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Repression of the *RHOH* gene by JunD

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RhoH is a member of the Rho family of small GTP-binding proteins that lacks GTPase activity. Since RhoH is constantly bound by GTP, it is thought to be constitutively active and controlled predominantly by changes in quantitative expression. RhoH is produced specifically in haematopoietic cells and aberrant expression has been linked to various forms of leukaemia. Transcription of the *RHOH* gene is the first level at which the quantitative levels of the RhoH protein are regulated. Previous studies have demonstrated that *RHOH* gene transcription is initiated by three distinct promoter regions designated P1, P2 and P3 that define the 5′ end of exons 1, 2 and 4 respectively. In the present study we report that the P3 promoter is largely responsible for *RHOH* gene transcription in the B-lymphocytic cell line Raji. The P3 promoter contains a minimal promoter region and a repressor region extending from −236 to +67 and +68 to +245 respectively, relative to the 5′ end of exon 4. Chromatin immunoprecipitation demonstrated that two AP1 (activator protein 1) sites in the minimal promoter region bind JunD. When *JUND* is overexpressed, the endogenous *RHOH* gene is repressed; however, when *JUND* is inhibited, expression of endogenous *RHOH* is induced both in the Raji cell line and AML (acute myeloid leukaemia) cells. In the HCL (hairy cell leukaemia) cell line JOK-1, induction of *RHOH* increases expression of the α isoform of protein kinase C. This downstream target of *RHOH* is also induced in AML cells by *JUND* inhibition. Collectively, these data indicate that JunD is an inhibitor of *RHOH* gene expression.

Key words: B-lymphocyte, differential promoter utilization, *JUND* proto-oncogene, reciprocal expression patterns, repression of transcription, *RhoH* gene promoter.

INTRODUCTION

RhoH is a small GTP-binding protein that is specifically but differentially expressed in haematopoietic cells. RhoH lacks the ability to hydrolyse GTP. Therefore, its function is not controlled by cycling between forms bound by GTP or GDP. Rather, the function of RhoH appears to be controlled by phosphorylation and its quantitative level of expression [1–5]. Mature B-lymphocytes and T-lymphocytes exhibit high levels of *RHOH* gene expression, in contrast with erythroid cells, mature myeloid cells and haematopoietic progenitors where *RHOH* expression is low. An increasing body of evidence indicates that RhoH plays an important role in a range of normal physiological processes and pathological conditions. The physiological functions of RhoH include the regulation of intracellular signal transduction in mast cells, T-lymphocytes and B-lymphocytes [5–9], integrin activation [10], leukotriene production [11], chemotaxis [3,12,13] and haematopoiesis [3,5,6,14]. A role for RhoH in the pathogenesis of malignancy has been implied from the rearrangement of its gene in follicular non-Hodgkin's lymphoma [15,16], nodal marginal zone lymphoma [17], splenic lymphoma with villous lymphocytes [18] and multiple myeloma [16]. In addition, the *RHOH* gene is subject to somatic hypermutation in a diverse set of B-lymphoid proliferative disorders. These include AIDS-related non-Hodgkin's lymphoma [19], DLBCL (diffuse large B-cell lymphoma) [20,21], B-cell post-transplant lymphoproliferative disorders [22], primary central nervous system lymphoma [23], MALT (mucosa-associated lymphoid tissue) lymphoma [24], primary cutaneous follicular center lymphoma and primary cutaneous large B-cell lymphoma, leg type [25]. As well as indirect evidence linking RhoH with malignancy, more direct linkage was first established in HCL (hairy cell leukaemia) where reconstitution of aberrant under-expression of RhoH limited disease progression in a xenograft mouse model [26]. Subsequently, low expression of *RHOH* mRNA was found to be an independent unfavourable prognostic factor in AML (acute myeloid leukaemia) [27]. Furthermore, high expression of *RHOH* mRNA has been shown to be associated with B-cell CLL (chronic lymphocytic leukaemia) and deletion of the *RHOH* gene in a genetic mouse model of CLL has been demonstrated to limit pathogenesis [28].

Within T-lymphocytes, the quantity of RhoH protein is regulated by a lysosomal degradation pathway that is activated following stimulation of the T-cell receptor complex [4]. In addition, the steady-state levels of *RHOH* mRNA are reduced in Th1 T-helper cells when stimulated with an anti-CD3ε (cluster of differentiation 3ε) antibody and in Jurkat T-lymphocytic cells when activated with PMA [2]. Changes in the steady-state levels of mRNA can be caused by mechanisms that regulate either gene transcription or transcript stability. If both or just one of these mechanisms control RhoH levels in T-lymphocytes is presently unknown.

Abbreviations used: AML, acute myeloid leukaemia; AP, activator protein; FBS, fetal bovine serum; CD3ε, cluster of differentiation 3ε; ChIP, chromatin immunoprecipitation; CLL, chronic lymphocytic leukaemia; DLBCL, diffuse large B-cell lymphoma; DTT, dithiothreitol; Ets1, E-twenty six 1; GEO, Gene Expression Omnibus; HCL, hairy cell leukaemia; HRP, horseradish peroxidase; LYF1, lymphoid factor 1; MEK1, mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase 1; MZF1, myeloid zinc finger 1; NFAT, nuclear factor of activated T-cells; NHL, non-Hodgkin's lymphoma; PAK1, p21-activating kinase 1; Q-RT–PCR, quantitative-reverse-transcription–PCR; PKCα, protein kinase Cα; siRNA, small interfering RNA; Sp1, specificity protein 1; TTF, translocation three four.

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Besides T-lymphocytes, mature B-lymphocytes represent the other major site of RhoH expression within the haematopoietic lineage [1–4]. However, in contrast with T-lymphocytes, very little is known about the mechanisms controlling RhoH expression in B-lymphocytes. The only insight gained to date is our finding that in B-lymphocytes transcription of the *RHOH* gene can be initiated at three distinct promoter regions and that one of these regions exhibits differential activity in the B-lymphocyte cell lines Raji and EH [1,26]. The Raji cell line was derived from a patient with Burkitt's lymphoma and expresses high steady-state levels of *RHOH* mRNA. EH cells were derived from a patient with HCL and mimic the under-expression of RhoH that characterizes this disease [26]. Our previous studies demonstrated that the P3 promoter of the *RHOH* gene, extending from nucleotide -236 to $+67$ relative to the 5′ end of exon 4, is 7.4-fold more active in Raji compared with EH cells [26]. Consequently, these results indicated that transcriptional repression of the *RHOH* gene could be the cause of RhoH under-expression in HCL.

In the present study, we report that, of the three promoters that can drive expression of the *RHOH* gene in B-lymphocytic cells, it is the P3 promoter that is preferentially utilized. Deletion studies indicate that the *cis*-acting elements critical for P3 promoter activity lie in a repressor region spanning nucleotides $+68$ to $+245$ relative to the 5′ end of exon 4 and an adjacent minimal promoter region spanning nucleotides -236 to $+67$. Within the minimal promoter region, two evolutionarily conserved binding sites for the transcription factor JunD were identified. Over-expression of JunD was found to inhibit transcription driven by the minimal promoter region when isolated and cloned into a plasmid vector. In contrast, knock-down of *JUND* either within Raji or AML mononuclear cells caused induced expression of endogenous *RHOH* mRNA. Finally, we determined that expression of PKCα (protein kinase Cα) is induced by either RhoH expression or *JUND* knockdown. Taken together, these data indicate that JunD binding to the P3 promoter of the *RHOH* gene represents a mechanism by which the quantitative levels of RhoH are controlled to effect downstream signalling pathways.

Interrogation of the GEO (Gene Expression Omnibus) database indicates that *JUND* mRNA is overexpressed in patients with HCL and AML where *RHOH* mRNA is low, but under-expressed in CLL where *RHOH* mRNA is high [37,38,42]. Furthermore, previous studies have demonstrated that, during physiological T-lymphocyte activation, JunD expression is induced, whereas that of RhoH is repressed [2,4,64]. Consequently, our finding that JunD represses transcription of the *RHOH* gene provides a mechanistic insight into their apparent reciprocal pattern of expression both in leukaemia and during normal physiological processes.

EXPERIMENTAL

Cell culture

The B-lymphocytic cell lines Raji and Namalwa were obtained from A.T.C.C. JOK-1 B-lymphocytic cells stably expressing the linearized plasmid pMEP-RhoH (JOK-RhoH) or the corresponding parental plasmid pMEP4 (JOK-Empty) were obtained as previously described [26]. The growth medium for all cell lines was composed of RPMI 1640, 10 % (v/v) FBS (fetal bovine serum), 100 units/ml penicillin and 100 μg/ml streptomycin sulphate. However, JOK-1 growth medium also contained 150 μg/ml of hygromycin, whereas Raji growth medium was supplemented with 1 % (v/v) non-essential amino acids and 1mM sodium pyruvate. Cultures were maintained in a humidified atmosphere of 5 % CO_2 at 37 °C and a density of 0.5–1.0×10^6 viable cells per ml. Complete medium changes were performed every 2–3 days.

Patients

Blood samples from two patients with AML were obtained from the Service des Maladies du Sang, Hôpital Huriez, Lille, France. These patients, designated AML#1 and AML#2, were classified by the French-American-British system as having stage 5 and stage 4 disease respectively. Both patients exhibited a normal karyotype and blood leucocyte counts ranging from $35–45 \times 10^6$ per millilitre. Patients AML#1 and AML#2 had 71 % and 68 % of their leucocytes assessed as blastic respectively. Peripheral blood was drawn after informed consent, then total mononuclear cells were isolated by Ficoll gradient centrifugation and frozen in liquid nitrogen. Donors provided informed consent in accordance with the Declaration of Helsinki and this study was approved by the Internal Review Board at the Tumorotheque of the Centre Hospitalier et Universitaire de Lille, Centre de Biologie, Lille, France.

Plasmid construction

The activity of the P3 promoter of the human *RHOH* gene was assessed using the expression vector pGL4.14[*Luc2/Hygro*] that contains a promoter-less firefly *LUCIFERASE* reporter gene (Promega). PCR that employed Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) was used to generate eight fragments of the P3 promoter. These fragments spanned nucleotides -1519 to $+245$, -583 to $+245$, -583 to $+67$, -380 to $+67$, -236 to $+67$, -167 to $+67$, -103 to $+67$ and -24 to $+67$ relative to the 5′ end of exon 4 [1]. Fragments with 3′ ends that mapped to nucleotide $+67$ were generated using a primer with the nucleotide sequence 5′-ATACAACAGTTGGGCTGGCAG-3′. Fragments with 3′ ends that mapped to nucleotide $+245$ were generated with a primer with the nucleotide sequence 5′-GAATGCCGAAGAAATGTCTTGACAG-3′. The 5′ ends of the P3 promoter fragments mapping to nucleotides -1519, -583, -380, -236, -167, -103 and -24 were generated with primers that had the respective nucleotide sequences; 5′-CAGCTCACTGCAACCTCAACC-3′, 5′-GGTAACTCCCAGCCCAGGTG-3′, 5′-ACCAAGATTGGTCAGTGGCATG-3′, 5′-GAAACATAGTCTCGGAGTAGGGTG-3′, 5′-TCTGCCTCTGATCTGGGGAAG-3′, 5′-CCTAAACCCACACAAATGAAACC-3′ and 5′-CTCTTTTGTCACATTCGGATTGC-3′. All the P3 promoter fragments generated by PCR were sub-cloned into the 'filled-in' HindIII site of pGL4.14[*Luc2/Hygro*] then validated by DNA sequencing (GATC Biotech, SARL, Mulhouse, France). The transfection control plasmid pRSV-βGal that directs constitutive expression of β-galactosidase was purchased from Promega. The plasmid pRSV-hjD that directs constitutive expression of JunD was provided by Yosef Shaul (Weizmann Institute of Science, Rehovot, Israel). The plasmid pRSV-Ng was used to control for any effects of pRSV-hjD not attributable to JunD overexpression. This negative control plasmid was produced by eliminating the entire JunD coding region from pRSV-hjD by digesting with SmaI followed by re-ligation of the isolated vector backbone. In previous studies, we have used pRSV-hjD and pRSV-Ng to elucidate molecular mechanisms that control expression of the *CD11C* gene [29].

siRNA (small interfering RNA)

Repression of *JUND* gene expression was achieved using siRNA purchased from Eurogentec. This siRNA has previously been shown to repress human *JUND* gene expression and consists of

the nucleotide sequence 5′-ACGCGAACCUGAGCAGCUAUU-3′ [30]. As a control for the siRNA targeted against transcripts encoding JunD, a siRNA was utilized with the nucleotide sequence 5′-AGACGGUGGUCAUUACCUAUU-3′. This control siRNA has no significant homology with any known human gene. The control siRNA was purchased from Eurogentec. Stock solutions (100 μM) of siRNA were stored at -80 °C. Prior to use, stocks were thawed and diluted to a final concentration of 10 μM in cell culture medium. The siRNA stock solutions were subjected to either one or two freeze-thaw cycles.

Transfection

Evaluation of the transcriptional activity of the *RHOH* P3 promoter was achieved by utilizing nine luciferase reporter constructs. The first of these constructs was the control parental vector pGL4.14[*Luc2/Hygro*]. The additional eight constructs comprised the pGL4.14[*Luc2/Hygro*] vector containing the P3 promoter regions extending from -1519 to $+245$, -583 to $+245$, -583 to $+67$, -380 to $+67$, -236 to $+67$, -167 to $+67$, -103 to $+67$ and -24 to $+67$ relative to the 5′ end of exon 4 [1]. A quantity (4.5 μg) of each of the P3 promoter constructs was combined with 0.5 μg of pRSV-βGal, then this mixture was transfected by electroporation into the Raji cell line using program M13 of the Nucleofector™ II apparatus and Cell Line Nucleofector Kit V (Lonza Group Ltd, Basel, Switzerland). In parallel to these experimental transfections, control transfections were performed with 0.5 μg of pRSV-βGal mixed with 4.5 μg of pGL4.14[*Luc2/Hygro*] empty of P3 promoter sequences. The influence of overexpression of JunD on recombinant *RHOH* was evaluated using the vector pGL4.14[*Luc2/Hygro*] into which was cloned the P3 promoter extending from nucleotides -236 to $+67$ relative to the 5′ end of exon 4. A quantity (2.5 μg) of this construct along with 0.5 μg of pRSV-βGal were mixed with 2.5 μg of the expression construct pRSV-hjD and in parallel with 2.5 μg of its empty parental vector pRSV-Ng. As controls for these experiments, parallel transfections were performed with the empty parental vector pGL4.14[*Luc2/Hygro*] from which the P3 promoter construct was derived. Raji cells were lysed 48 h after electroporation by a 5 min incubation at room temperature (25 °C) with 250 μl of lysis buffer [91 mM K_2HPO_4, 8 mM KH_2PO_4, 0.2 % Triton X-100 and 1 mM DTT (dithiothreitol)]. The lysate was cleared by centrifugation (18 000 ***g*** at 20 °C) and 50 μl of supernatant was mixed with 100 μl of luciferase buffer [25 mM Tris phosphate, pH 7.5, 8 mM $MgCl_2$, 1 mM DTT, 1 % (v/v) Triton X-100, 15 % (v/v) glycerol, 1.8 mM ATP and 45 μM luciferin]. Luciferase activity was then quantified using a Lumat LB 9501 luminometer (Berthold Technologies). This same apparatus was used to quantify the level of β-galactosidase activity with a Luminescent β-galactosidase Detection Kit II (Clontech). All luciferase and β-galactosidase quantifications were determined in duplicate. The level of β-galactosidase activity was taken as reflective of transfection efficiency and used to correct the luciferase assay results. The influence of JunD overexpression on the endogenous *RHOH* gene was evaluated in the same way as its influence on recombinant *RHOH*, except that *LUCIFERASE* plasmids were omitted and 5 μg not 2.5 μg of each of the plasmids pRSV-hjD and pRSV-Ng were used. In addition, β-galactosidase levels were used to correct Q-RT–PCR (quantitative-reverse-transcription–PCR) analyses of total RNA and not luciferase activities. The influence of JunD repression on transcription of the endogenous *RHOH* gene in Raji cells was evaluated by electroporation using 500 nM of siRNAs targeting transcripts encoding JunD or a control siRNA with no human gene homology. Each siRNA was mixed with 500 nM of BLOCK-iT™ Fluorescent Oligo for electroporation (Invitrogen). At 48 h after siRNA electroporation, cells were washed once in PBS and fluorescence intensity was measured using a COULTER® EPICS® XL-MCL™ flow cytometer (Coulter). This intensity was taken as reflecting the level of transfection efficiency and used to correct Q-RT–PCR analyses of total RNA that was subsequently extracted. Transfection of total mononuclear cells isolated from AML patients was undertaken by first thawing the stored frozen cells, then washing them twice in RPMI 1640 containing 10 % FBS. Next, cells were cultured for 2 h in the same medium to increase viability and transfection efficiency. Cells (5×10^6) were mixed with 500 nM of BLOCK-iT™ Fluorescent Oligo for electroporation (Invitrogen) and either 500 nM of a siRNA directed against *JUND* mRNA or 500 nM of a control siRNA with no human gene homology. These cells were then electroporated using the Human CD34 + Cell Nucleofector® kit from Lonza according to the manufacturer's instructions. At 48 h after siRNA electroporation, fluorescent intensity was measured and used to correct Q-RT–PCR analyses of subsequently extracted total RNA.

Western blotting

Proteins were isolated from cell cultures using M-PER® Mammalian Protein Extraction Reagent (Thermo-Fisher Scientific) and their concentrations were evaluated using the Protein Assay from Bio-Rad Laboratories. Proteins were reduced using the NuPAGE® Sample Reducing Reagent and 30 μg was immediately subjected to electrophoresis through 12 % (v/v) NuPAGE® Gels using NuPAGE® Mops SDS Running Buffer (Invitrogen). After electrophoresis, proteins were transferred on to nitrocellulose filters using the iBlot® Dry Blotting System from Invitrogen. Next, filters were soaked for 2 h at room temperature in PBS containing 3 % (w/v) non-fat dried skimmed milk powder then incubated overnight at 4 °C in this same blocking buffer containing rabbit polyclonal antibody sc-74 directed against JunD (Santa Cruz Biotechnology) or either rabbit monoclonal antibody 60A8 or C37F9 directed against c-Jun or JunB respectively (Cell Signaling Technology). Filters were then washed and incubated for 1 h at room temperature with a donkey anti-rabbit secondary antibody conjugated with HRP (horseradish peroxidase) that was purchased from Amersham Biosciences. Filters were again washed and HRP was visualized using the ECL (enhanced chemiluminescence) Western Blotting System (Amersham Biosciences). The protein loading of gels was evaluated by hybridization with the mouse monoclonal antibody AC-15 directed against β-actin (Sigma–Aldrich) and a sheep anti-mouse secondary antibody conjugated with HRP (Amersham Biosciences).

ChIP (chromatin immunoprecipitation)

Evaluation of the occupancy of the *RHOH* gene promoter by JunD was achieved using ChIP assays performed using the ChIP-IT™ kit (Active Motif). Unless otherwise stated, all reagents, buffers and supplies were included in the kit. Briefly, Raji cells were cross-linked with 1 % (v/v) formaldehyde for 10 min at room temperature. After washing and treatment with glycine Stop-Fix solution, the cells were resuspended in lysis buffer and incubated for 30 min on ice. The cells were homogenized, and nuclei were resuspended in shearing buffer and subjected to optimized sonication shearing conditions (15–25 pulses of 20 s each; between pulses the samples were allowed to cool on ice for 30 s) to yield DNA fragments of 200–1000 bp in size. The

sonicator instrument employed was a Vibra Cell™ (Sonics & Materials). Sheared chromatin was pre-cleared with Protein G beads and incubated overnight at 4 °C with 2 μg of a negative control human IgG antibody, an antibody directed against human RNA Polymerase II or the sc-74-x rabbit polyclonal antibody directed against human JunD (Santa Cruz Biotechnology). Protein G beads were then added to the antibody–chromatin mixtures and incubated for 1.5 h at 4 °C. After extensive washings, the immunoprecipitated DNA was removed from the beads with an elution buffer. To reverse cross-links and remove RNA, NaCl was added to a final concentration of 0.2 M and RNase A to a final concentration of 100 ng/μl. Samples were then incubated overnight at 65 °C. Next, the samples were treated with 180 ng/μl of proteinase K for 2 h at 42 °C and DNA purified using DNA purification mini-columns. The diluted purified DNA was subjected to semi-quantitative PCR analysis. Binding of human RNA Polymerase II to the appropriate region of the *GAPDH* gene promoter was used as a positive control for the efficiency of the ChIP procedure as recommended by the manufacturer of the ChIP-IT™ kit (Active Motif).

Analytical PCRs

Total RNA was extracted from the mononuclear cells of AML patients and from the cell lines Raji, JOK-RhoH, JOK-Empty and Namalwa by using the High Pure RNA Isolation Kit (Roche Diagnostics). The procedure included a DNAse-I treatment to eliminate genomic DNA contamination. The amounts and quality of RNA were evaluated by UV spectroscopy using Nanodrop technology (Thermo Scientific). The cDNA templates for Q-RT–PCR were prepared as previously described [26]. Evaluation of *PKCα* expression and the relative expression of *RHOH* gene transcripts initiated by promoters P1, P2 and P3 was achieved using SYBR® Green technology (Applied Biosystems). The quantity and quality of the cDNA templates were evaluated by analysis of *ABL* mRNA in accordance with the recommendations of Europe Against Cancer [31]. PCR efficiencies were evaluated by generating linear regression curves using cDNAs from Raji or mononuclear cells. Each PCR reaction was performed in a 10 μl total volume using 0.4 μl of each of the sense and anti-sense primers at 10 μM, approximately 50 ng of cDNA template and 5 μl of 2 × UDG Master Mix (Life Technologies). Transcripts of the *PKCα* gene were quantified using the primers *PKCα-S* and *PKCα-AS* that have the respective nucleotide sequences 5′-GATGGATGGAGTCACGACCAG-3′ and 5′-AGGACGCCATAGGCCCA-3′. Previous studies have validated the efficacy of this primer pair in determining *PKCα* expression [32]. Transcripts specifically initiated by the P1 promoter were quantified using primer *21* located inside exon 1 coupled with primer *482* located inside exon 6. These primers had the respective nucleotide sequences 5′-CCCTTGACCCAAAGATACTGCTC-3′ and 5′-TCTCTGTCGGCTTCTACTCCAAG-3′. Transcripts initiated by promoter P2 were quantified using primer *482* coupled with primer *125* located inside exon 2 that had the sequence 5′-AACACTGCAGGGCCATTTG-3′. Transcripts initiated by promoter P3 were quantified using primer *41* located inside exon 4 coupled with primer *299* located inside exon 6. These primers had the respective nucleotide sequences 5′-CCCTGCCAGCCCAACTG-3′ and 5′-AGAGCGTTCTCCACGGCTT-3′. The primer *41* sequence is only present in transcripts initiated at promoter P3 since it is removed by splicing in transcripts initiated at promoter P1 or P2. Transcripts originating from the *ABL* gene were quantified using the primers *ABL-F* and *ABL-R* with the respective sequences 5′-TGGAGATAACACTCTAAGCATAACTAAAG-G-3′ and 5′-GATGTAGTTGCTTGGGACCCA-3′ [31]. The relative expression level of *RHOH* gene transcripts initiated specifically upstream of exons 1, 2 and 4 was calculated by dividing their quantitative level of expression by the quantitative level of *ABL* gene expression. Relative expression levels were determined from two independent cultures of Raji cells and each cDNA synthesis was analysed by three independent quantifications performed in triplicate. Evaluation of the sum total of all *RHOH* gene transcripts was achieved as we have previously described using Q-RT–PCR and TaqMan technology (Applied Biosystems) [26]. The B-cell line Namalwa that expresses high levels of ABL and *RHOH* mRNA was used to produce calibration curves that evaluated PCR efficiency for each primer pair. Semi-quantitative PCR was used to analyse immunoprecipitated chromatin. In these studies, the degree to which JunD binds *RHOH* at the consensus AP1 (activator protein 1) site named AP1#1 was assessed using the primer pair 5′-AGAAACATAGTCTCGGAGTAGGGT-3′ and 5′-AAGAGATTAGCAAGTTCCTGT-3′. JunD binding to the AP1 site named AP1#2 was assessed using the primer pair 5′-GGCACAGGAACTTGCTAATCTC-3′ and 5′-CGAG-AATATTAACTGATTCACACTGGAAGAACTG-3′. The binding of RNA polymerase II to the promoter region of the *GAPDH* gene was assessed using a sense-strand primer with the nucleotide sequence 5′-TACTAGCGGTTTTACGGGCG-3′ coupled with an antisense-strand primer with the nucleotide sequence 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′. The products of semi-quantitative PCR were visualized by agarose gel electrophoresis.

Microarray analysis

Total RNA was isolated from the B-lymphocytic cell lines JOK-RhoH and JOK-Empty using the RNAeasy kit from Qiagen that included a DNAse I treatment. Control of quality was evaluated using an Agilent 2100 Bioanalyser (Agilent Technologies). Total RNA (0.5 μg) was then processed and analysed on Human Whole Genome Agilent 44K 60-mer oligonucleotide microarrays using the kits for cRNA amplification, labelling, fragmentation, hybridization and washing recommended by the manufacturer for the Two-Color Microarray-Based Gene Expression protocol (Agilent Technologies). For each cell line, two biological samples were studied by dye-swap analysis. Microarrays were scanned using an Agilent DNA Microarray G2505B scanner, the expression data was extracted by Feature Extraction (version 9.1.3.1) and then processed by GeneSpring version 7.3 (Agilent Technologies) for normalization, filtering and statistical analysis using the Student's *t* test. Significance was assessed at a P value of 0.05.

Bioinformatic tools

Cross-species sequence homology analysis of the *RHOH* gene was performed using the computer program GenomeVISTA [33]. This is an automatic server that allows the user to find candidate orthologous regions for a draft or finished DNA sequence from any species on a base genome and provides detailed comparative analysis. A computational strategy is used where query sequences are anchored on the base genome by local alignment matches and then globally aligned to candidate regions by the program AVID [34]. The VISTA family of tools are developed and hosted by the Genomics Division of Lawrence Berkeley National Laboratory and the United States Department of Energy Joint Genome Institute (Walnut Creek, CA, U.S.A.). The sequence of the P3 promoter of humans

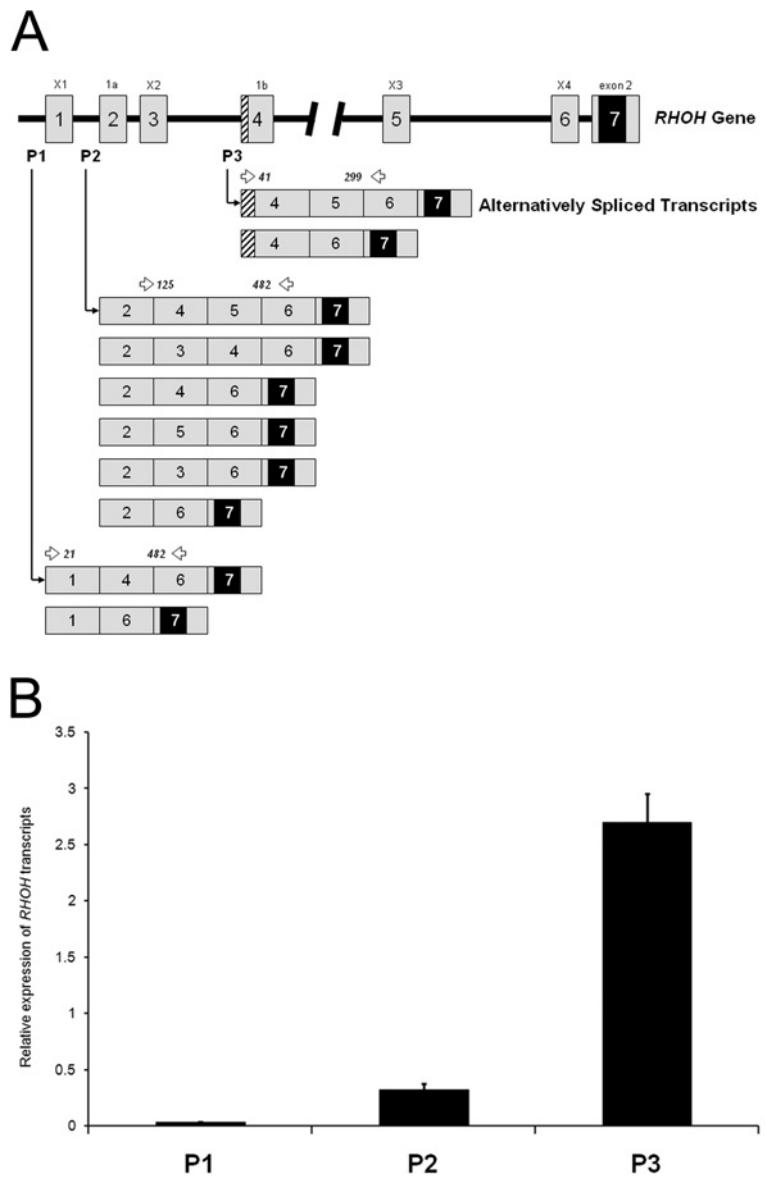

Figure 1 Transcription of the *RHOH* gene in Raji B-lymphocytic cells is initiated primarily at the P3 promoter

(**A**) Schematic representation of the *RHOH* gene and its transcripts. The sizes of the six introns and seven exons that comprise the *RHOH* gene are not presented to scale. Previously the six exons were named X1, 1a, X2, 1b, X3, X4 and Exon 2 [1]. These original exon names are indicated above the gene schematic. In the present study we rename the *RHOH* exons 1–7. These new names are indicated within the boxes that denote exons. The size of the fourth intron between exons 4 and 5 is 37.88 kb [1]. Owing to its large size compared with the other *RHOH* introns, intron 4 is presented interrupted with a hash mark. The coding region is entirely present within exon 7 and is depicted by a black box. Transcription of the *RHOH* gene can be initiated at exon 1 by promoter P1, at exon 2 by promoter P2 or at exon 4 by promoter P3. The hatched box within exon 4 indicates a region that is present in mature transcripts originating from P3 but that is spliced out of transcripts originating from P1 and P2. The structures of the mature transcripts resulting from differential splicing of primary transcripts are depicted immediately below the gene schematic. Numbered boxes denote the originating exons. Converging open arrows indicate the position and name of the primers used to determine the relative usage of promoters P1, P2 and P3. (**B**) Relative utilization of *RHOH* gene promoters P1, P2 and P3 by Raji B-lymphocytic cells. The expression of *RHOH* gene transcripts specifically initiated by the promoter regions P1, P2 and P3 was assessed by Q-RT–PCR using SYBR® Green technology and the primers depicted in (**A**). PCR efficiencies were established for primer pairs that specifically detect transcripts originating from the *ABL* gene and from the *RHOH* gene at promoters P1, P2 and P3. This was achieved by performing five 10-fold serial dilutions of one Raji reference cDNA then quantifying in triplicate each dilution with each primer pair. Linear regression curves were then constructed for each primer pair by plotting the log of cDNA quantity against the cycle number at which amplification crossed an arbitrary threshold. The slope of the regression curves was taken as reflecting PCR efficiency and used to correct the quantifications of *ABL* transcripts and *RHOH* transcripts specifically initiating at promoter P1, P2 or P3. The relative expression of *RHOH* transcripts initiating at each of these promoters was then calculated by dividing their quantitative level of expression by the quantitative level of *ABL* gene expression. Histograms represent the mean ± S.E.M. of two independent cultures of Raji cells and three independent quantifications of each cDNA synthesis performed in triplicate.

was aligned to the equivalent sequence in the genomes of mouse (chr5:66254636–66255114 [+]), rat (chr14:45055696–45056166 [−]), rhesus monkey (chr5:35224232–35224710 [+]), horse (chr3:80658339–80658821 [−]) and dog (chr3:75142670–75143127 [−]). Putative transcription factor binding sites within the P3 promoter were identified using the analytic program Match™ version 1.0 Public (BIOBASE Biological Databases, Wolfenbüttel, Germany). This software uses a library of mononucleotide weight matrices from the database TRANSFAC® 6.0 that contains experimentally proven eukaryotic transcription factor-binding sites. Interrogation of the GEO database determined the relative expression of *C-JUN*, *JUNB* and *JUND* in Raji cells, the relative expression of *RHOH* and *JUND* in HCL patients, the expression of *RHOH* in DLBCL

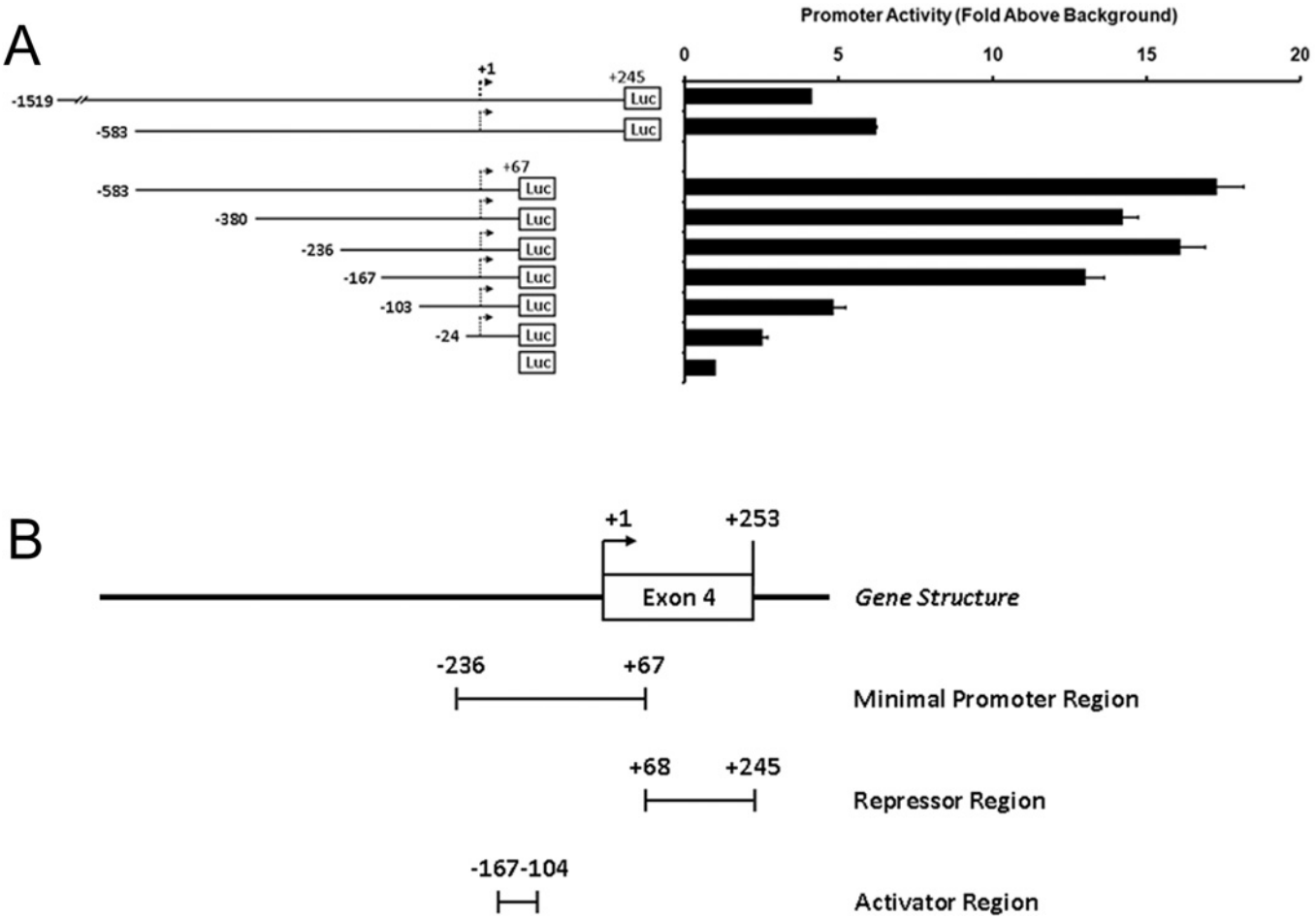

Figure 2 The P3 promoter of the *RHOH* gene contains both activator and repressor elements

(**A**) Two series of fragments of the P3 *RHOH* promoter were generated by PCR and cloned immediately upstream of the promoter-less *LUCIFERASE* gene present in the plasmid vector pGL4.14 [*Luc2/Hygro*]. The two fragments of the first series had a common 3′ end mapping to nucleotide + 245 and 5′ ends mapping to nucleotides − 1519 or − 583 relative to the 5′ end of exon 4 (bent arrow). The second series of fragments had a common 3′ end mapping to nucleotide + 67 and 5′ ends mapping to nucleotides − 583, − 380, − 236, − 167, − 103 or − 24. Raji B-lymphocytic cells were transfected with the empty parental vector or independently with the *RHOH* P3 promoter constructs each mixed with the plasmid pRSV-βGal that constitutively expresses β-galactosidase. Transfected cells were cultured for 48 h then assayed for β-galactosidase and luciferase activity. The levels of β-galactosidase activity were taken as reflective of transfection efficiency and used to correct the luciferase results. After this correction, the level of *LUCIFERASE* reporter gene activity directed by each *RHOH* P3 fragment above the background level directed by the empty parental vector was calculated. Each histogram represents the mean ± S.E.M of three independent experiments. (**B**) Transfection of P3 promoter fragments indicates that the minimal region of the P3 promoter that supports high-level expression extends from nucleotides − 236 to + 67. This region is designated the minimal promoter region. Within the minimal promoter, there is an activator region that extends from nucleotides − 167 to − 104. Immediately downstream of the minimal promoter region, there is a repressor region that extends from nucleotides + 68 to + 245.

compared with normal B-lymphocytes and the relative expression of *JUND* in AML and CLL mononuclear cells compared with normal controls [35,36]. The GEO database was created and is administered by the National Center for Biotechnology Information and serves as a public repository for a wide range of high-throughput experimental data. These data include single and dual channel microarray-based experiments measuring mRNA, miRNA (microRNA), genomic DNA and protein abundance, as well as non-array techniques such as serial analysis of gene expression. The specific DataSet interrogated for expression of *JUND* mRNA in AML patients compared with normal volunteers was GDS1059 [37]. The equivalent DataSet interrogated for CLL patients was GDS1454 [38]. The DataSets interrogated for the relative expression of *C-JUN*, *JUNB* and *JUND* in Raji cells were GDS181 and GDS989 [39,40]. The DataSet interrogated for the expression of *RHOH* in DLBCL compared with normal B-lymphocytes was GDS3516 [41]. The Series record interrogated for relative expression of *JUND* and *RHOH* in HCL patients was GSE2350 [42].

RESULTS

Preferential usage of the P3 promoter of the *RHOH* gene by B-lymphocytic cells

Previous studies have established that transcription of the *RHOH* gene can be initiated at three distinct sites [1] (Figure 1A). These sites define the 5′ ends of non-coding exons 1, 2 and 4. The promoter regions responsible for these initiation events are designated P1, P2 and P3 respectively. We used Q-RT–PCR to determine whether the different promoters of the *RHOH* gene exhibit preferential usage in the B-lymphocytic cell line Raji (Figure 1B). This analysis demonstrated that promoter P3 is <8-fold more active than the P1 and P2 promoters combined.

The P3 promoter is controlled by both repressor and activator elements

The human P3 promoter extending from − 1519 to + 245 relative to the 5′ end of exon 4 was cloned into a *LUCIFERASE* reporter

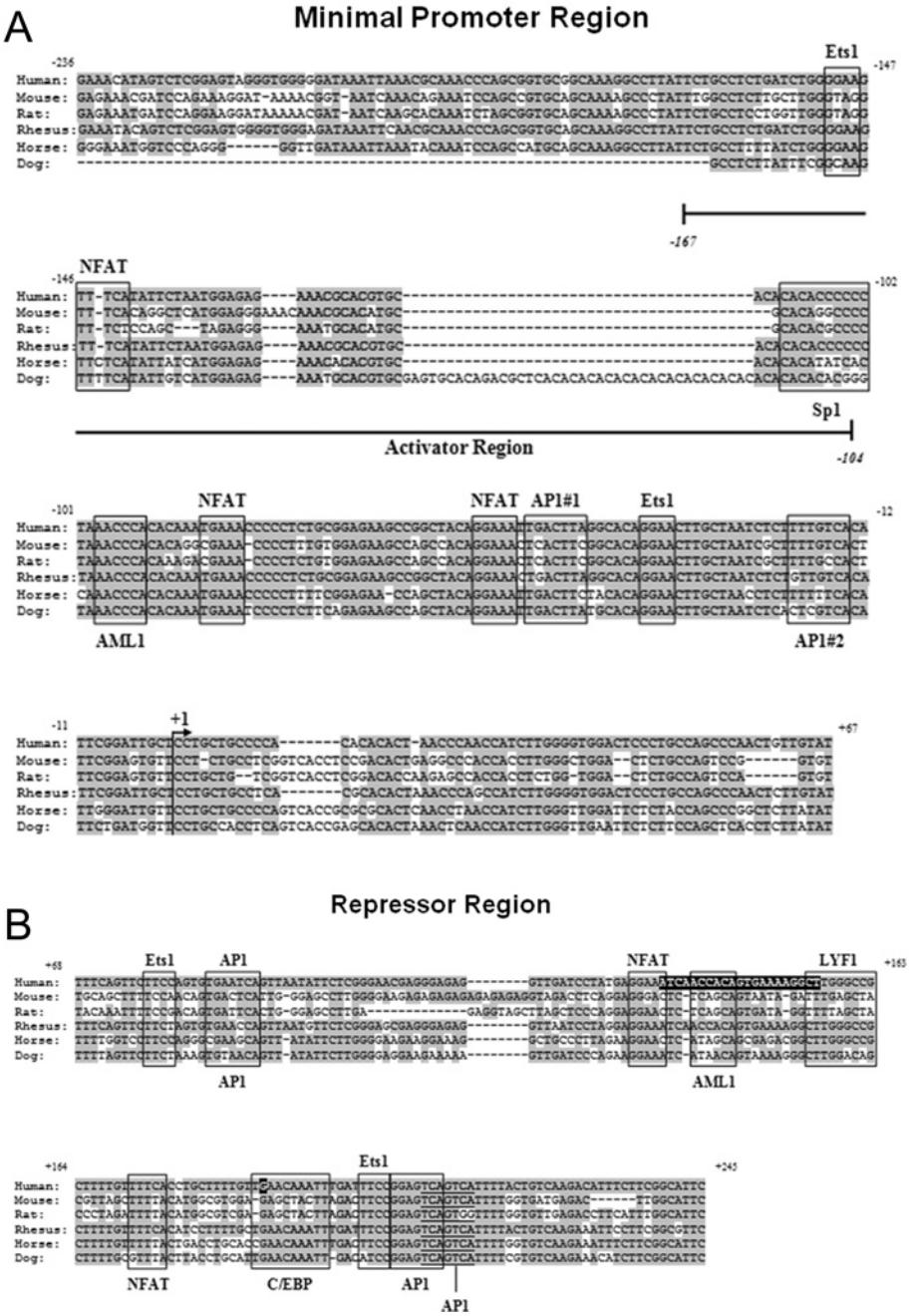

Figure 3 Binding sites for potential activator and repressor transcription factors within evolutionarily conserved regions of the *RHOH* P3 promoter

(**A**) The nucleotide sequence of the human minimal promoter region extending from nucleotides − 236 to + 67 relative to the 5′ end of exon 4 [1] was compared with the nucleotide sequence of the equivalent region in the mouse, rat, rhesus monkey, horse and dog genomes. Compared with the human sequence, those of the other species exhibit 72.3 %, 70.3 %, 95.7 %, 82.8 % and 64 % identity respectively. Identical sequences are depicted by grey shading. The activator region extending from nucleotides − 167 to − 104 is delineated with a black bar. Evolutionarily conserved potential binding sites for transcription factors are boxed. Consensus binding sites on the sense strand are annotated above the species alignments while those on the anti-sense strand are annotated below the alignments. The upstream AP1 site extending from nucleotides − 50 to − 44 is named AP1#1 and the downstream site extending from − 20 to − 14 is named AP1#2. The transcription initiation site that defines the 5′ end of exon 4 is designated + 1 and marked with a bent arrow. (**B**) The nucleotide sequence of the human repressor region extending from nucleotides + 68 to + 245 was compared with the nucleotide sequence of the equivalent region in the mouse, rat, rhesus monkey, horse and dog genomes. Compared with the human sequence, those of the other species exhibit 61 %, 57 %, 94 %, 76 % and 76 % identity respectively. Identical sequences are depicted by grey shading. Evolutionarily conserved potential binding sites for transcription factors are boxed or underlined. Mutations that are sometimes found in patients with DLBCL are indicated by white text highlighted with black boxes [20]. The upstream mutation termed Ly3 consists of the deletion of nucleotides + 136 through + 156 and their replacement with the nucleotide sequence CTA. The downstream mutation termed 470 consists of the replacement of a G nucleotide with a C. Mutation 470 does not alter potential transcription factor binding. However, Ly3 results in disruption of the potential binding sites for NFAT, AML1 and LYF1 and the creation of a potential binding site for MZF1.

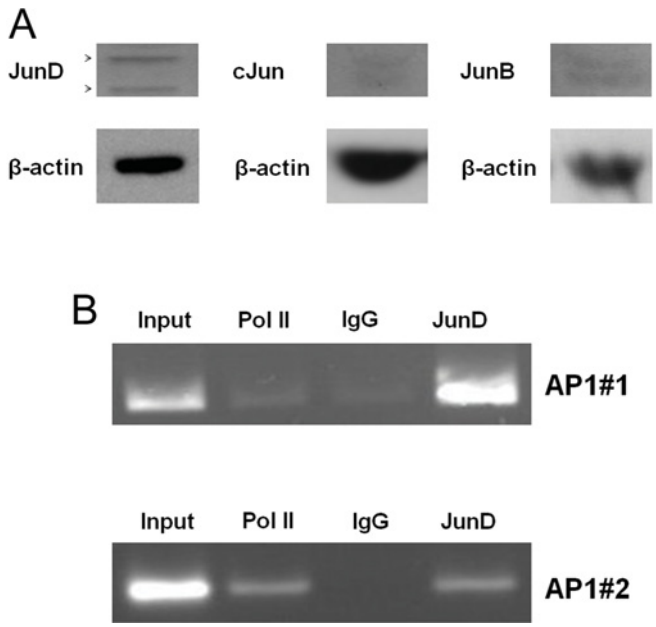

Figure 4 A minimum of two sites within the endogenous *RHOH* P3 promoter bind JunD, and one of these sites also binds RNA polymerase II

(**A**) Western blot analyses of total protein extracts prepared from Raji cells using antibodies that bind human JunD, c-Jun, JunB or a control antibody that binds β-actin. Arrowheads indicate the two JunD isoforms of 39 and 34 kDa that are generated by alternative translation initiation [57]. (**B**) Chromatin extracted from Raji cells was immunoprecipitated with a negative control non-immune IgG antibody, an antibody directed against human RNA polymerase II (Pol II) and an antibody directed against human JunD. The ability of these antibodies to interact with proteins bound by the consensus AP1 sites AP1#1 and AP1#2 within the minimal promoter region was assessed by semi-quantitative PCR. A sample of sheared Raji genomic DNA that was not subject to immunoprecipitation was used as a positive PCR control (Input). PCR products were visualized by agarose gel electrophoresis. One of two independent experiments is depicted.

plasmid to identify individual *cis*-acting elements that control its function. The $-1519/+245$ region was chosen as a starting point for our analysis since -1519 to -1 contains the most evolutionarily conserved section of the intron that separates exon 4 from exon 3 and $+1$ to $+245$ represents all but the last eight nucleotides of exon 4. These last eight nucleotides were excluded since they contain nucleotides involved in RNA splicing and therefore have the potential to confound reporter gene studies. The *LUCIFERASE* reporter plasmid containing the $-1519/+245$ region was found to direct transcription in Raji cells only 4-fold above the background level directed by the plasmid empty of *RHOH* gene sequences (Figure 2A). A modest increase in expression to 6-fold above background occurred when nucleotides -1519 to -584 were deleted, indicating this region does not contain *cis*-acting elements of significance. On the basis of this finding, we now concentrated on the P3 promoter region spanning nucleotides -583 to $+245$. Deletion of nucleotides $+68$ to $+245$ dramatically increased transcriptional activity to 17-fold above background, indicating this region contains repressor *cis* elements. Consequently, the span of nucleotides mapping from $+68$ to $+245$ was designated a repressor region (Figure 2B). Next, serial 5′ truncation of the $-583/+67$ region was performed. The 5′ ends of this series mapped such that successive constructs represented the progressive deletion of distinct regions of evolutionary conservation. Significant and progressive reductions in transcriptional activity occurred when nucleotides downstream of -236 were deleted. Therefore the region spanning nucleotides -236 to $+67$ was taken to represent the minimal promoter region (Figure 2B). Deletion of nucleotides -167 to -104 dramatically reduced transcriptional activity, indicating this region contains activating *cis* elements. Consequently, the $-167/-104$ span of nucleotides was designated an activator region (Figure 2B).

Identification of evolutionarily conserved elements within the *RHOH* P3 promoter that represent potential transcription factor-binding sites

Critical *cis*-acting control elements are often conserved during evolution. Therefore, in order to identify such elements within the P3 promoter, the human sequence was aligned with the equivalent DNA sequences from mouse, rat, rhesus monkey, horse and dog (Figure 3). The human sequence was then analysed for consensus transcription factor-binding sites and these sites mapped over the cross-species alignment. These *in silico* procedures focused on the repressor region spanning nucleotides $+68$ to $+245$ and the minimal promoter region spanning nucleotides -236 to $+67$ that included the $-167/-104$ activator region. Within the $-167/-104$ activator region, single evolutionarily conserved consensus binding sites were identified for Ets1 (E-twenty six 1), NFAT (nuclear factor of activated T-cells) and Sp1 (specificity protein 1) (Figure 3A). Each of these factors has been reported to have the potential to function as a transcription activator [43–45]. Outside the activator region, but within the minimal promoter region, single evolutionarily conserved potential binding sites for AML1 and Ets1 were identified along with two potential binding sites for NFAT and two for the AP1 family of transcription factors. The potential AP1-binding site mapping most distal to the 5′ end of exon 4 was designated AP1#1 and the proximal site was designated AP1#2 (Figure 3A). Within the $+68/+245$ repressor region, evolutionarily conserved consensus binding sites were identified for Ets1, NFAT, AML1, LYF1 (lymphoid factor 1) and members of the C/EBP (CCAAT/enhancer-binding protein) and AP1 families (Figure 3B). All these factors have the potential to act as transcription repressors [46–54]. Mutations sometimes found in the repressor region of patients with DLBCL disrupt binding sites for NFAT, AML1 and LYF1 while creating a potential binding site for MZF1 (myeloid zinc finger 1) [20,55]. Interrogation of the microarray studies deposited with the GEO database indicate that *RHOH* is expressed at higher levels in DLBCL compared with normal B-lymphocytes [41]. Therefore it is intriguing to speculate that this induction is due to the disruption of repressor elements within the P3 promoter and their replacement with a binding site for the transcription activator MZF1.

JunD binds the endogenous P3 promoter

The putative *cis*-acting elements most commonly identified by *in silico* analysis of the P3 promoter are potential binding sites for the AP1 family of transcription factors. Two such elements map within the minimal promoter region and four map within the repressor region (Figure 3). AP1 is a group of dimers composed of members of the Jun, Fos and ATF (activating transcription factor) families of proteins [56]. The majority of AP1 dimers contain c-Jun, JunB or JunD, which comprise the members of the Jun family. Analysis of the GEO database indicates that, in Raji cells, mRNA encoding JunD predominates over those encoding c-Jun and JunB [39,40]. Western blot analysis indicates that this relative expression profile is reflected at the protein level (Figure 4A). Since JunD appears to be the member of the AP1 family that is expressed at the highest level in Raji cells, ChIP was used

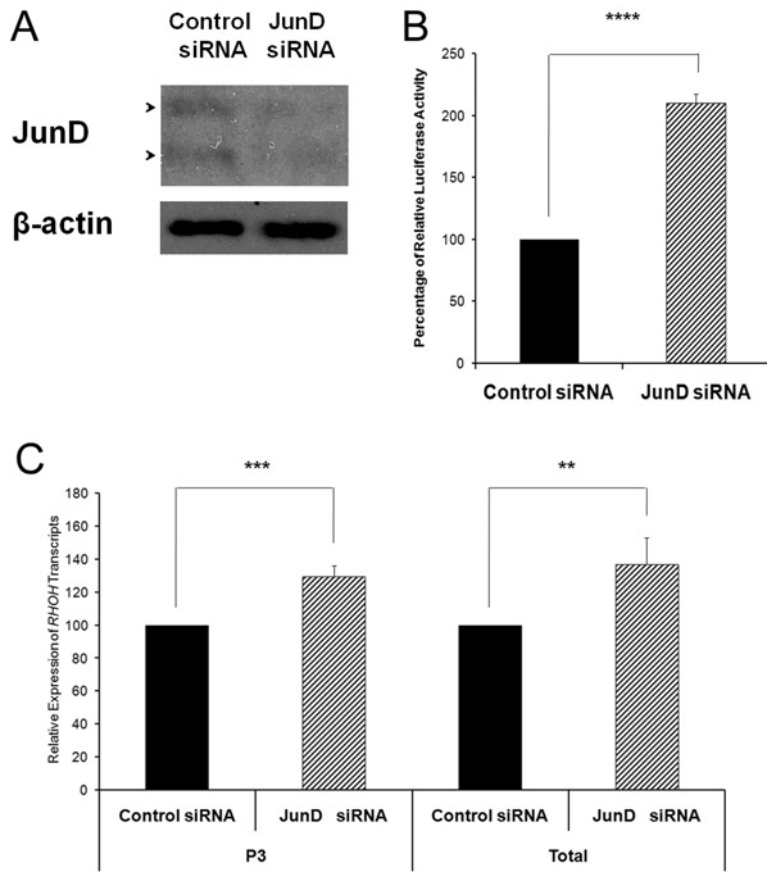

Figure 5 JunD is necessary to repress the *RHOH* gene

(**A**) Raji cells were transfected with the pGL4.14 [*Luc2/Hygro*] plasmid containing the −236/+67 region of the *RHOH* P3 mixed with either a siRNA that specifically targets human *JUND* mRNA (JunD siRNA) or a control siRNA that targets no known human gene (Control siRNA). Next, cells were cultured for 48 h, then total protein was isolated and analysed by Western blotting using an antibody that binds human JunD or a control antibody that binds β-actin. Arrowheads indicate the two JunD isoforms generated by alternative translation initiation [57]. (**B**) The pGL4.14 [*Luc2/Hygro*] plasmid, either empty or containing the −236/+67 region of the *RHOH* P3 promoter, was transfected into Raji cells in the presence of siRNA that specifically targets human *JUND* mRNA (JunD siRNA) or a control siRNA that targets no known human gene (Control siRNA). The β-galactosidase expression plasmid pRSV-βGal was also included in each transfection to control for transfection efficiency. Transfected cells were cultured for 48 h, harvested and luciferase and β-galactosidase assays were performed. The levels of β-galactosidase activity were taken as a measure of transfection efficiency and used to correct the levels of luciferase activity. Following this correction, the level of luciferase reporter gene activity directed by the −236/+67 plasmid in the presence of the control siRNA was divided by the luciferase activity directed by the empty parental plasmid also in the presence of the control siRNA. This fold-above background expression was assigned a value of 100 % (Control siRNA). The fold-above background level of luciferase activity directed by the −236/+67 plasmid in parallel transfections containing siRNA directed against *JUND* mRNA (JunD siRNA) is expressed as a percentage of this value. Each histogram represents the means ± S.E.M. of three independent experiments. A one-sided Student's *t* test was used to determine the degree of significance between expression of the −236/+67 plasmid in the presence of targeting siRNA compared with the control siRNA. $^{****}P > 0.001$. (**C**) Raji cells were transfected with either a siRNA that specifically targets human *JUND* mRNA (JunD siRNA) or a control siRNA that targets no known human gene (Control siRNA). BLOCK-iT™ Fluorescent Oligo for electroporation (Invitrogen) was also included in each transfection to control for transfection efficiency. Following transfection, cells were cultured for 48 h, fluorescence intensity was measured and total RNA was isolated. The expression of *RHOH* gene transcripts specifically initiated by the P3 promoter was assessed by Q-RT–PCR using SYBR® Green technology and the primer pair 41 and 299 as described for Figure 1(B). The Q-RT–PCR results were normalized against fluorescence intensity to correct for variations in transfection efficiency and the level of *RHOH* expression in the presence of control siRNA was assigned a value of 100 %. The level of *RHOH* expression in the presence of JunD siRNA was calculated as a percentage of this value. The expression of the sum total of *RHOH* transcripts was determined using TaqMan technology as previously described [26]. Histograms represent the means ± S.E.M. of three transfections of Raji cells and three independent quantifications of each cDNA synthesis performed in triplicate. A one-sided Student's *t* test was used to determine the degree of significance between promoter activity in the presence of siRNA targeting JunD compared with control siRNA. $^{***}P < 0.01$ and $^{**}P < 0.05$.

to determine if this transcription factor binds the endogenous *RHOH* P3 promoter (Figure 4B). This analysis established that JunD binds each of the two AP1 sites within the minimal promoter region.

JunD is both necessary and sufficient to repress transcription driven by the P3 promoter

The binding of JunD to the P3 promoter of the endogenous *RHOH* gene suggests this transcription factor might play a role in controlling *RHOH* expression. In order to begin to determine this role, siRNA that inhibits expression of JunD was transfected into Raji cells and its affect on expression of both the cloned and endogenous *RHOH* gene was determined. These analyses indicated that, on average, JunD repression increased transcription driven by the cloned minimal promoter region, the endogenous P3 promoter and all *RHOH* promoters combined by 110 %, 29 % and 37 % respectively (Figure 5). Consequently, it appears that JunD is necessary to affect repression of the *RHOH* gene. This conclusion was confirmed when a plasmid that directs constitutive expression of JunD was employed. For this, transcriptional activity of the cloned minimal promoter region, the endogenous P3 promoter and all *RHOH* promoters combined was reduced on average by 14 %, 18 % and 26 % respectively (Figure 6). These results indicate that JunD is not only necessary but also sufficient to

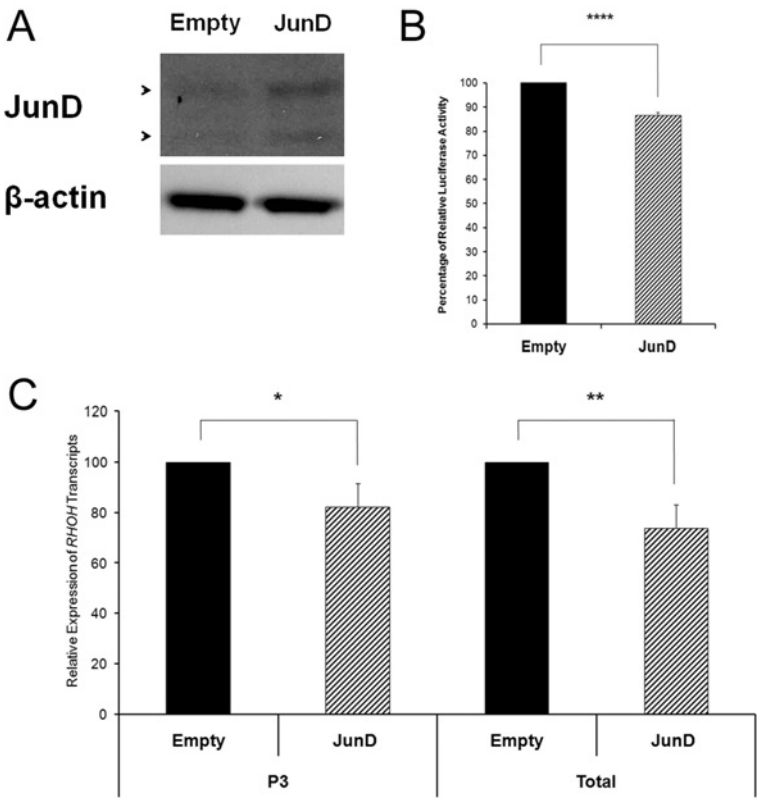

Figure 6 JunD is sufficient to repress the *RHOH* gene

(**A**) Raji cells were transfected with the pGL4.14 [*Luc2/Hygro*] plasmid containing the −236/+67 region of the *RHOH* P3 mixed with either the plasmid pRSV-hjD that constitutively expresses human JunD (JunD) or its parent pRSV-Ng that is empty of JunD coding sequences (Empty). Next, cells were cultured for 48 h then total protein was isolated and analysed by Western blotting using an antibody that binds human JunD or a control antibody that binds β-actin. Arrowheads indicate the two JunD isoforms generated by alternative translation initiation [57]. (**B**) The pGL4.14 [*Luc2/Hygro*] plasmid, either empty or containing the −236/+67 region of the *RHOH* P3 promoter was transfected into Raji cells mixed with either pRSV-hjD or pRSV-Ng. The β-galactosidase expression plasmid pRSV-βGal was also included in each transfection to control for transfection efficiency. Following transfection, cells were cultured for 48 h, harvested and luciferase and β-galactosidase assays were performed. The levels of luciferase activity were normalized against β-galactosidase levels to correct for variations in transfection efficiency. Following this correction, the level of luciferase activity directed by the −236/+67 reporter plasmid in the presence of pRSV-Ng was divided by the luciferase activity directed by the empty reporter plasmid also in the presence of pRSV-Ng. This fold-above background expression was assigned a value of 100% (Empty). Next, the level of luciferase activity directed by the −236/+67 reporter in the presence of pRSV-hjD was divided by the luciferase activity directed by the empty reporter also in the presence of pRSV-hjD. The percentage that this fold-above background expression represents relative to expression in the presence of pRSV-Ng is presented (JunD). Each histogram represents the means ± S.E.M. of three independent experiments. A one-sided Student's *t* test was used to determine the degree of significance between expression of the −236/+67 plasmid in the presence of Jun expression plasmids compared with their corresponding empty parents. $^{****}P < 0.001$. (**C**) Raji cells were transfected with either the plasmid pRSV-hjD (JunD) or its parent pRSV-Ng (Empty). The β-galactosidase expression plasmid pRSV-βGal was also included in each transfection to control for transfection efficiency. The expression of total and P3-specific *RHOH* transcripts was determined as described for Figure 5(C), except that β-galactosidase activity not fluorescence intensity was used to correct for variations in transfection efficiency. Histograms represent the means ± S.E.M. of three transfections of Raji cells and three independent quantifications of each cDNA synthesis performed in triplicate. A one-sided Student's *t* test was used to determine the degree of significance between promoter activity in the presence of the JunD expression plasmid compared with its corresponding empty parent. $^{**}P < 0.05$ and $^{*}P < 0.1$.

affect *RHOH* repression. The levels of *RHOH* repression that are affected by JunD induction are lower than those of *RHOH* induction elicited by JunD inhibition. This could indicate that JunD requires ancillary factors to exert its full function and that these factors are in limited supply.

JunD effects repression of the endogenous *RHOH* gene in mononuclear cells isolated from patients with AML

Immortalized cell lines cultured *in vitro* for many years may not reflect the function of native human cells. Consequently, we sought to validate our finding in the cell line Raji that JunD is a *RHOH* repressor with a functional analysis of leucocytes isolated from volunteer patients. *RHOH* mRNA has been shown to be expressed at abnormally low levels in the mononuclear cells of patients diagnosed with AML [27]. In addition, interrogation of the GEO database indicates that, compared with normal mononuclear cells, those of AML patients tend to overexpress *JUND* mRNA [37]. Therefore in AML cells there appears to be a correlation between low expression of RhoH and high expression of JunD. Consequently, AML cells were transfected with siRNA directed against JunD in order to determine if repression of JunD could affect induction of the sum total of *RHOH* transcripts irrespective of whether they initiated at the P1, P2 or P3 promoter (Figure 7). JunD siRNA caused mononuclear cells isolated from two different AML patients to both exhibit significant increases in expression of total *RHOH* mRNA. The increases of 132% and 89% in AML cells compared favourably with the 37% increase in total *RHOH* transcripts that was caused by JunD siRNA in Raji cells.

Both RhoH induction and JunD repression cause increased expression of *PKCα*

HCL is characterized by both overexpression of JunD and under-expression of RhoH [26,29]. JOK-1 is a B-lymphocytic cell

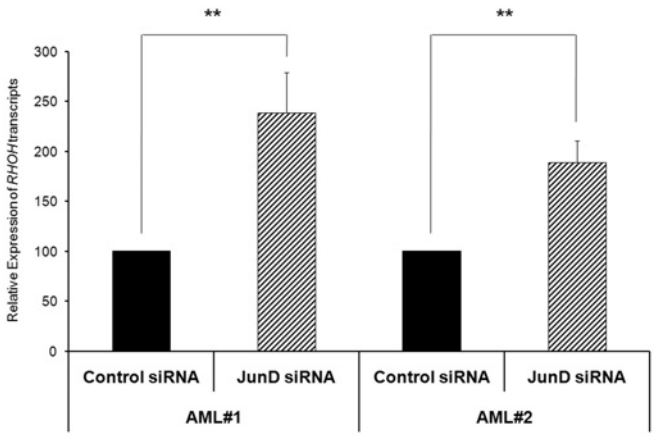

Figure 7 JunD affects repression of the endogenous *RHOH* gene in AML mononuclear cells

Mononuclear cells isolated from two different AML patients (AML#1 and AML#2) were transfected with either a siRNA that specifically targets human *JUND* mRNA (JunD siRNA) or a control siRNA that targets no known human gene (Control siRNA). Each transfection included BLOCK-iT™ Fluorescent Oligo for electroporation (Invitrogen) to control for transfection efficiency. Transfected cells were cultured for 48 h and then the fluorescence intensity was measured and total RNA was isolated. The expression of the sum total of all *RHOH* transcripts was assessed for both patients by Q-RT–PCR using TaqMan technology as described previously [26]. Relative expression of all *RHOH* transcripts was calculated by dividing their quantitative level of expression by the quantitative level of *ABL* gene expression. Relative *RHOH* levels were then normalized against fluorescent intensity to correct for variations in transfection efficiency. The relative *RHOH* expression level obtained in the presence of the Control siRNA was assigned a value of 100 % and the expression levels obtained in the presence of JunD siRNA were expressed as a percentage of this value. Histograms represent the means ± S.E.M. of three measurements made after two transfections. A one-sided Student's *t* test was used to determine the degree of significance between *RHOH* expression in the presence of siRNA targeting JunD compared to control siRNA. $^{**}P < 0.05$.

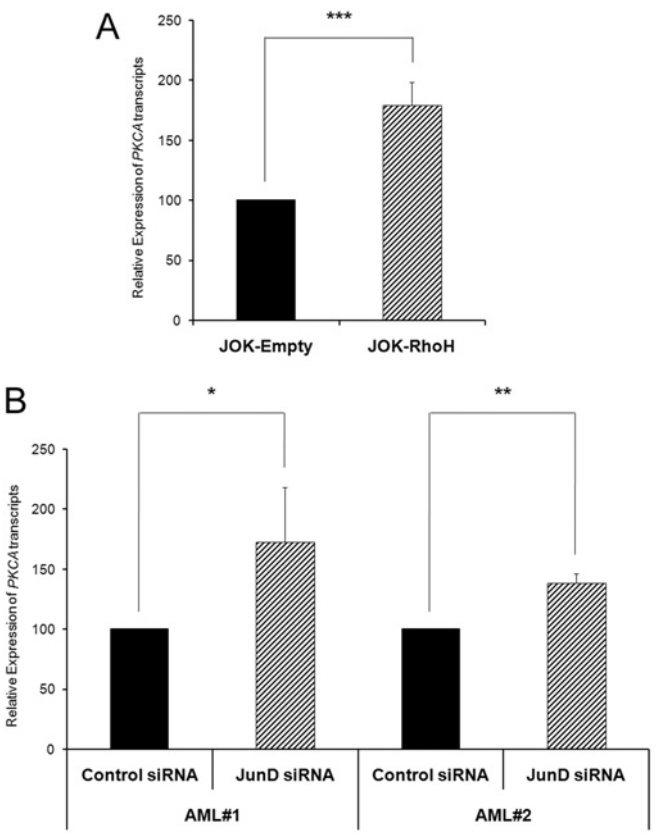

Figure 8 RhoH induction or JunD repression cause increased expression of *PKCα*

(**A**) Pools of the HCL cell line JOK-1 were generated that stably contain in their genome either an empty expression vector (JOK-Empty) or the same vector constitutively expressing *RHOH* (JOK-RhoH) [26]. These two pools were analysed for expression of *PKCα* (*PKCA*) by Q-RT–PCR that employed SYBR® Green technology in the same way as described in Figure 1(B). Relative *PKCα* expression levels were calculated by dividing their quantitative level of expression by the quantitative level of *ABL* gene expression. The relative expression of *PKCα* in JOK-Empty was assigned a value of 100 % and *PKCα* expression in JOK-RhoH was expressed as a percentage of this value. Histograms represent the means ± S.E.M. of three independent cultures of JOK-Empty and JOK-RhoH and three independent quantifications of each cDNA synthesis performed in triplicate. A one-sided Student's *t* test was used to determine the degree of significance between *PKCα* expression in JOK-RhoH compared with JOK-Empty. $^{***}P < 0.01$. (**B**) Mononuclear cells from two different patients with AML were transfected with either a siRNA that specifically targets human *JUND* mRNA (JunD siRNA) or a control siRNA that targets no known human gene (Control siRNA). Following transfection, cells were cultured for 48 h and total RNA was isolated. The expression of *PKCα* (*PKCA*) was assessed by Q-RT–PCR using SYBR® Green technology in the same way as described for Figure 1(B). Relative expression of *PKCα* was calculated by dividing its quantitative level of expression by the quantitative level of *ABL* expression. The relative *PKCα* expression level obtained in the presence of the Control siRNA was assigned a value of 100 % and the expression levels obtained in the presence of JunD siRNA were expressed as a percentage of this value. Histograms represent the means ± S.E.M. of three measurements made after two transfections. A one-sided Student's *t* test was used to determine the degree of significance between *PKCα* expression in the presence of siRNA targeting JunD compared with control siRNA. $^{**}P < 0.05$ and $^{*}P < 0.1$.

line derived from a patient with HCL. In a previous study we determined that JOK-1 exhibits a low expression of *RHOH* mRNA [26]. Pools of JOK-1 were generated that stably contain in their genome either an empty expression vector or the same vector constitutively expressing *RHOH*. These pools of JOK-1 were designated JOK-Empty and JOK-RhoH respectively. JOK-RhoH produces xenograft tumours that, on average, are 70 % smaller than those produced by JOK-Empty [26]. Comparison of the transcriptomes of JOK-Empty and JOK-RhoH by microarray analysis was performed in order to identify downstream targets of RhoH signalling. For each cell line, two biological samples were studied by dye-swap analysis. The result of this analysis identified a 56 % increase in the expression of the mRNA encoding PKCα. Using a one-sided Student's *t* test, the probability value for this increase was calculated at $P = 0.016$. Q-RT–PCR analysis confirmed the microarray study by demonstrating that expression of *PKCα* was increased by 85 % in JOK-RhoH compared with JOK-Empty (Figure 8A). Given that JunD is a repressor of RhoH, it would be expected that inhibition of JunD would increase expression of *PKCα* due to increased expression of RhoH. Consistent with this expectation, increases of 70 % and 37 % in *PKCα* expression were elicited in mononuclear cells isolated from two AML patients when they were transfected with siRNA targeted against *JUND* mRNA (Figure 8B). Consequently, JunD appears to be a functionally relevant repressor of RhoH since its inhibition both upregulates RhoH and also at least one of its downstream signalling targets.

DISCUSSION

RHOH mRNA was originally identified in a NHL (non-Hodgkin's lymphoma) cell line as a fusion with transcripts originating from the *BCL6/LAZ3* gene [15,63]. Since the fused transcripts resulted from a translocation of chromosomes 3 and 4, RhoH was initially named TTF for translocation three four. The *TTF* gene was subsequently found to encode a novel member of the Rho family of small GTP-binding proteins. Consequently, TTF was renamed RhoH to indicate a Rho protein following in the discovery

sequence designated by the Gene Nomenclature Committee of the Human Genome Organization (European Bioinformatics Institute, Cambridge, U.K.) [63]. That RhoH was also found to be expressed specifically in haematopoietic cells was conveniently coincident with its alphabetic designation.

Following its original identification as a participant in a NHL translocation, the association between aberrant RhoH expression and haematopoietic malignancy has strengthened. In AML, low expression of *RHOH* mRNA was shown to be a predictor of worse prognosis in both overall and disease-free survival [27]. B-lymphocytes derived from patients with HCL also exhibit low expression of *RHOH* transcripts [26]. A direct link between this low expression and HCL pathogenesis was demonstrated in a xenograft mouse model where reconstitution of high levels of RhoH reduced primary tumour burden by 70 % and protected against splenomegaly and mortality [26]. The B-lymphocytes of patients with CLL express abnormal levels of *RHOH* mRNA [28]. However, unlike AML and HCL where *RHOH* mRNA is unusually low, in CLL it is unusually high. As with HCL, a direct link between the quantitative level of RhoH expression and disease pathogenesis has been established in CLL using an animal model. Specifically, deletion of the *RHOH* gene in the *Eμ-TCL1*^{Tg} mouse model of CLL was found to delay accumulation of leukaemic cells in peripheral blood and the leukaemic burden in the peritoneal cavity, bone marrow and spleen [28]. Taken together, the analyses of malignant leucocytes indicate that the function of RhoH is controlled to a significant degree by its quantitative level of expression. Indeed, such control has been proposed to be largely responsible for RhoH function, since the protein is expressed in a constitutively active conformation [2,14].

Despite the likelihood that RhoH levels are critical to its function, only one study to date has defined a mechanism by which these are regulated [4]. In that study T-lymphocytes were shown to down-regulate RhoH protein levels by lysosomal degradation following stimulation of the T-cell receptor complex with an anti-CD3ε antibody. In the present study, we report for the first time a mechanism that controls *RHOH* expression in B-lymphocytic cells and mononuclear cells isolated from patients with AML. We find that in the B-lymphocytic cell line Raji and AML mononuclear cells *RHOH* is repressed by JunD binding its P3 promoter. Interestingly, RNA polymerase II exhibits binding to the JunD site that maps between only 14 and 20 nucleotides upstream of the transcription initiation site defined by the P3 promoter. Therefore, it is possible that JunD affects repression of the P3 promoter either by passive steric hindrance of RNA polymerase loading or active direct binding.

The finding that JunD represses *RHOH* is consistent with the observation in HCL of abnormal constitutive expression of JunD [29] but under-expression of *RHOH* [26]. *In silico* analysis of microarray studies deposited in GEO confirms the reciprocal expression of *JUND* and *RHOH* mRNA in HCL [42]. In addition, further analysis of the GEO database indicates that compared with normal mononuclear cells those of AML and CLL patients tend to over- and under-express *JUND* mRNA respectively [37,38]. Since AML and CLL patients respectively under- and over-express *RHOH* mRNA, a consistent inverse correlation appears to exist between *RHOH* and *JUND* expression. This correlation also holds in physiological rather than pathological circumstances, since *RHOH* expression is reduced during activation of normal T-lymphocytes [2,4], whereas *JUND* expression is induced [64]. Consequently, our finding that JunD acts as a *RHOH* repressor provides a mechanistic connection between their reciprocal expression both in physiology and pathology. This connection indicates a reinforcing feedback loop may exist in which RhoH down-regulation by JunD relieves RhoH inhibition of Rac1 that is then able to activate JunD through PAK1 (p21-activating kinase 1) and MEK1 (mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase 1) (Figure 9) [2,61,62]. However, a counter-balancing feedback loop may also exist between JunD and RhoH. This possibility arises since both RhoH induction and JunD inhibition induce *PKCα*. The kinase produced by *PKCα* can activate the AP1 family, of which JunD is a member [58–60]. Therefore, JunD could inhibit its own activator through RhoH repression (Figure 9). Our data cannot exclude the possibility that JunD could also directly inhibit *PKCα* by binding its *cis*-acting control elements. However, to date the only transcription factors implicated in *PKCα* regulation are AP2, Sp1, Ets1 and SOX9 [65,66].

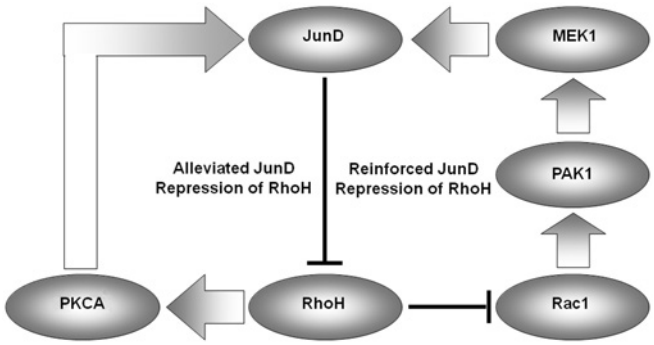

Figure 9 Potential feedback between JunD and RhoH

Our studies indicate that JunD is a repressor of RhoH and its downstream effector PKCα (PKCA). Previous studies have demonstrated that PKCα can activate the AP1 family of transcription factors, of which JunD is a member [58–60]. In addition, RhoH inhibits the function of Rac1 that activates PAK1, that in turn is an activator of MEK1 [2,61]. MEK1 has been shown to increase expression of JunD [62]. Consequently, potentially two counter-balancing feedback loops exist between RhoH and JunD.

RhoH plays an important role in haematopoiesis by driving leukocyte differentiation and inhibiting leukocyte survival and proliferation [3,5,6]. In myeloid cells, PKCα has also been shown to promote differentiation and inhibit proliferation [67,68]. In haematopoietic progenitor cells, PKCα has been shown to limit survival and decrease proliferation [69]. Therefore, the finding that RhoH is a positive regulator of *PKCα* expression is consistent with an intracellular signalling cascade involved in producing short-lived differentiated leukocytes with a limited capacity to divide. Consequently, it is not surprising that leucocytes lacking these characteristics are hallmarks of AML where RhoH expression has been demonstrated to be low [27] and HCL where both RhoH and PKCα expression are low [26,70]. However, in CLL, RhoH is expressed at abnormally high levels [28]. Initially, this would appear to contradict the apparent beneficial role played by RhoH in haematopoiesis. However, CLL is characterized by over-expression of active protein kinase Cβ II [70]. Protein kinase Cβ antagonizes the function of PKCα [67]. Therefore, even though RhoH expression is high in CLL, its function is likely confounded by inhibition of its down-stream effector.

AUTHOR CONTRIBUTION

Bruno Quesnel and Céline Berthon provided anonymized patient samples and clinical records. Carl Simon Shelley designed and interpreted experiments, provided research funds and wrote the paper. Jean-Pierre Kerckaert and Martin Figeac performed microarray experiments. Laure Delestré performed the majority of experiments, helped design and interpret experiments and helped write the paper. Sylvie Galiègue-Zouitina designed and interpreted experiments, provided research funds and helped write the paper.

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