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PU.1-dependent regulation of UCH L1 expression in B-lymphoma cells

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Abstract

Elevated levels of ubiquitin C-terminal hydrolase L1 (UCH L1) have been detected in a variety of malignancies, and recent studies show the oncogenic capacity of overexpressed UCH L1 *in vivo* in animal models. Here we demonstrate that expression of endogenous UCH L1 is significantly higher in B-lymphoma cells than in transformed cells of epithelial and fibroblastic origin. The specific hematopoietic transcription factor PU.1 induces UCH L1 expression through direct activation of the uch l1 promoter. Using chromatin immunoprecipitation (ChIP) assays and direct mutagenesis we identified PU.1 binding sites on the uch l1 promoter, at least three of which are involved in this activation. We also show that the viral transcriptional co-activator EBNA2 dramatically increases PU.1-dependent up-regulation of endogenous UCH L1 expression. Finally, inhibition of PU.1 expression with specific shRNA resulted in reduction of UCH L1 mRNA and protein levels in Epstein–Barr virus (EBV)-transformed B-cells. We propose that the ubiquitin-editing enzyme UCH L1 is a multifunctional pro-oncogenic factor involved in development and progression of certain lymphoid malignancies, including EBV-associated lymphomas.

Keywords

Transcription factor PU.1; ubiquitin C-terminal hydrolase L1; lymphomas

Introduction

There are few known representatives of the small group of deubiquitinating enzymes named ubiquitin C-terminal hydrolases, UCHs. They are relatively small proteins (25–75 kDa) with approximately 50% sequence homology among the members [1]. Among them is ubiquitin

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C-terminal hydrolase L1 (UCH L1), a cysteine hydrolase that contains the typical active-site triad of cysteine, histidine, and aspartic acid and catalyzes hydrolysis of C-terminal esters and amides of ubiquitin [2]. Normally UCH L1 is expressed abundantly (and almost exclusively) in brain tissue. Although the physiological function of UCH L1 in neurons is still unclear, mutations in the UCH L1 gene have been associated with Parkinson and Alzheimer diseases [3]. UCH L1 has also been shown to function as a ubiquitin ligase in its dimeric form [4], and as a stabilizer of mono-ubiquitin in neurons [5].

Recent studies indicate that UCH L1 is involved in regulation of cytoskeletal dynamics: it affects tubulin polymerization [6,7], and alters the dynamics of actin filaments during bacterial invasion [8]. Moreover, the recently discovered association of UCH L1 with mitotic spindle microtubules suggests a functional role of general significance for this protein during mitosis [7].

Increased levels of UCH L1 have been detected in cancers of different cellular and tissue origin such as lung [9], colorectal [10], bladder [11], and breast cancer [12], suggesting its involvement in oncogenesis. Indeed, several studies demonstrate that inhibition of UCH L1 expression reduces the tumorigenic phenotype of transformed cells [13–15]. Recently two independent *in vivo* studies provided strong evidence that UCH L1 is an oncogene: transgenic mice with overexpressed UCH L1 develop tumors [16], and pulmonary metastasis of cancer cells in nude mice can be suppressed by inhibition of UCH L1 expression [15]. These distinct studies indicate that this multifunctional protein of the ubiquitin system is involved in diverse cellular processes, and that the specific physiological roles of UCH L1 and regulation of its expression in transformed cells need further analyses.

Elevated levels of UCH L1 RNA in malignant tumor cells indicate that the *uch l1* gene is subject to regulation during cellular transformation by oncogenic transcription factors. The minimal *uch l1* promoter region has been mapped to a 233 bp region that possesses binding sites for neuron-specific transcription factors such as OCT and PSN, which regulate UCH L1 expression in neurons [17]. Indeed, B-Myb, a transcription factor implicated in regulation of the cell cycle, has been shown to stimulate expression of murine *uch l1* on the promoter level *in vitro* and *in vivo* [18]. Additionally, we have shown that the β -catenin/T-cell factor (TCF) transcription complex stimulates expression of the *uch l1* gene [19].

UCH L1-expressing transgenic mice are susceptible to spontaneous lymphomas, and UCH L1 overexpression accelerated lymphomagenesis in E μ -myc transgenic mice [16]. High levels of UCH L1 were also observed in transformed cells of lymphoid origin, including Burkitt lymphomas [13,20].

Analysis of the *uch l1* promoter sequence revealed five putative PU.1 binding sites. The transcription factor PU. 1 belongs to the Ets family and contains the Ets domain as a DNA-binding region [21]. Expression of PU.1 is restricted in the hematopoietic system: it activates gene expression during myeloid and B-lymphoid cell development [22,23]. Studies in PU.1 knockout mice have shown that it is required for the development of macrophages, B-cells, and neutrophils, as well as dendritic cells [24,25]; PU.1 has been shown to regulate the expression of a growing list of genes in each of these lineages [23]. PU.1 functions as an

oncogene in Friend virus-induced erythroleukemia and as a tumor suppressor in acute myeloid leukemias [26,27]. Additionally, PU.1 is up-regulated in a majority of lymphomas including B-cell, Hodgkin, follicular, and mantle cell [28,29].

First isolated from African Burkitt lymphoma, Epstein-Barr virus (EBV) is a DNA tumor virus that causes or is closely associated with several malignancies, including B-cell lymphomas in which it acts as an etiologic agent or cofactor [30]. EBV can infect and transform resting B-cells both *in vitro* and *in vivo*, confirming its B-lymphotrophic nature. EBV-positive lymphomas include virtually all B-cell post-transplant lymphoproliferative diseases and acquired immunodeficiency syndrome (AIDS)-associated lymphomas, a subset of Hodgkin lymphomas, and rare T-cell lymphomas [31]. The endemic Burkitt lymphomas from Africa are 95% EBV-positive [32]. Among the EBV latency genes, EBV nuclear antigen 2 (EBNA2) is crucial for the transformation process [33]. EBNA2 is the major viral transcriptional activator in latently infected transformed B-cells, and it activates not only viral but also cellular promoters via other transcription factors, one of which is PU.1 [34,35].

In this study we show that the essential hematopoietic transcription factor PU.1 directly up-regulates the *uch l1* gene in transformed B-cells, and that the EBV transactivator EBNA2 further enhances PU.1-dependent activation of UCH L1 expression. We also show that suppression of PU.1 levels reduces endogenous UCH L1 expression in transformed B-cells, providing evidence that PU.1 contributes to UCH L1 expression in these cells at physiological levels.

Materials and methods

Cell culture

All adherent cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma) and penicillin–streptomycin. Burkitt lymphoma cell lines (LCLs) BL30 and BL30-EBV, X-50/7, Raji, and KR4 lymphoblastoid cells were cultured in RPMI 1640 medium plus 10% heat-inactivated FBS and 100 units/mL penicillin–streptomycin. All cell lines were maintained at 37°C in 5% CO₂ in air.

Plasmid constructs

Wild-type pAG-EBNA2-HA was a gift from Dr. Paul Ling [36], wild type pECE-PU. 1 a gift from Dr. Alan Friedman [37], and PU.1 siRNA construct a gift from Dr. Mark Kaplan [38]. pGL3-UCH L1 promoter reporter construct was amplified and cloned as described earlier [14]. pET-32a PU.1 was a gift from Dr. Michael Ostrowski [39].

Transient transfections and luciferase reporter assay

For luciferase assays, cells were plated in six-well plates and transiently transfected with the use of Eugene HD (Roche Diagnostics) with UCHL1p-LUC promoter plasmid, and effector plasmid (for concentrations refer to figure legends). The total amount of DNA in all transfections was kept constant with empty vector. Luciferase assays were performed 48 h post-transfection as specified by the manufacturer (Promega). All reporter assay results are

from three independent experiments prepared in triplicate and have been normalized for β -galactosidase activity.

Reverse transcriptase-PCR

NIH 3T3 cells were transiently transfected with a total of 2 μ g of DNA with Fugene HD reagent (Roche Diagnostics) according to the manufacturer's instructions. Cells were collected 48 h post-transfection for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted with the use of Agilent's Total RNA isolation mini kit as per the manufacturer's instructions (Agilent Technologies). Five hundred nanograms of total RNA were used for RT-PCR reactions with Qiagen's one step RT-PCR kit (Qiagen) as per the manufacturer's instructions at an annealing temperature of 55°C. Samples were analyzed on 1% agarose gel. Primers used were: UCH L1: 5'-GGATGGCC ACCTCTATGAAC-3', 5'-AGACCTTGGCAGCG TCCT-3'; GAPDH: 5'-AGGTGAAGGTCGG AGTCAACG-3', 5'-AGGGGTCATTGATGGCA ACA-3'.

Quantitative real-time PCR

Total RNA was extracted from BL30 or BL30-EBV cells and LCLs stably expressing PU. 1 shRNA or GFP (green fluorescent protein) shRNA. The reverse transcription reaction was performed with 500 ng total RNA using the iScript cDNA synthesis kit per the manufacturer's instructions (Bio-Rad). A 1:25 dilution of cDNA was used in the quantitative real-time (QRT)-PCR reaction. QRT-PCR was carried out in a 15 μ L reaction mixture with gene-specific primers for UCH L1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (also used in RT-PCR) using an iQ-SYBR green kit (Bio-Rad). PCR conditions were 95°C for 3 min, 45 cycles at 95°C for 15 s, and 55°C for 45 s on an ABI HT 7600 PCR instrument. All samples were assayed in triplicate. Differences in expression of UCH L1 were evaluated using a relative quantification method in which expression of UCH L1 was normalized to the reference gene GAPDH.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was carried out using an Active Motif ChIP-IT enzymatic kit (Active Motif) as per the manufacturer's instructions. KR4 cells were fixed with 37% formaldehyde (1% final concentration) for 10 min at 37°C; the reaction was stopped with cold 0.125 M glycine solution for 5 min at room temperature. The cells were then washed twice with phosphate buffered saline solution (PBS) and collected in 0.5 mL of digestion buffer with 1 $\times$ protease inhibitors. Chromatin was sheared with shearing enzyme for 10 min at 37°C to obtain an average of 200–1000 bp fragments. Sheared chromatin was incubated overnight at 4°C with Protein G magnetic beads, and PU.1 antibody (Santa Cruz-352 X). Immunoprecipitation was performed as per the manufacturer's instructions; cross-linking was reversed by incubating immunoprecipitated complexes with 5 M NaCl and RNase A (final concentration 25 μ g/mL) for 2 h at 65°C followed by Proteinase-K (final concentration 50 μ g/mL) treatment for 2 h at 42°C. PCR was performed with 5 μ L precipitated DNA with primer pairs flanking consensus PU.1 sites in the uch l1 promoter. PCR conditions were: one cycle at 95°C for 2 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min, and a final extension at 72°C for 10 min. The primers used in the

reaction were: 1500 bp upstream: 5'-CAGTTCCCAAGCTCAGTG-3', 5'-CCGGACACATAACCCAGT-3'; site 1: 5'-TC ACTTAAAAACGAACCTCG-3', 5'-GAGTCTCT GAAGCATTTGAG-3'; site 2/3: 5'-CAACAAATC CCGTCTCCACA-3', 5'-TCGCCGGTGAGATA ATCTGG-3'; site 4/5: 5'-GTACCCATCTGGC CGCGACC-3', 5'-CCATCGGCTTGAGCTGCA TC-3'.

Site-directed mutagenesis

The uch l1 promoter construct was mutated at different PU. 1 binding sites with a Quik-Change Site Directed Mutagenesis Kit (Stratagene). The PU.1 binding site GGAA was mutated to CTTC and exactly the same mutations were introduced at all the binding sites. Four nucleotide changes were introduced into the parental plasmid by oligonucleotide primers carrying the specific mutations. The construct was mutated at a single PU.1 binding site or two adjacent binding sites at a time. The parental DNA template was then digested with *DpnI*. All mutations and the integrity of the remainder of the promoter were confirmed by DNA sequencing.

Western blotting

Total cell lysates were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare), blocked in 5% milk-Tris-buffered saline solution, and incubated at 4°C overnight with UCH L1 (1:7500; Invitrogen), PU.1 (1:500; SCBT), and GAPDH (1:5000; Sigma) antibodies followed with horseradish peroxidase-conjugated secondary antibody. Proteins were detected with a Super Signal West Pico Chemiluminescence Detection Kit (Pierce Biotechnology) and exposed to Kodak XAR-5 film.

Purification of recombinant PU.1

pET-32a PU.1 was transformed into BL21(DE3) pLysS (Stratagene). The cultures were incubated at 37°C and induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were lysed in 100 μ L of FastBreak lysis buffer (Promega) and PU.1 was purified with His-Magne Beads (Promega), as per the manufacturer's instructions. PU.1 was eluted with 500 mM imidazole, 10% glycerol, and 100 mM HEPES [4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid; pH 7.5]. The purity of PU.1 was confirmed by densitometric analysis of Coomassie blue-stained 10% SDS-PAGE gel.

Electrophoretic mobility shift assay

Mobility shift assays (Invitrogen, E-33075) were performed using the following double-stranded oligonucleotides containing PU.1 binding sites and surrounding sequences from the human uch l1 promoter [Figure 2(A)]: site 1: 3'-ACTTAACATG-GAGGAGGAATTGTCTA AGGTAA-5'; site 2: 5'-TTGGGCGAAGTTTCTGGAAACCCATCCT TTCACCA-3'; site 3: 3'-CTGCTCCATACACT-CAAGGAACACCCACCA ACAAAT-5'; site 4: 5'-GAACCAAGCGAGGGGGAAACGGACGGTCG CGGCC-3'; site 5: 5'-CA GAAATAGCCTAGGGAAAGACGAAAAACAGCT AG-3'.

Each PU.1 site was generated using single-stranded oligonucleotides. The oligomers (3 mg/mL) were annealed to complementary oligomer in 3 mM sodium citrate, pH 7.0, and 30 mM NaCl at 95°C for 5 min and then cooled slowly to room temperature for several hours.

The annealed oligomers (100 pg) were incubated with or without recombinant PU.1 (200 ng) in presence of 5×binding buffer (Invitrogen; E33075) for 20 min at room temperature. At the end of the reaction, DNA loading dye was added and the reaction was separated on 4% PAGE in Tris-acetate–ethylenediaminetetraacetic acid (EDTA) (TAE) buffer at 200 V. The gel was then stained with SYBR green DNA stain for 20 min, washed with double-distilled H₂O, and visualized using a fluorescence imager at 473 nm excitation and 520 nm emission wavelength settings. Next, for detecting PU.1, proteins were transferred onto a PVDF membrane and Western blot analysis was performed for PU.1 with anti-PU.1 antibody (SCBT).

Immunoprecipitation

KR4 cells were lysed with buffer containing 50 mM of Tris-HCl, pH 7.6, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, and complete protease inhibitor mixture (Roche Diagnostics). After pre-clearing, cell lysates were incubated with PU.1 (SCBT) or control immunoglobulin G (IgG) for 1 h at 4°C; immune complexes were then incubated with protein A/G Sepharose beads (Santa Cruz) at 4°C overnight, washed four times with protein lysis buffer, and then eluted from protein G Sepharose with 2 × Laemmli's buffer.

Results

UCH L1 levels are elevated in transformed B-cell lines

We compared UCH L1 expression in cells of different origins. Comparison of different transformed B-cell lines with transformed epithelial and fibroblastic cell lines demonstrated that UCH L1 expression is substantially increased in transformed B-cells versus the other cell lines (Figure 1), which suggests a specific role for UCH L1 in lymphoid cancers.

Direct binding of PU.1 on uch l1 promoter in vitro

The uch l1 promoter sequence analysis had revealed five PU.1 putative binding sites. A schematic representation of the PU.1 sites is shown in Figure 2(A). We performed electrophoretic mobility shift assays (EMSAs) to determine whether PU.1 can bind directly to the uch l1 promoter (Figure 2) using recombinant PU.1 purified from *Escherichia coli* [Figure 2(B)] and oligos corresponding to the putative PU.1 sites on the uch l1 promoter. Detection of DNA complexes with SYBR green DNA stain [Figure 2(C)] and Western blot analysis with PU.1 antibody [Figure 2(D)] showed that PU.1 caused a shift in the mobility of dsDNA oligonucleotides representing binding at each of the five PU.1 sites on the promoter, indicating that PU.1 directly binds to the uch l1 promoter.

UCH L1 is regulated at the transcriptional level through PU.1 binding sites in transformed B-cell lines

We also tested whether PU.1 could bind to the endogenous uch l1 promoter with ChIP assays (see 'Materials and methods'). Non-immunoprecipitated DNA was used as input DNA and normal IgG antibody as negative control. The ChIP data indicate that PU.1 was capable of binding the uch l1 promoter on PU.1 sites [Figure 3(A)]; since sites 2 and 3 are located close to each other, a single PCR reaction was performed to detect binding on these sites. Although the GC-rich character of the uch l1 promoter sequence somewhat impeded PCR reactions for sites 4/5, binding was detected at the combined sites. Additionally, we also performed PCR for a region 1500 bp upstream of site 1 on the uch l1 promoter as a control for non-specific binding. Our ChIP results demonstrate that PU.1 could bind to at least three of the five sites on the uch l1 promoter.

To confirm that PU.1 induces uch l1 promoter activity through its binding sites, we made site-directed mutations in the parental uch l1 promoter plasmid by creating a total of five uch l1 promoter plasmids, each with single-site mutations, and an additional construct in which all five PU.1 binding sites were mutated from GGAA to CTTC. An identical mutation was previously shown to prevent binding of PU.1 to the cathepsin G gene [40]. We then examined how these mutations affected PU.1's ability to activate the uch l1 promoter. NIH 3T3 cells were co-transfected with control or pECE-PU.1 expression plasmid, along with either wild-type or mutated UCHL1p-LUC construct. All luciferase assays were performed as described in 'Materials and methods.' We found that PU.1 induced the wild-type uch l1 reporter construct by 2.5-fold. Additionally, while individual mutation in each PU.1 binding site reduced promoter activity, there was a more profound inhibitory effect when all five PU.1 binding sites were mutated [Figure 3(B)]. These results indicate that the expression of UCH L1 is regulated directly by PU.1.

UCH L1 expression is up-regulated in primary B-lymphocytes immortalized with EBV

Previous studies have shown that EBV-transformed peripheral blood mononuclear cells (PBMCs) analyzed after 90 days of infection had increased the enzymatic activity of at least six cellular deubiquitinating enzymes (DUBs) including UCH L1 [41]. To investigate how UCH L1 expression is increased in such cells upon EBV infection, we examined the expression of UCH L1 in two sets of PBMCs before and after EBV infection [Figure 4(B)]. UCH L1 was not detected in primary PBMCs; however, it was readily detectable at high levels in the EBV-transformed cells. Thus, upregulation of UCH L1 RNA [Figure 4(A)] and protein levels [Figure 4(B)] after EBV immortalization likely accounts for the increased UCH L1 activity.

Infection of Burkitt lymphoma cells with EBV further increases UCH L1 expression

We next investigated whether the induction was related to EBV products expressed during type III EBV latency. African Burkitt lymphomas are characterized by translocations that activate the expression of *c-myc* and are almost always EBV-positive. The basal levels of UCH L1 are elevated in such lines [41]. Rarely, Burkitt lymphoma (BL) and cell lines derived from them are EBV-negative, but they can be infected with EBV *in vitro*. We took advantage of one such cell line, BL30, and its B95.8-infected counterpart, BL30-EBV [42],

to compare endogenous levels of UCH L1 and then to determine whether EBV type III latency products affect UCH L1 expression. First, we examined whether the type III latency state of BL30-EBV cells would alter basal uch l1 promoter activity by comparing activity in these two cell lines with the use of UCHL1p-LUC construct [19]. As shown in Figure 5(A), uch l1 promoter activity is higher in EBV-positive BL30 cells.

We next analyzed UCH L1 RNA and protein levels in these cell lines with QRT-PCR and Western blot analysis. Although UCH L1 RNA [Figure 5(B)] and protein [Figure 5(C)] were readily detectable in EBV-negative BL30 cells, levels were consistently higher in EBV-infected cells. To further confirm that changes observed in UCH L1 expression were due to EBV products and not due to changes in PU.1 expression, we analyzed PU.1 RNA and protein levels in these cell lines with QRT-PCR and Western blot analysis. Previous studies [43,44] and our data indicate that PU.1 expression does not change with EBV infection of B-cells [Figures 5(D) and 5(E)].

The results with these representative cell lines indicate that in BL cells, whether infected with EBV or not, UCH L1 levels are elevated. These results also show that EBV infection of BL30 cells induced uch l1 promoter activity and further increased UCH L1 expression, presumably due to EBV type III latency products.

PU.1 forms endogenous complex with EBNA2 and induces endogenous UCH L1 expression

Our results suggest that one or more type III EBV latency products increase UCH L1 expression in EBV-immortalized B-cells. It has been shown that EBNA2 interacts with PU.1 and plays an important role in EBNA2-mediated transcriptional activation [34,35]. To confirm the interaction between EBNA2 and PU.1, we performed IP with PU.1 antibody from KR4 LCLs. Association between EBNA2 and PU.1 was detected by performing Western blot analysis for EBNA2 [Figure 6(A)]. We tested whether EBNA2 activates the uch l1 promoter by co-transfecting NIH 3T3 cells with PU.1 or EBNA2-HA, or EBNA2-HA and PU.1 together, or control plasmid along with UCHL1p-LUC reporter or UCHL1p-LUC construct mutated at all five PU.1 binding sites [Figure 6(B)]. While EBNA2 and PU.1 separately activated the wild-type uch l1 promoter to a modest level, co-expression of EBNA2 and PU.1 resulted in synergistic activation of the promoter, indicating that EBNA2 acts as a transcriptional co-activator for PU.1. However, PU.1 was not able to induce the uch l1 promoter mutated at all five PU.1 sites. EBNA2 by itself slightly increased activity of the mutated promoter but not as strongly as the wild-type uch l1 promoter. In the case of EBNA2 and PU.1 co-expression, induction was reduced by 50% as compared with wild-type uch l1 promoter [Figure 6(B)].

We next investigated whether EBNA2 and PU.1 induces endogenous UCH L1 expression in NIH 3T3 cells, which express very low levels of this enzyme. Although expression of EBNA2 and PU.1 separately did not induce detectable levels of endogenous UCH L1 (data not shown), together they substantially increased UCH L1 expression at both RNA and protein levels [Figures 6(C) and 6(D)]. These data indicate that PU.1 and EBNA2 cooperate in up-regulation of UCH L1 expression.

Inhibition of PU.1 in B-lymphoma cells reduces endogenous UCH L1 expression

To validate the role of PU.1 as a hematopoietic transcription factor for UCH L1 expression in lymphoid cells, we suppressed PU.1 levels in EBV-transformed B-cells with specific shRNA (Figure 7). Inhibition of endogenous PU.1 expression resulted in visible reduction of endogenous UCH L1 RNA [Figure 7(A)] and protein [Figure 7(B)] levels. In addition, the analysis of UCH L1 expression in LCLs stably expressing second PU.1 shRNA confirmed these results (data not shown). These results demonstrate that PU.1 contributes to the UCH L1 expression in B-lymphoma cells.

Discussion

Several recent studies provide evidence that UCH L1 is an oncogene involved in the development of certain types of lymphoma [13,14,16], raising the question whether its expression in lymphoid malignancies can be regulated by specific lymphoid factors. In this study we show that the myeloid/lymphoid transcription factor PU.1 contributes substantially to UCH L1 expression in B-lymphoma cell lines. The addition of PU.1 to putative binding site oligonucleotides generated slowly migrating PU.1/uch l1 promoter complexes (Figure 2), indicating that PU.1 induces the *uch l1* gene through direct binding to specific sites on the uch l1 promoter. Since there are five PU.1 binding sites, it is possible that PU.1 may bind competitively or sequentially to these sites, and we believe that further analysis of these sites is required to determine the binding affinity of PU.1 on each site. It is important to note that competition assays were not performed, because SYBR green will stain any free DNA and will interfere with DNA gel analysis.

The ChIP analysis showed that PU.1 forms an endogenous complex on the uch l1 promoter [Figure 3(A)]. While mutations in single PU.1 binding sites reduced PU.1-mediated induction of the uch l1 promoter, mutations in all five PU.1 sites practically abolished PU.1-dependent activation [Figure 3(B)]. We suggest that PU.1 binding sites can complement each other in PU.1-mediated induction of the uch l1 promoter. PU.1 is known to regulate gene expression in a dose-dependent manner, and levels of PU.1 determine whether progenitor cells will differentiate into lymphoid or myeloid origin [22,45]. Interestingly, we observed that lower concentrations (up to 250 ng/well) of PU.1 induced uch l1 promoter activity whereas higher concentrations (more than 500 ng/well) of PU.1 rather inhibited activity (data not shown). PU.1 is not the only example of such concentration-dependent effects: low or high concentrations of p53 transcription factor can inhibit or activate the epidermal growth factor receptor (EGFR) promoter [46]. It is possible that multiple PU.1 binding sites on the uch l1 promoter are associated with different levels of UCH L1 expression during the process of hematopoietic differentiation. It will also be interesting to see whether PU.1 occupies the same sites on the uch l1 promoter in cells of lymphoid versus myeloid origin.

Tumor viruses are important oncogenic agents, and EBV is prominently linked to lymphoid cancers: after establishing latent infection in B-cells, EBV latency products dysregulate major signaling pathways, which results in cell transformation [30,47]. Our study shows that immortalization of primary B-cells induces, and infection of Burkitt lymphoma cells by EBV increases, UCHL1 expression (Figures 4 and 5). These results complement those of

previous studies showing that UCH L1 deubiquitinating activity is increased in EBV-transformed cells [41]. We do not know whether expression of UCH L1 induced after EBV immortalization is a result of EBV infection alone or due to other factors, a subject of interest for future studies. However, we think that the physiological effect of EBV-mediated up-regulation of the *uch l1* gene in EBV-infected B-cells is probably not induction of the initial UCH L1 expression, but rather preservation of certain levels of UCH L1 protein after immortalization, and as a result, maintenance of the transformed status of the cells [14].

Various transcriptional factors, including viral, have been identified as interaction partners of PU.1 in positive or negative regulation of transcription [48]. Partners of PU.1 include c-Jun and c-Fos (co-activators), C/EBP- δ (synergistic), AML-1/ETO (antagonistic), c-Myb (cooperative), and EBNA2 viral protein (synergistic) [34,48]. Our results show that co-expression of PU.1 and EBNA2 activates the *uch l1* promoter construct more vigorously than does either factor separately [Figure 6(B)]. More important, together PU.1 and EBNA2 induce endogenous UCH L1 expression in cells that do not express either of these products [Figure 6(C)]. For now we can only speculate that either over-expressed PU.1/EBNA2 complex has greater ability than PU.1 alone to transactivate the promoter or that co-expression of EBNA2 initiates a synergistic chain of events that activates the *uch l1* promoter indirectly, through other transcription factors. The *uch l1* promoter has binding sites for other transcription factors such as c-Jun/c-Fos, GATA2, and c-Myb, which are known to be PU.1 partners also, that might be playing a role in regulation of the promoter [48]. Additionally, the *uch l1* promoter has binding sites for other oncogenic transcription factors such as nuclear factor κ B (NF κ B), AP2, E2F4, and POU, which we believe might participate in inducing UCH L1 expression in transformed cells in the absence of EBV infection.

We show that suppression of endogenous levels of PU.1 in B-lymphoma cell lines reduces UCH L1 expression (Figure 7), which confirms that PU.1 is indeed a positive regulator of the *uch l1* gene at physiological levels. We think that suppression of UCH L1 expression is decreased by 40% because of interplay with other transcription factors. It is also important to note that SpiB, an isoform of PU.1, is expressed in B-cells, and can bind to PU.1 binding sites to mediate transcription [49]. We believe that SpiB works as a compensatory mechanism to maintain UCH L1 expression in EBV-transformed cells in the absence of PU.1.

In addition to PU boxes the *uch l1* promoter contains binding sites for several oncogenic transcription factors such as AP-1 and c-Myc. Since translocation and constitutive expression of c-Myc is a hallmark of Burkitt lymphomas [50–52], c-Myc-dependent activation of the *uch l1* gene might be one of the reasons why UCH L1 levels are elevated in primary EBV-negative Burkitt lymphoma cells (Figure 5). To summarize our findings, we put forward a simplified model of a mechanism for *uch l1* promoter activation by PU.1 (Figure 8). PU.1 and its partners enhance PU.1-mediated induction of the *uch l1* promoter in transformed B-cells. Since PU.1 is a hematopoietic-specific transcription factor, implication of PU.1 in direct regulation of the *uch l1* promoter accounts at least in part for UCH L1 expression in lymphoid cancers, including EBV-associated lymphomas.

The observation that UCH L1 expression is significantly greater in B-lymphoma cells compared with transformed cells of non-lymphoid origin (Figure 1) suggests that UCH L1 might play a specific role in the development or progression of lymphoid malignancies. Increased motility is one of the basic characteristics of invasive lymphoid cancers [53–55]. Dynamic changes in cell shape during adhesion and migration are tightly controlled by the lymphoid cell cytoskeletal network [56–61], and recent studies implicate UCH L1 in regulation of cytoskeletal dynamics [7,8]. These observations might explain the high levels of UCH L1 in such malignancies, and highlight this multifunctional protein of the ubiquitin system as a new target for anti-cancer therapy in specific lymphoid cancers.

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Figure 1.

High levels of UCH L1 protein detected in B-lymphoma cells. Total cell lysates from four epithelial (U2OS, 293, HeLa, and C33A, lanes 1–4), four fibroblastic (NIH3T3, GM0010A, Cos7, and GM00637F, lanes 5–8), and four B-lymphoblastoid (BL30, X-50/7, KR4, and Raji, lanes 9–12) cell lines were separated in SDS-PAGE and probed with UCH L1 and GAPDH antibodies.

Figure 2.

PU.1 directly binds to the *uch11* promoter at each of five PU.1 sites *in vitro*. (A) Schematic illustration of *uch11* promoter with five putative PU.1-binding sites. (B) Purified recombinant PU.1 separated on SDS-PAGE and detected with Coomassie blue stain; 4 μ g of BSA was used as loading control. (C) High molecular weight DNA-protein complexes of *uch11* promoter and PU.1 stained with SYBR green EMSA to visualize DNA. (D) Western blot analysis was performed on the same gel for visualization of PU.1 in complex with *uch11* promoter with PU.1 antibody.

Figure 3. Mutations in PU.1-binding sites abolish PU.1-mediated activation of the *uch11* promoter. (A) ChIP/PCR analysis was performed to determine binding of PU.1 factor to the putative binding sites on *uch11* promoter with the use of specific PU.1 antibody in KR4 LCLs. Normal IgG was used as negative control in immunoprecipitations. PCR reactions were performed with primers targeting PU.1-binding sites (see ‘Materials and methods’), and amplified DNA products were resolved in 2% agarose gel. (B) NIH 3T3 cells were co-transfected with control or PU.1 (250 ng/well) expression vectors, along with UCHL1p–

LUC wild-type and mutant reporter plasmids (500 ng/well). Luciferase assays were performed 48 h post-transfection. The data represent three independent experiments prepared in triplicate and normalized to β -galactosidase activity.

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Figure 4.

UCH L1 expression is induced in primary B-cells after EBV immortalization. Total RNA and protein were extracted from uninfected PMBCs from two donors (I and II) and from the same PBMCs after EBV immortalization. (A) Reverse-transcription PCR analysis was performed with UCH L1-specific primers and GAPDH primers used as internal control. (B) Western blot analysis was performed with UCH L1 antibody. β -Actin served as loading control.

Figure 5.

Type III EBV latency products induce UCH L1 expression in a Burkitt lymphoma cell line latently infected with EBV. uch l1 promoter activity was determined in paired uninfected and EBV-infected BL30 cell lines. (A) Cells (2×10^5) were nucleofected with UCHL1p–LUC reporter construct. Luciferase assays were performed 48 h post-transfection. The data represent three independent experiments prepared in triplicate and normalized to β -galactosidase activity. (B) Total RNA was extracted from BL30 and BL30-EBV cells, and real-time PCR analysis with specific primers for UCH L1 was performed. GAPDH was used as internal control. (C) Western blot analysis for UCH L1 protein levels in lysates from BL30 and BL30-EBV cells was performed with UCH L1 antibody. GAPDH was used as loading control. Quantification is shown for UCH L1 protein expression. (D) Total RNA was extracted from BL30 and BL30-EBV cells, and real-time PCR analysis with specific primers for PU.1 was performed. GAPDH was used as internal control. (E) Western blot analysis for UCH L1 protein levels in lysates from BL30 and BL30-EBV cells was performed with PU.1 antibody. GAPDH was used as loading control.

Figure 6.

PU.1 and EBNA2 form endogenous complex and synergistically induce endogenous UCH L1 expression. (A) PU.1 was immunoprecipitated from total lysates of KR4 LCLs, IPs were resolved in 12% PAGE and probed with EBNA2 antibody. Rabbit normal immunoglobulins were used as control for IPs. (B) NIH 3T3 cells (low endogenous level of UCH L1) were used for all experiments. Cells were co-transfected with control (1000 ng/well) or PU.1 (250 ng/well) or EBNA2-HA (750 ng/well) or EBNA2-HA (750 ng/well) and PU.1 (250 ng/well) together, along with UCHL1p-LUC wild-type or with five PU.1 binding sites mutated (500 ng/well). Control DNA was used as filler where necessary. Luciferase assays were performed 48 h post-transfection and are normalized to β -galactosidase activity. (C) Next, cells were co-transfected with EBNA2-HA (750 ng/well) and PU.1 (250 ng/well) expression vector or control. Total RNA and protein were extracted 48 h post-transfection, RT-PCR was performed to detect expression of UCH L1 RNA levels with UCH L1-specific primers. (D) Western blot analysis was used to detect endogenous UCH L1 protein levels with UCH L1 antibody. GAPDH served as loading control.

Figure 7.

Inhibition of PU.1 expression results in reduction of UCH L1 levels in transformed B-cells. PU.1 shRNA or GFP shRNA was stably nucleofected in the EBV-transformed B-cell line KR4. Cells were selected and maintained in medium with puromycin (1 μ g/mL). (A) Total RNA was extracted from cells expressing GFP shRNA and PU.1 shRNA, and real-time PCR analysis with specific primers for PU.1 and UCH L1 was performed. GAPDH was used as internal control. (B) Western blot analysis for UCH L1 protein levels was performed in cell

lysates from cells expressing GFP shRNA and PU.1 shRNA with PU.1 and UCH L1 antibody. GAPDH was used as loading control.

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Figure 8.

Working model: PU.1 up-regulates uch l1 expression in B-cell lymphomas. In B-lymphomas, the hematopoietic specific factor PU.1 induces UCH L1 expression. Induced expression of UCH L1 is then used to activate oncogenic pathways [14].