTAD1 cells that adhere to the culture dish. (**b**) The percentage of attached cells was determined by measuring the total protein content of floating cells isolated directly from the media and from cells that remained attached to the culture dish. The assay was performed with triplicate plates; error bars represent standard error.

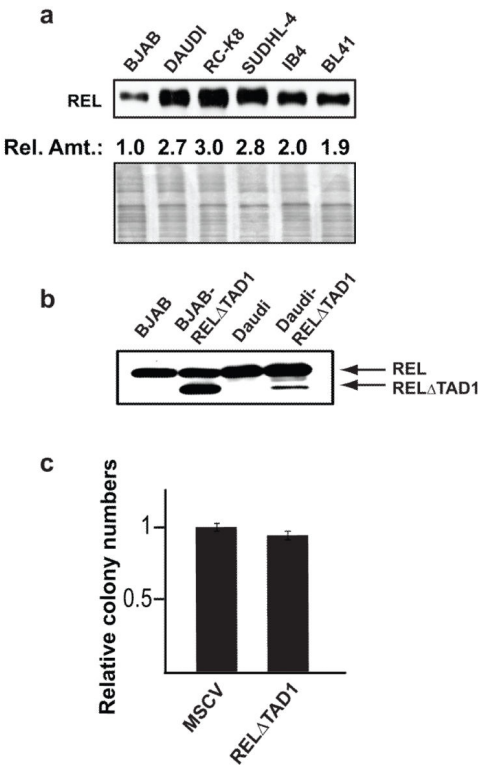

Figure 5.

Expression of REL in several human B-lymphoma cell lines. (**a**) The following human B-lymphoma cell lines were used: BJAB (EBV-negative Burkitt-like lymphoma), SUDHL-4 (DLBCL), RC-K8 (DLBCL), IB4 (umbilical cordblood B-cell lymphoblastoid line infected with EBV), Daudi (EBV-positive Burkitt's lymphoma), and BL41 (Burkitt's lymphoma). Lysates were prepared from actively growing cells, and 20 μg of total protein was analyzed by anti-REL Western blotting (top). At the bottom is shown a Coomassie blue-stained gel of equalized total protein extracts. Rel. Amt. indicates the relative amount of REL in each cell type, as compared to BJAB cells (1.0), determined by scanning of the film in the top panel. (**b**) Anti-REL Western blotting of control BJAB cells, BJAB-RELΔTAD1 cells, control Daudi cells, and a Daudi-RELΔTAD1 cell line. (**c**) Relative soft agar colony forming ability of control vs Daudi-RELΔTAD1 cells. Assays were performed as in Figure 1c, values are the averages of 5 experiments performed with triplicate plates, and were normalized to the number of colonies obtained with control Daudi cells (1.0).

Table 1

Tumor-forming abilities of BJAB-RELΔTAD1 and BJAB-MSCV cells in SCID mice

Cell type	*Number of mice injected*[a]	*Number of tumors*[b]	*% Tumors formed*[c]
BJAB-MSCV	7	6	43%
BJAB-RELΔTAD1	7	11	79%

Using a chi-square test, a *P*-value = 0.05 was obtained for the difference in tumor number between control BJAB-MSCV and BJAB-RELΔTAD1 cells.

[a] Mice were injected above both right and left hind limbs (two injections/mouse) with 5×10^6 cells/site.

[b] Tumors were monitored for up to 7 weeks post-injection.

[c] Percentage of tumor formation (tumors/14 injection sites × 100).

Table 2

mRNAs upregulated at least ten-fold in BJAB-RELΔTAD1 cells

Gene	*Gene function*	*Fold upregulated*	*P-value*	*ABC-gene* [a]	*NF-κB target* [b]
NFAM1	B-cell receptor signaling	121.2	0.00001		
NCAM2	Neural adhesion	80.7	≤1E-46		
CXCR7	Chemokine receptor signaling	77.5	2.28E-29		
FSTL5	Calcium ion binding	72.6	≤1E-46		
THC2683057	Apoptosis	61.9	3.42E-07		
CB123670	-	59.7	≤1E-46		
MARCKS	Actin cytoskeleton	49.4	2.18E-35	+	
C10orf10	Progesterone signaling	39.4	≤1E-46		
BC128163	Protease inhibitor	37.7	2.61E-28		
SEMA3A	Neuron development	37.3	≤1E-46		
MLPH	Actin binding	35.9	7.87E-42		
SOCS2	Regulates cell growth	35.1	3.10E-08		
AFAP	Inflammation	33.5	≤1E-46		
ZC3H12C	Zinc ion binding	32.8	8.26E-23		
IRF4	T-cell activation	32.2	≤1E-46	+	+
CX3CL1	Chemokine ligand	32.1	7.48E-39		
PCOLCE2	Heparin binding	30.3	≤1E-46		
INPP4B	Signaling phosphatase	28.7	≤1E-46		
CD44	Cell adhesion	26.3	8.24E-40	+	+
CLIC2	Chloride ion binding	25.6	2.25E-41		
PLD1	Signal transduction	25.5	≤1E-46		
ESR1	Estrogen signaling	25.1	6.11E-25		
REL	Transcription factor	25.1	≤1E-46		+
VCAM1	Cell adhesion	24.4	2.98E-38		+
PTGER4	Prostaglandin signaling	22.2	≤1E-46		
CUTL2	Transcription	21.5	1.54E-38		

Gene	*Gene function*	*Fold upregulated*	*P-value*	*ABC-gene* [a]	*NF-κB target* [b]
FLJ42709	-	21.5	≤1E-46		
THC2665663	-	21.1	≤1E-46		
CCL22	Inflammation signaling	20.7	≤1E-46	+	+
SERPINB10	Endopeptidase inhibitor	20.6	8.05E-30		
DMD	Actin binding	19.3	3.24E-18		
FLJ20605	Oxidation/reduction	19.1	≤1E-46		
GFRA1	Receptor signaling	18.8	4.64E-34		
PTPRN2	Phosphatase	17.0	9.63E-31		
MSR1	Receptor-mediated endocytosis	16.4	≤1E-46		
CAMK4	Calcium ion binding	16.2	1.42E-15		
C1orf133	-	15.6	1.84E-08		
SPATA16	Spermatogenesis	15.4	2.98-13		
LOC653117	-	15.3	≤1E-46		
AK027257	-	14.8	1.07E-08		
PTPN3	Signaling phosphatase	14.3	≤1E-46		
ST8SIA6	Protein trafficking	14.2	1.95E-20		
BCL2	Anti-apoptosis	13.6	≤1E-46	+	+
SERTAD4	-	13.6	≤1E-46		
KCNMB1	Calcium-activated potassium channel activity	13.5	≤1E-46		
MNDA	Transcription	13.4	1.95E-16		
THC2649506	-	13.3	9.17E-19		
AF086044	-	13.0	5.15E-17		
KIF26B	Microtubule binding	12.7	5.35E-11		
ADAMDEC1	Integrin binding	12.6	≤1E-46		
SDPR	Protein binding	12.6	2.64E-32		
LOC51760	Transporter activity	12.5	3.69E-41		
FBLN1	Extracellular matrix structural constituent	12.5	≤1E-46		

Gene	*Gene function*	*Fold upregulated*	*P-value*	*ABC-gene* [a]	*NF-κB target* [b]
X86816	Estrogen signaling	12.2	6.03E-19		
BDKRB1	Bradykinin receptor activity	11.8	1.27E-24		+
CCL17	Chemokine activity	11.8	2.32E-33		+
SGPP2	Hydrolase activity	11.8	2.37E-27		
TPCN2	Calcium channel activity	11.1	1.29E-22		
A_23_P106814	-	11.0	6.65E-24		
ZBTB32	Transcription	10.9	≤1E-46		
FLJ42342	-	10.9	3.07E-35		
LOC124220	Sugar binding	10.6	≤1E-46		
D4S234E	Dopamine receptor binding	10.5	2.51E-08		
LOC646627	Phospholipase inhibitor	10.4	1.25E-21		
EPB41L4B	Cytoskeleton protein binding	10.3	2.79E-10		
ENST00000321715	-	10.2	2.06E-32		
TP73L	DNA binding	10.1	6.1E-44		

[a] ABC-gene refers to a gene classified as being overexpressed in ABC-DLBCL (Alizadeh *et al.*, 2000; Shipp *et al.*, 2002; Wright *et al.*, 2003; Feuerkhake *et al.*, 2005; Ngo *et al.*, 2006).

[b] NF-κB targets are classified based on www.nf-kb.org

Table 3

ABC and GCB genes whose expression is altered in RELΔTAD-BJAB cells

Gene type	*Total number of genes*	*No.upregulated*	*No. downregulated*	*No. unchanged*
All ABC-specific genes	102	31 (30%)	12 (12%)	59 (58%)
ABC-NF-κB targets	29	17 (59%)	2 (7%)	10 (34%)
All GCB-specific genes	62	6 (10%)	15 (24%)	41 (66%)
GCB-NF-κB targets	3	2 (67%)	1 (33%)	0 (0%)

Gene lists were obtained using previously classified ABC and GCB-specific genes (Alizadeh *et al.*, 2000; Rosenwald *et al.*, 2002; Wright *et al.*, 2002; Feuerkhake *et al.*, 2005; Ngo *et al.*, 2006) and NF-κB targets were obtained from www.nf-kb.org. See Tables S2 and S3 for complete gene lists, references and annotations. Listed are the numbers of genes that are upregulated, downregulated or unchanged in RELΔTAD1 cells compared to BJAB-MSCV cells within a given subset. The genes with altered expression were classified based on a *P*-value cut-off of 0.005. Genes were grouped into ABC-specific genes, ABC-specific NF-κB targets, GCB-specific genes and GCB-specific NF-κB target genes. To validate the patterns of ABC and GCB gene expression distribution in BJAB-RELΔTAD1 cells, we calculated the *P*-value of the two gene sets (ABC, 31 and 12 versus GCB, 6 and 15) using a two-tailed chi-square test at 95% confidence using Graphpad Prism 4 software (Graphpad Prism Software, San Diego, CA). These two gene sets differed with a highly significant *P*-value (0.0009). That is, the percentage of upregulated ABC-genes and the percentage of downregulated GCB-genes in BJAB-RELΔTAD1 cells are significantly different from one another.

Table 4

Gene Ontology classifications for upregulated genes in the BJAB-RELΔTAD1 cells

Gene ontology	*No. of genes*	*P-value*
Protein function		
Intrinsic to plasma membrane	73	2.80×10^{-10}
Extracellular region part	52	9.70×10^{-9}
Cell communication	157	3.10×10^{-8}
Signal transduction	144	1.30×10^{-7}
Biological adhesion	45	7.60×10^{-7}
Immune response	50	2.00×10^{-6}
Membrane part	205	5.50×10^{-6}
Protein binding	227	3.60×10^{-6}
Biological function		
Diseases and disorders		
Immunological disease	75	1.12×10^{-10}– 1.99×10^{-3}
Connective tissue disorder	52	5.07×10^{-9}– 1.50×10^{-3}
Molecular and cellular functions		
Cellular growth and proliferation	144	4.88×10^{-10}– 1.88×10^{-3}
Cell to cell signaling and interaction	114	1.73×10^{-9}– 1.50×10^{-3}
Physiological system development and function		
Immune and lymphatic system development and function	84	2.01×10^{-10}– 1.98×10^{-3}
Tissue morphology	71	2.01×10^{-10}– 1.88×10^{-3}

Gene Ontology grouping of the functions of the upregulated genes (563 annotated total) in RELDTAD1 cells. Shown at the top are the protein functions of the GO-terminology groupings with the lowest *P*-values (http://david.abcc.ncifcrf.gov/). In the bottom half of the table are the biological groupings of 421 significantly upregulated genes that were annotated in the Ingenuity Pathways Analysis program (www.ingenuity.com). Shown are the classifications based on the lowest *P*-values. Ranges of *P*-values refer to the fact that multiple subcategories are included in these classifications.