

# Gene-expression analysis identifies novel *RBL2/p130* target genes in endemic Burkitt lymphoma cell lines and primary tumors

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Burkitt lymphoma (BL) is a B-cell tumor whose characteristic gene aberration is the translocation t(8;14), which determines *c-myc* overexpression. Several genetic and epigenetic alterations other than *c-myc* overexpression have also been described in BL. It has been demonstrated that the *RBL2/p130* gene, a member of the retinoblastoma family (pRbs), is mutated in BL cell lines and primary tumors. The aim of this study was to investigate the biologic effect of *RBL2/p130* in BL cells and its possible role in lym-

phomagenesis. Therefore, we reintroduced a functional *RBL2/p130* in BL cell lines where this gene was mutated. Our results demonstrated that *RBL2/p130*-transfected cells regain growth control. This suggests that *RBL2/p130* may control the expression of several genes, which may be important for cell growth and viability. Gene-expression analysis revealed a modulation of several genes, including *CGRRF1*, *RGS1*, *BTG1*, *TIA1*, and *PCDHA2*, upon *RBL2/p130* reintroduction. We then monitored their expres-

sion in primary tumors of endemic BL as well, demonstrating that their expression resembled those of the BL cell lines. In conclusion, these data suggest that, as *RBL2/p130* modulates the expression of target genes, which are important for cell growth and viability, its inactivation may be relevant for the occurrence of BL. (Blood. 2007;110:1301-1307)

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## Introduction

Burkitt lymphoma is an aggressive B-cell tumor that occurs in several clinical forms, with a high growth rate and a large fraction of cycling cells.<sup>1</sup> The World Health Organization (WHO) classification recognizes 3 subsets of BL: endemic, sporadic, and immunodeficiency associated. Each affects different populations and can present in different forms.<sup>2</sup> All subtypes of BL are characterized by translocation of genetic material between the long arm of chromosome 8 (8q24) and the immunoglobulin heavy chain region on chromosome 14, resulting in t(8;14). The translocation may infrequently occur between 8q24 and the kappa light chain locus on chromosome 2 (t(2,8)) or between 8q24 and the lambda light chain locus on chromosome 22 (t(8,22)). Regardless of which of these translocations occurs, the result is deregulation of the oncogene *c-myc*. The mechanism by which *c-myc* deregulation results in lymphomagenesis is still unclear. In experimental models, deregulation of *c-myc* results in slowing of differentiation, impaired cell-cycle exit, and increased tumor angiogenesis.<sup>3</sup> However, genetic and epigenetic alterations other than *c-myc* have been described in different forms of BL, suggesting that *c-myc* translocation is not the only genetic alteration implicated in BL molecular pathogenesis and that different mechanisms may be involved in the different subtypes of BL.<sup>4</sup> Subsequent tumor progression involves selection for additional genetic and epigenetic changes, including *p53* point mutation and *p16INK4a* gene silencing by promoter methylation.<sup>5</sup> Genetic alteration occurring in a subset of BL, including mutations

in *p73*, *Bax*, and *Bcl6*, may promote cell growth and/or antagonize apoptosis.<sup>6,7</sup> These genes belong to the pathways of pRb/p105 and p53 and may confer a growth advantage or resistance to apoptosis, resulting in the enhancement of cell growth rate typical of BL.

pRb/p105 is frequently targeted in many types of cancer but appears functional, normally expressed, and phosphorylated in BL.<sup>8</sup> The retinoblastoma family is composed of 3 members, pRb/p105, p107, and pRb2/p130.<sup>9</sup> Many of the sequence similarities among these genes reside in a homologous functional domain known as the pocket region. This particular region mediates the interaction with E2F/DP members and viral oncoproteins. There are fundamental differences in the specific mechanisms of growth inhibition employed by the proteins,<sup>10</sup> because they exert growth arrest properties in different cell lines. It has been previously demonstrated that the pRb-related gene *RBL2/p130* is mutated in its nuclear localization signal in BL cell lines and primary tumors.<sup>11,12</sup> This raises the possibility that inactivation of this member of the pRb family renders BL cells more susceptible to transformation by activated *c-myc*. However, it has been shown that the *RBL2/p130* gene is mutated in most of the cases of endemic BL and to a lesser extent in sporadic BL.<sup>11,12</sup> In contrast, in AIDS-related BL, the wild-type *RBL2/p130* gene is highly expressed.<sup>11-13</sup> This finding already suggests that different pathogenetic mechanisms are involved in different BL subtypes.

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We therefore planned an experimental model to (1) assess whether restoration of pRb2/p130 function may affect the cell cycle and proliferation rate in endemic BL-derived cell lines and (2) identify pRb2/p130 target genes deregulated in BL as a consequence of *RBL2/p130* mutation.

To achieve this goal, we reintroduced a wild-type *RBL2/p130* gene in BL cell lines—thus determining the synthesis of a normal and functional pRb2/p130 protein—to analyze whether pRb2/p130 may regain its biologic function of growth suppressor. To identify the target genes regulated by pRb2/p130, BL cells were transiently transfected with the *RBL2/p130* gene and the complete gene-expression profile was then assessed. Microarray analysis on these samples revealed that the expression of several genes was modulated, depending on *RBL2/p130* reintroduction. Finally, the expression of modulated genes after *RBL2/p130* reintroduction in BL cell lines was also evaluated in primary tumors of endemic BL in comparison with *RBL2/p130*-transfected and wild-type BL cell lines.

## Patients, materials, and methods

Approval was obtained from the institutional review board of the Department of Human Pathology and Oncology of the University of Siena, Italy, for these studies. Informed consent was obtained in accordance with the Declaration of Helsinki. The present study has been approved by the Ethical Committee of the Kenyatta National Hospital, Nairobi, Kenya.

### Primary tumors

Fine-needle aspirate using a 25-gauge needle attached to a 10-cm<sup>3</sup> syringe from 6 patients with clinical manifestations of endemic BL before treatment were obtained in a standard clinical manner at the Department of Pathology of the Kenyatta National Hospital. Immediately upon collection, fine-needle aspirates within the needle hub were rinsed in 200  $\mu$ L RNA later (Ambion, Austin, TX). The diagnosis of BL was confirmed by morphology on cytologic slides stained with May-Grunwald Giemsa (Figure 2A); by immunophenotyping with monoclonal antibodies against CD20, CD10, Bcl2, Bcl6, and pRb2/p130; by in situ hybridization for EBER; by polymerase chain reaction (PCR) amplification for HIV detection; and by fluorescence in situ hybridization (FISH) for c-myc translocation, as previously described.<sup>11</sup> Clinical and pathological characteristics of these cases are summarized in Table 1.

### Plasmids

The pCDNA3-*RBL2/p130* vector has been previously described.<sup>13</sup>

### Cell lines

The human Daudi and Raji endemic BL-derived cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Both cell lines carry a t(8;14) chromosome translocation.<sup>14,15</sup> Cells were cultured in RPMI supplemented with 10% FBS, 1% L-glutamine, penicillin/streptomycin, with 5% CO<sub>2</sub>, at 37°C. For the proliferation assay, cells were

counted each day for 4 days. Statistical significance was assessed by the analysis of variance (ANOVA) test.

### Mutational analysis

Mutational analysis of the *RBL2/p130* gene was performed in Raji cells by direct sequencing of PCR products. Briefly, genomic DNA extracted from Raji cells was amplified by PCR and sequenced using the Big Dye terminator kit (Applied Biosystems, Foster City, CA), following manufacturer's instructions. Mutation of exon 21 of the *RBL2/p130* gene was observed and then reconfirmed on different PCR products. Primer sequences were as follows: exon 21 forward: TGGTTTAGCACACCTCTTCAC; reverse: GCTTAGCACAAACCCTGTTC.

### Transfections

Transient transfections of both Raji and Daudi cells were performed by nucleofection using an Amaxa apparatus, program T16, and solution T (Amaxa, Cologne, Germany). The empty vector was transfected as a negative control. To evaluate the expression profile of genes of interest, cells were harvested 24, 48, and 72 hours from transfection for RNA and protein extraction.

### Adenoviral production and transduction

Ad-CMV-*RBL2/p130*, Ad-CMV, and Ad-CMV- $\beta$ -Gal were generated and purified with CsCl. A viral titer of  $22 \times 10^9$  plaque-forming units (pfu) per milliliter was determined with a plaque assay for the Ad-CMV and Ad-CMV-*RBL2/p130* viruses. A viral titer of  $40 \times 10^9$  pfu/mL was determined for the Ad- $\beta$ -Gal virus.

### Flow cytometry

Daudi and Raji cells—either overexpressing pRb2/p130 or containing the empty vector—and the untransfected Daudi and Raji cells (as controls) were used for flow cytometry. Briefly, 48 hours from transfection cells were pelleted by centrifugation, washed with PBS, and analyzed by flow cytometry for cell-cycle analysis (FACStar Plus; BD Biosciences, Mountain View, CA).

### RNA extraction

RNA from cell lines and primary tumors was extracted using the kit RNA easy (Qiagen, Valencia, CA), following the manufacturer's instructions.

### Real-time RT-PCR

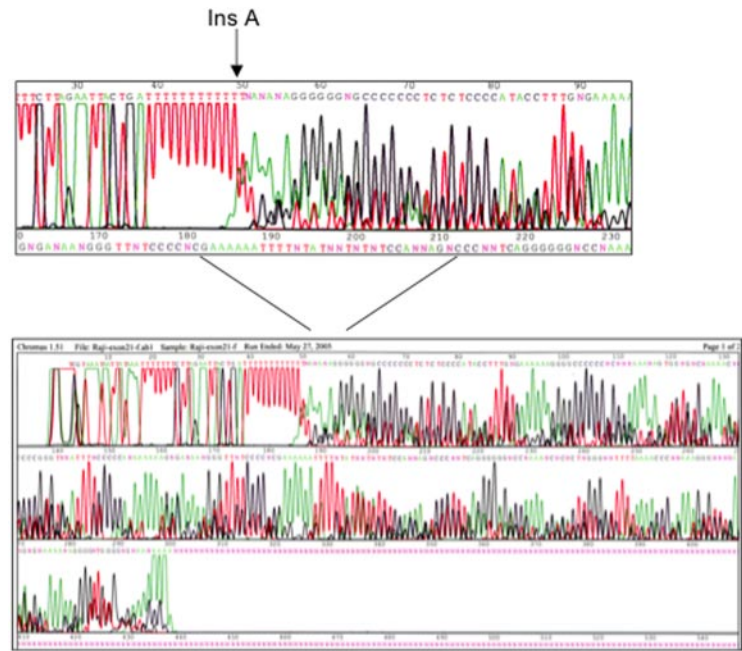
A total of 400 ng of RNA was reverse transcribed for 1 hour at 42°C using avian myeloblastosis virus (AMV) (Promega, Madison, WI) and RNasin (Promega) as enzymes. One microliter of the cDNA was amplified by real-time PCR using the Opticon II (MJ Research, Waltham, MA). The DYNAMO SYBR green 1 kit (Finnzyme, Espoo, Finland) was used according to the manufacturer's instructions. The primers were specifically designed between 2 adjacent exons using the AutoPrime program. HPRT was used as a control gene, and mRNA levels for each gene were rationalized to those for hypoxanthine-guanine phosphoribosyl transferase (HPRT) using the  $\Delta\Delta$ Ct method. Primer sequences were as follows:

**Table 1. Patient characteristics**

Case no.	Age of patients, y	Sex	EBER	HIV	CD20	CD10	BCL6	BCL2
1	7	M	+	–	+	+	+	–
2	16	M	+	–	+	+	+	–
3	6	M	+	–	+	+	+	–
4	10	F	+	–	+	+	+	–
5	3	M	+	–	+	+	+	–
6	5	M	+	–	+	+	+	–

All patients were diagnosed with BL and had tumors located in the jaw region.  
+ indicates present; –, absent

**Figure 1. Mutational analysis of the *RBL2/p130* gene.** Exon 21 has been sequenced and reveals an A insertion in the 20–21 intron-exon boundary.



*RBL2/p130*: left 5'-GGATACTGGCATTCTGTGTAA-3', right 5'-ATTTC-CAGATAGTAAGCCCA-3'; *cyclin E1*: left 5'-AGATTGCAGAGCT-GTTTG-3', right 5'-GAAATGATACAAGGCCGAAG; *cyclin A2*: left 5'-GAAGACGAGACGGGTTC-3', right 5'-AAAGCCAGGGCATCT-TCAC; *E2F4*: left 5'-CAGAACAGCTCCATTCTCC-3', right 5'-AGAGGGTATCTCCAGCAAAG; *HPRT*: left 5'-AGCCAGACTTGTG-TGGATTG-3', right 5'-TTTACTGGCGATGTC AATAGG.

For microarray validation of the genes of interest, Assays-on-demand from Applied Biosystems was used.

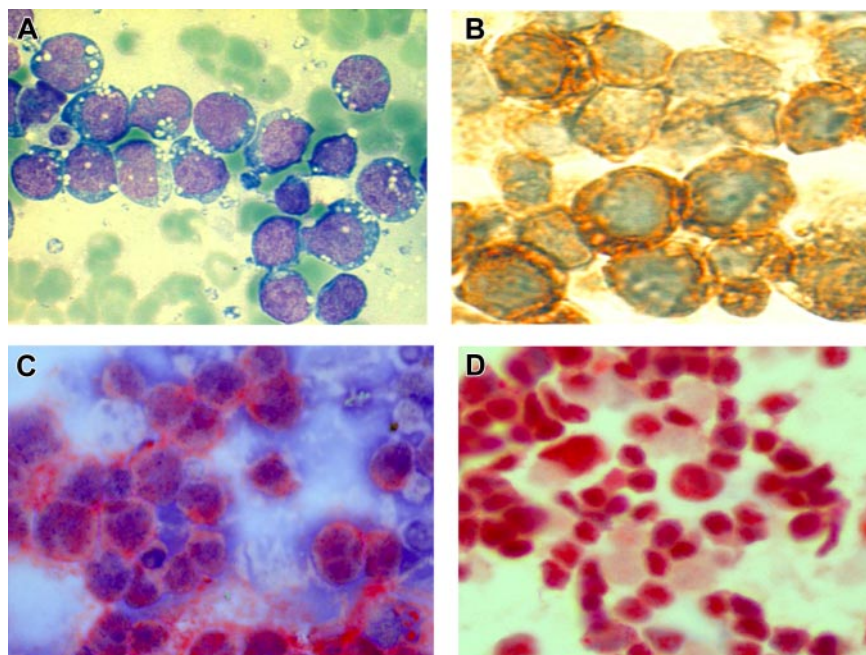
**Generation of gene-expression profiles**

Double-strand cDNA was generated from 5 µg of total RNA using the Super-Script Choice System (Invitrogen and Life Technologies, Carlsbad, CA) and a poly-dT oligonucleotide that contained a T7 RNA polymerase initiation site. The double-strand cDNA was used as a template to generate

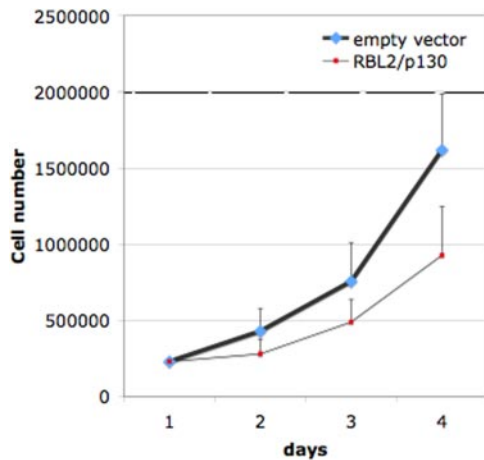
biotinylated cRNA by in vitro transcription using the MEGascript T7 High Yield Transcription kit (Ambion), biotin-11-CTP, and biotin-11-UTP (PerkinElmer, Waltham, MA). The biotinylated cRNA was purified by the RNeasy Kit (Qiagen) and fragmented according to the Affymetrix protocol; 15 µg of fragmented cRNA was hybridized to HG-U133 A microarrays (Affymetrix, Santa Clara, CA). The gene-expression values were determined by MAS 5 algorithm in GCOS 1.2 (Affymetrix). Experiments were repeated twice.

**Gene-expression profiles analysis**

Gene-expression analyses were performed by using GeneSpring GX 7.3.1 (Agilent Technologies, Santa Clara, CA) and Genes@Work (Santa Clara, CA) platforms as previously reported.<sup>16</sup> Briefly, unsupervised hierarchical clustering was generated using an algorithm based on the average-linkage method.<sup>17,18</sup> Only genes displaying a 2-fold average



**Figure 2. Morphology and immunohistochemistry of BL primary tumors and cell lines.** (A) May-Grunwald Giemsa staining of cytologic smears showing the characteristic intracytoplasmic vacuoles. (B) Immunohistochemistry of BL primary tumors showing cytoplasmic localization of the pRb2/p130 protein suggestive of mutations within the nuclear localization signal. Immunohistochemistry of BL cells transfected with either the empty vector (C) or *RBL2/p130* (D). Cytoplasmic localization of the pRb2/p130 protein is observed in empty vector-transfected cells whereas *RBL2/p130*-transfected BL cells show normal nuclear expression of pRb2/p130. Images were viewed by means of a Zeiss Aski Axioskop 40 microscope equipped with a Zeiss Axiocam H Rc (panels A, C, and D, 40×/0.65 NA; panel B, 63×/0.80 NA) (Zeiss, Vertrieb, Germany) and processed using Axiovision (Zeiss) software. Original magnification: panels A, C, and D, × 750; panel B, × 970.



**Figure 3. Proliferation rate of BL cell lines either transfected with the empty vector or with *RBL2/p130*.** Cells were counted for 4 days, and proliferation rate was assessed. Each experiment was performed in duplicate, and the average of 5 different experiments is reported.  $P = .006$ . Error bars represent standard deviation.

change in the expression level across the whole panel were chosen to generate the hierarchical clustering. The expression value of each selected gene is normalized to have a zero mean value and unit standard deviation. The distance between 2 individual samples was calculated by Pearson distance with the normalized expression values.

To perform the supervised gene-expression analysis, we used either GeneSpring 7.3.1 platform or Genes@Work software platform, which is a gene-expression analysis tool based on the pattern discovery algorithm structural pattern localization analysis by sequential histograms.<sup>19,20</sup> In the matrix, each column represents a sample and each row represents a gene. The color scale bar shows the relative gene-expression changes normalized by the standard deviation (0 is the mean expression level of a given gene). For ANOVA/ $t$  tests, 1-way tests with a false discovery rate below 0.05, multiple testing corrections (Bonferroni), and a family-wise error rate of 0.01 or below were adopted.

EASE Software (Portland, OR) was applied to establish whether specific cell functions and biologic processes, defined according to the gene ontology,<sup>21,22</sup> were significantly represented among the deregulated genes.

## Results

### The *RBL2/p130* gene is mutated in Raji cells

It has been already demonstrated that the *RBL2/p130* gene is mutated in several BL cell lines and primary tumors.<sup>11</sup> In particular, Daudi cells carry a heterozygous mutation within exon 21, which

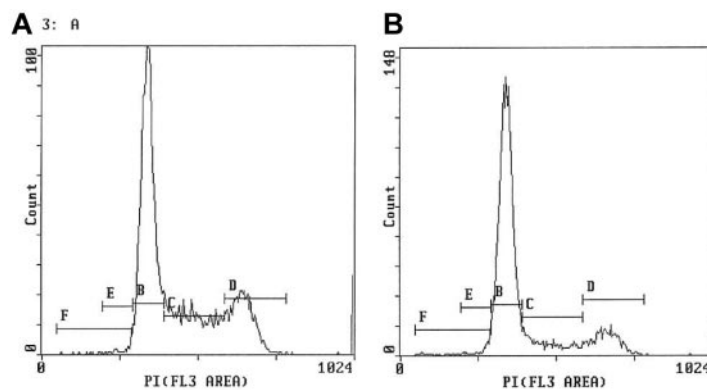
leads to a frame shift. No information was available about possible mutations of *RBL2/p130* in Raji cells, even though an aberrant cytoplasmic localization of the pRb2/p130 protein was observed. Mutational analysis of *RBL2/p130* in this cell line revealed an A insertion at the intron-exon boundary of exon 21, within a splice site, which impairs splicing site recognition (Figure 1).

### pRb2/p130 reintroduction reduces the proliferation rate of BL cells

To evaluate the biologic effect of pRb2/p130, we reintroduced a wild-type *RBL2/p130* gene in 2 BL cell lines, Daudi and Raji cells, where it was demonstrated that this gene was mutated<sup>12</sup> (present work). Hyperexpression of pRb2/p130 in BL cells after *RBL2/p130* transfection was detected by reverse transcription (RT)-PCR in real time (data not shown), and restoration of the physiological nuclear expression of the protein was observed by immunohistochemistry (Figure 2). The *RBL2/p130* gene was inserted in Daudi and Raji cells, either by adenoviral infection or by transient transfection, and cells were monitored until the fifth day after pRb2/p130 reintroduction. Overall, the proliferation rate of both pRb2/p130-expressing BL cell lines was strongly reduced compared with the proliferation rate of wild-type or empty vector-containing BL cells (Figure 3). In addition, flow-cytometry analysis software (FACS) demonstrated that pRb2/p130-expressing BL cells were mostly blocked in  $G_0$  whereas wild-type BL cells were proliferating cells (Figure 4; Table 2), thus suggesting that *RBL2/p130* reintroduction in these 2 BL cell lines restored its growth suppressor activity.

### *RBL2/p130* reintroduction alters the expression levels of cell-cycle regulatory molecules

To assess whether the wild-type *RBL2/p130* inserted in BL cell lines was able to affect the expression of regulatory molecules, we monitored the mRNA level of several regulators differentially expressed in the  $G_1$  and S phases. In normally dividing cells, the transition from the  $G_1$  to the S phase is characterized by the accumulation of active cyclin E1-CDK2 complex,<sup>23,24</sup> and cyclin A2 is associated with cellular proliferation.<sup>25</sup> In particular, we analyzed the expression levels of cyclin E1 and cyclin A2, which are subsequently expressed with a specific kinetic pattern (Table S1, Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). We found that empty vector-transfected Daudi and Raji cells showed a progressive decrease of cyclin E1 levels and concomitant increase of cyclin A2 expression at 24 and 48 hours. An analog profile was observed in wild-type cells (not shown). This pattern is consistent with S phase progression. On the other hand, in both BL cell lines



**Figure 4. Analysis of the cell cycle of BL cell lines, transduced either with *RBL2/p130* (B) or with the empty vector (A), as a negative control, obtained by FACS analysis.** Briefly, each cell-cycle phase is distinguishable on the basis of DNA content because propidium iodide (PI) stoichiometrically binds to DNA, making it possible to discriminate between dividing and nondividing cells. Each cell-cycle stage is graphically represented as a distinct peak on the graph. Percentages of cells in each cell-cycle stage are reported in Table 2. Notably, *RBL2/p130* induces an increase in the  $G_0/G_1$  fraction, thus suggesting a reduction of the proliferation rate.

**Table 2. Percentage of cells in each cycle stage**

	BL-empty vector	BL-RBL2/p130
G <sub>0</sub> /G <sub>1</sub> , %	52.2	70.7
S, %	25.7	12.3
G <sub>2</sub> /M, %	19.2	13.3
Apoptosis/necrosis, %	1.86	3.25

transfected with *RBL2/p130* there was increased expression of cyclin E1 at 24 hours, again declining at 48 hours, suggesting possible G<sub>1</sub> phase prolongation. In accordance with this hypothesis, cyclin A2 expression was not sustained at 48 hours, suggesting a consequent impairment of the S phase (Table S1, Figure S1).

To further investigate this phenomenon, we then studied the expression of E2F4, a member of the E2F transcription factor family that positively regulates cell-cycle progression. pRb2/p130 specifically binds and inactivates E2F4, thus preventing S phase progression.<sup>26</sup> We observed that wild-type but not *RBL2/p130*-transfected Daudi and Raji cells expressed high and progressively increasing levels of E2F4 (Table S1, Figure S1), as occurs in proliferating cells, again suggesting a difference in terms of cell-cycle stage between *RBL2/p130*-transfected and wild-type BL cells.

#### Reintroduction of *RBL2/p130* modulates the gene-expression profile in BL cells

To identify target genes regulated by pRb2/p130 that may be responsible for the differences observed in terms of cell growth and viability, the gene-expression profile of transiently transfected BL cells was obtained by DNA microarray analysis to identify possible early *RBL2/p130* target genes.

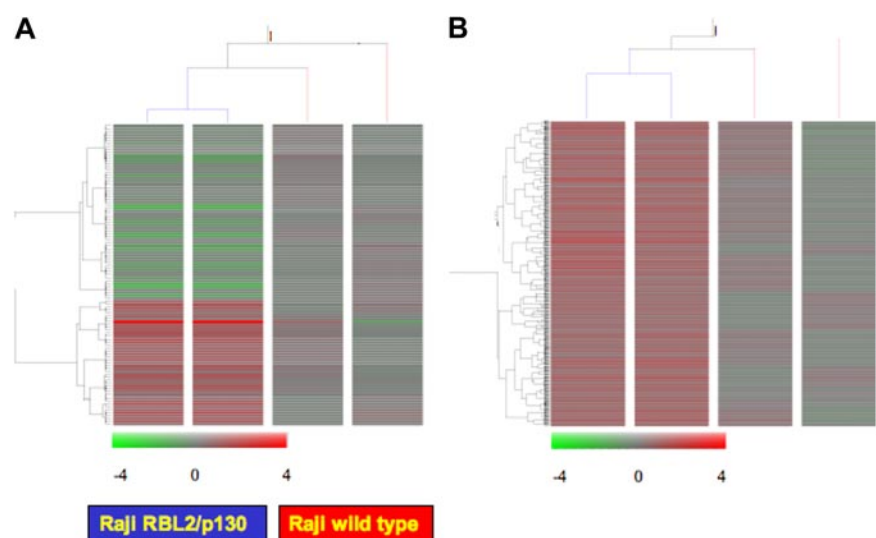
First, hierarchical cluster analysis promptly separated *RBL2/p130*-transfected and wild-type BL cells (not shown). Secondly, a supervised approach identified 103 genes differentially expressed in *RBL2/p130*-transfected and wild-type BL cells (Figure 5A). As expected, the 2 gene lists were significantly overlapping ( $P < .001$ ). However, subsequent analysis focused on genes recognized by supervised comparison because this is the most powerful tool for identifying differentially expressed genes. Forty-three turned out to be overexpressed in the *RBL2/p130*-transfected cells, whereas 60 were downregulated. These genes included molecules involved in cell-cycle

regulation, cell proliferation, B-cell activation, apoptosis, and homophilic cell adhesion. Genes belonging to the functional category of protein binding were significantly overrepresented in the panel (Table 3). We then looked for genes whose expression level behaved like that of *RBL2/p130* in *RBL2/p130*-transfected cells, and we found 586 genes (Figure 5B). Subsequently, these were classified according to relevant functional categories such as nucleic acid binding, transcription corepressor activity, transcription factor binding, transcription cofactor activity, immune cell activation, cell activation, and transcription regulator activity. The latter turned out to be significantly overrepresented also when considering the number of genes belonging to each category that were included within the GeneChip (Affymetrix) ( $P < .05$ ).

Among the regulated genes in Table 3, we then focused on selected ones due to their potential biologic interest and representing different functional categories, such as *CGRRF1*, *BTGI*, *RGS1*, *TIA1*, and *PCDHA2*, which were studied by real-time RT-PCR (Figure 6). *CGRRF1*, which physiologically determines cell-cycle arrest, was confirmed to be overexpressed in *RBL2/p130*-transfected cells. This finding is consistent with the hypothesis of loss of cell growth control due to pRb2/p130 inactivation in BL. This result was also indirectly confirmed by the down-regulation of *BTGI*, which sustains cell proliferation. Intriguingly, *BTGI* also plays a role in cell migration and angiogenesis.<sup>27</sup> In addition, we observed the up-regulation of *RGS1* upon *RBL2/p130* reintroduction, suggesting a possible involvement of pRb2/p130 in B-cell activation/differentiation. We also observed a dramatic down-regulation of *PCDHA2*, which is responsible for homophilic cell adhesion. This finding is consistent with our observation of an altered adhesion pattern in stable *RBL2/p130*-transfected BL cells (unpublished, January 2007). Finally, we found *TIA1* to be down-regulated upon *RBL2/p130* reintroduction.

#### Expression of *RBL2/p130*-regulated genes in primary cases of BL

The expression of these *RBL2/p130* target genes was also analyzed in primary tumors of endemic BL, which showed a cytoplasmic localization of the pRb2/p130 protein as observed in BL cell lines and primary tumors, which suggests a mutation in the nuclear localization signal, as previously reported (Figure 2B).<sup>12</sup> Interestingly, the expression patterns resembled the ones of untransfected BL cell lines rather than *RBL2/p130*-transfected cells, suggesting the involvement of such genes in primary tumors as well (Figure 6).



**Figure 5. Gene-expression analysis of *RBL2/p130* transfected vs untransfected cells.** (A,B) Supervised analysis (*t* test ANOVA,  $P < .01$ ), wild-type versus *RBL2*-transfected Raji cells, 103 genes modulated after transfection (A). Genes with Pearson correlation of at least 0.95 to *RBL2/p130* (B).

**Table 3. Significant modulated genes after *RBL2/p130* reintroduction**

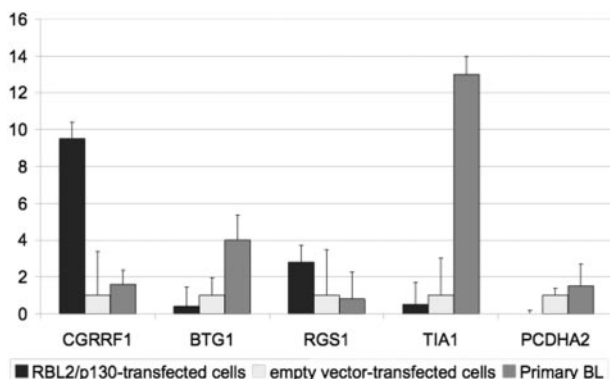
Common	P	Description
RBL2	.007	Retinoblastoma-like 2 (p130)
CGRRF1	.001	Cell growth regulator with ring finger domain 1
TIA1	.010	TIA1 cytotoxic granule-associated RNA binding protein
VEGF	.992	Vascular endothelial growth factor
HDAC4	.961	Histone deacetylase 4
PDCD10	.963	Programmed cell death 10
PCDHA2	.984	Protocadherin alpha 5
RGS1	.987	Regulator of G-protein signaling 1
BTG1	.984	B-cell translocation gene 1, antiproliferative
CASP1	.956	caspase 1, apoptosis-related cysteine protease (interleukin-1, beta, convertase)
CCNL1	.994	Cyclin L1
CLK1	.962	CDC-like kinase 1
CD84	.987	CD84 antigen (leukocyte antigen)
CLK4	.975	CDC-like kinase 4
DPT	.981	Dermatopontin
ING1	.976	Inhibitor of growth family, member 1
MS4A1	.003	Membrane-spanning 4-domains, subfamily A, member 1
MAD	.965	MAX dimerization protein 1
USP10	.984	Ubiquitin-specific protease 10
VEGF	.992	Vascular endothelial growth factor
WIG1	.999	p53 target zinc finger protein
YAF2	.994	YY1-associated factor 2
	.961	T-cell receptor precursor; human T-cell receptor rearranged beta-chain V-region (V-D-J) mRNA, complete cds.

## Discussion

In this paper we investigated whether reintroduction of a wild-type *RBL2/p130* gene in 2 BL cell lines carrying a mutated nonfunctional form of the gene had a significant impact on cell cycle control and proliferation. In addition, we analyzed the gene-expression profile of *RBL2/p130*-transfected cells, looking for possible pRb2/p130 early target genes. First of all, our results indicate that the expression of a functional pRb2/p130 in Daudi and Raji cells is able to reduce the proliferation rate of these cell lines. Interestingly, FACS analysis showed that *RBL2/p130*-transfected cells were mostly blocked in G<sub>0</sub>-G<sub>1</sub> phase. This finding prompted us to investigate the possible role of 2 main regulators of the G<sub>1</sub>/S phases, cyclins E1 and A2. Cyclins E and A are known to be more directly involved in the entry and progression of cells through S phase. Cyclin E regulates G<sub>1</sub>/S transition<sup>28,29</sup> whereas the cyclin A level increases during S until G<sub>2</sub> phases.<sup>30</sup> We observed a differential expression of cyclin E1 and cyclin A2 upon *RBL2/p130*

reintroduction in the 2 BL cell lines. In particular, the reintroduction of functional *RBL2/p130* seemed to determine a cell-cycle shift from S to G<sub>1</sub> in agreement with its supposed role as a negative regulator of cell growth and proliferation (Figure 4). Intriguingly, the remarkable effect on E2F4 regulation (typically increasing during the S phase) further supported this hypothesis. Secondly, to better understand the molecular mechanisms underlying these phenomena, we performed a gene-expression profiling of wild-type and *RBL2/p130*-transfected BL cell lines. Supervised analysis identified several *RBL2/p130*-dependent genes belonging to relevant functional categories, such as cell proliferation, cell cycle regulation, apoptosis, B-cell activation, and homophilic cell adhesion. Furthermore, the expression of selected genes was validated by real time RT-PCR. In particular, reintroduction of *RBL2/p130* led to the up-regulation of genes responsible for cell cycle arrest, such as *CGRRF1*, as well as to the down-regulation of genes involved in cell proliferation, such as *BTG1*. In addition, the up-regulation of *RGS1* suggested an involvement of pRb2/p130 in B-cell activation/differentiation. In fact, *RGS1* is expressed in mature B cells partially differentiated toward plasma cells, and its regulation in B lymphocytes likely significantly impacts lymphocyte migration and function.<sup>31</sup> Of note, this finding is in line with our previous observation of the possible post-germinal center origin of some BLs.<sup>32</sup> Finally, the dramatic down-regulation of *PCDHA2* suggests that pRb2/p130 may also affect homophilic cell adhesion; this assumption is in line with our observation that stable *RBL2/p130*-transfected BL cells display a significantly different aggregation pattern if compared with wild-type cells (unpublished, January 2007).

The relevance of these genes in BL was also confirmed by their deregulation in primary cases of BL. In particular, we observed that the expression of these genes was consistent with that of wild-type BL cell lines, in contrast with *RBL2/p130*-transfected cells. Yet, a more pronounced trend was observed in the case of *TIA1*, because its expression level in primary tumors was markedly higher than that observed in cell lines. Such diversity may be dependent on the different microenvironment of the growth of cell lines and primary



**Figure 6. Quantitative RT-PCR of BL cell lines, either transfected with the empty vector or *RBL2/p130*, versus BL primary tumors to validate microarray analysis upon *RBL2/p130* reintroduction. Error bars represent standard deviation.**

tumors. However, the results are quite preliminary, and future studies will lead to a more detailed characterization of the pathways and mechanisms targeted by the reintroduction of *RBL2/p130*.

Recently, a molecular signature able to distinguish sporadic BL and diffuse large B-cell lymphoma was identified.<sup>33,34</sup> Of note, this signature did not include the *RBL2/p130* target genes identified in our study, suggesting that *RBL2/p130* deregulation may not be exclusive to BL pathogenesis but that it may represent a common feature of various B–non-Hodgkin lymphomas (NHLs).<sup>35</sup> On the other hand, differences between the eBL and sBLs have been acknowledged. In fact, endemic BLs (eBL) and sporadic BLs (sBLs) differ in clinicopathological manifestations, association with Epstein-Barr virus (EBV) and environmental cofactors, and cell kinetics characteristics.<sup>2,36,37</sup> Furthermore, a recent investigation suggests that EBV-positive (mostly endemic) and EBV-negative (mostly sporadic) BLs have different cells of origin.<sup>32</sup> This diversity may well suggest distinct pathogenetic mechanisms and also account for different molecular signatures.

Future analyses of gene-expression profiling in different subsets of BLs are warranted to better define the complete gene-expression profile and better clarify the molecular mechanisms that underlie lymphomagenesis.

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## Authorship

Contribution: G.D.F., E.L., A.O., and G.C. performed experiments; D.L. and P.P.P. performed microarray analysis; P.P.C. performed experiments with viruses; C.B., J.N. and W.M. collected primary tumors; L.L. and G.D.F. designed the overall study; L.L. coordinated the work; G.D.F. wrote the paper; M.H. performed microarrays; P.T., H.S., A.G., and S.P. contributed their expertise in the field and fruitful discussion.

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