BMI-1 IS INDUCED BY THE EPSTEIN-BARR VIRUS ONCOGENE LMP1, AND REGULATES THE EXPRESSION OF VIRAL TARGET GENES IN HODGKIN’S LYMPHOMA CELLS

Dutton A¹, Woodman CB¹, Chukwuma MB¹, Last JIK¹, Wei W¹, Vockerodt M¹, Baumforth KRN¹, Flavell JR¹, Rowe M¹, Taylor AMR¹, Young LS¹, Murray PG¹#

¹ Cancer Research UK Institute for Cancer Studies, The Medical School, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

#Corresponding author. Dr PG Murray, CRUK Institute for Cancer Studies, The Medical School, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.
Tel: +44 (0) 121 414 4021
Fax: +44 (0) 121 414 4486
Email: P.G.Murray@bham.ac.uk

Keywords: Hodgkin’s lymphoma, Polycomb Group proteins, Bmi-1, latent membrane protein-1, Epstein-Barr virus, ataxia telangiectasia mutated.

Running Title: EBV LMP1 induces Bmi-1 expression

Editorial Note: AD performed research/wrote paper. CBW analysed data/wrote paper. MBC performed immunohistochemistry. JIKL performed ATM immunoblotting. WW analysed data. MV analysed data. KRNB assisted with microarray work. JF generated L591/SD3 cells. MR supervised work with BL cell lines. AMRT supervised ATM studies. LSY designed research/wrote paper. PGM designed research/wrote paper.

Acknowledgment: We are grateful to Leukaemia Research Fund, UK for supporting this project.
ABSTRACT

Polycomb Group (PcG) proteins are chromatin modifiers which are necessary for the maintenance and renewal of embryonic and adult stem cells. However, over-expression of the PcG protein, Bmi-1, causes lymphomas in transgenic mice. We show that Bmi-1 is upregulated in Hodgkin’s lymphoma (HL) cells by the Epstein-Barr virus oncogene, latent membrane protein-1 (LMP1), and that this upregulation is mediated by NF-κB signalling; we also show that Bmi-1 is upregulated by NF-κB in EBV-negative HL cells. Downregulation of LMP1 and Bmi-1 decreased the survival of HL cells, suggesting that Bmi-1 may mediate the pro-survival effects of LMP1-induced NF-κB signalling in HL cells. Transcriptional targets of Bmi-1 were identified following its knockdown in a HL cell line. We show here that both Bmi-1 and LMP1 downregulate the ataxia telangiectasia mutated (ATM) tumor suppressor. We conclude that Bmi-1 contributes to LMP1-induced oncogenesis in HL.
INTRODUCTION

Classical Hodgkin’s lymphoma (HL) is derived from germinal center (GC) B cells, and is characterised by malignant Hodgkin/Reed-Sternberg (HRS) cells in a background of non-malignant ‘reactive’ cells [1]. The Epstein-Barr virus (EBV) is present in HRS cells in approximately half of all HL cases where it expresses a restricted set of virus latent genes; these include the major EBV oncogene, latent membrane protein-1 (LMP1) [2]. By mimicking a constitutively active CD40 receptor, LMP1 activates signaling pathways, such as NF-κB, which enhance B cell survival and are essential for EBV-induced transformation [3, 4].

Polycomb Group (PcG) genes are necessary for the maintenance and renewal of embryonic and adult stem cells, embryogenesis and cell cycle regulation [5, 6]. Two polycomb repressive complexes, PRC1 and PRC2, are required for the initiation and maintenance of gene silencing, respectively [7, 8, 9]: Bmi-1/PCGF4 (B lymphoma Mo-MLVinsertion region/ polycomb group ring finger 4) is a component of the PRC1 [10, 11]. Bmi-1 induces lymphoid proliferation and the development of lymphomas in transgenic mice [12-15]. Bmi-1 is highly expressed in high-grade large B-cell lymphomas, mantle cell lymphoma, and in non-lymphoid malignancies, for example, colorectal cancer and non small cell lung cancer [16-18]. Although Bmi-1 is highly expressed in HRS cells [19-21], its regulation and contribution to the pathogenesis of HL is unknown. We show here that Bmi-1 is a transcriptional target of LMP1; that the expression of Bmi-1 promotes the survival of HL cells and that Bmi-1 induces transcriptional changes in HL cells which include the downregulation of the ataxia telangiectasia mutated (ATM) gene.
MATERIALS AND METHODS

The work undertaken in the study received ethical approval from the South Birmingham Research Ethics Committee (LREC no.0844).

Cell lines and tissue samples

EBV negative cell lines from mixed cellularity (MC) HL (KM-H2) and nodular sclerosis (NS) HL (L428) [22] were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2mM L-glutamine and 1% penicillin-streptomycin solution (Sigma-Aldrich, UK). An EBV positive cell line from a patient with NS HL, L591, and an EBV negative clone of this line (L591-SD3), were grown in the same way [23]. Paraffin-embedded HL biopsies were obtained from Queen Elizabeth Hospital, Birmingham, UK and their EBV status determined by immunohistochemical staining for LMP1 [24].

Transient transfection of HL lines

Transfection of HL-derived cell lines was performed using the Nucleofector unit supplied by Amaxa GmbH and described by Schakowski et al. [25]. In brief, 2x10^6 KM-H2 cells and 4x10^6 of other HL cell lines were pelleted at 15000rpm for 9 minutes: following resuspension in 100µL of freshly prepared Nucleofector solution kit T (Amaxa, cat no: VCA-1002), 2µg of plasmid DNA were added to KM-H2 and 4µg to the other HL cells. Subsequently, KM-H2 cells were pulsed using program T-01, and the other HL cells using program U-09; cells were then incubated in culture media and analysed after 24 and 48 hours.

Reverse transcriptase polymerase chain reaction

Total RNA was extracted from cell lines using the StrataPrep Total RNA Microprep Kit (Stratagene, USA, cat no. 400805), according to the protocol of the manufacturer. cDNA
was synthesised using gene-specific primers in a reverse transcription reaction using AMV reverse transcriptase (Roche, UK). Gene transcripts were amplified using the following primers: Bmi-1 forward, 5’-GCCTTCTCTGCTATGTCTGAA-3’, Bmi-1 reverse, 5’-CTGATGAACACACACCAACTT-3’; LMP1 forward, 5’-ACAATGCCTGTCCGTGCAAA-3’, LMP1 reverse, 5’-CTTCAGAAGAGACCTTCTCT-3’; GAP-DH forward, 5’-GGTGAAGGTCGGAGTCAACGGA-3’, GAP-DH reverse, 5’-GAGGGATCTCGCTCCTGGAAGA-3’; HK2 forward, 5’-AGGGGATCTCGCTCCTGGAAGA-3’, HK2 reverse, 5’-GGTGGACAGGATACGAGAAAA-3’; ATM forward, 5’– GTGGGTATTCCGACTTTGTT-3’, ATM reverse, 5’-GTGGGTATTCCGACTTTGTT-3’.

PCR was performed using ‘hot start’ with Red Hot Taq DNA Polymerase (Abgene, UK); this comprised an initial 2 minute denaturation at 94°C, followed by 25 cycles, consisting of a denaturing step for 30 seconds at 94°C, an annealing step for 1 minute at 45°C and an extension for 1 minute at 72°C. For semi-quantitative analysis 15, 20 and 25 PCR cycles were performed. PCR products were visualised on 2% agarose gels.

Western blot analysis

HL cell lines were washed in cold PBS and lysed in 80µl of lysis buffer (20mM Tris HCL buffer pH 7.4; 150mM NaCl; 1mM EDTA; 1mM EGTA; 1% TritonX-100). Protein concentrations were determined using the Dc protein assay (Bio-Rad, Hercules, CA); and proteins were transferred to nitrocellulose membranes following their separation by 10% SDS-PAGE. After a 1-hour incubation in blocking solution (5% milk in PBS, 0.01% Tween 20), blots were probed overnight with primary antibody for Bmi-1 (Upstate
Laboratories, clone 229F6; mouse monoclonal diluted 1/1000), LMP1 (CS1-4, prepared in house; mouse monoclonal diluted 1/50), HK2 (Santa Cruz, clone-14, goat polyclonal diluted 1/200) or actin (Santa Cruz, clone-2, mouse monoclonal 1/500). 50µg of whole cell lysates separated by 6% SDS-PAGE were immunoblotted with ATM antibody (11G12 mouse monoclonal, 1:500 [26]). After washing in PBS, the secondary peroxidase-labelled antibody (Dako, UK) was added at 1:1000. Proteins were visualised using the enhanced chemiluminescence (ECL) technique (Amersham, UK).

Inhibition of NF-κB activity

HL cells were incubated with the NF-κB inhibitor tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK; Sigma-Aldrich, UK) for 6 hours at a 1:500 dilution (100mM stock) in growth medium; this proteasome inhibitor prevents the degradation of IkB-alpha and inhibits the processing of the p105 subunit of NF-κB. Following treatment, cells were centrifuged, washed twice in cold PBS, (pH 7.6), and resuspended in RNA extraction buffer or protein lysis buffer. HL cells were also transiently transfected with an expression vector encoding a mutant form of IkBα. As phosphorylation of this mutant IkBα is prevented by the substitution of alanine for serine residues at positions 32 and 36, it has a dominant negative phenotype which prevents the activation of NF-κB by sequestering it in the cytoplasm [27]. Finally, HL cells were transfected with pSG5 vectors expressing either wild type LMP1 or a mutant LMP1 (AxAxA 386 stop) that lacks the CTAR1 and CTAR2 domains, both of which are responsible for NF-κB activation [4].

RNA interference
LMP1 expression was knocked down using exogenously supplied oligonucleotides (5’UUUGCACGGACAGGCAUUG 3' and 3’AAACGUGCCUGUCCGUAAC 5’), designed and manufactured by Eurogentec Ltd., (Belgium). Prior to transfection, 30µl of each of the RNA oligonucleotide solutions (50µM) were combined with 15µl of annealing buffer (final buffer concentration 100mM potassium acetate, 30mM HEPES-KOH pH 7.4, 2mM magnesium acetate). These solutions were incubated for 1 minute at 90°C, briefly centrifuged and incubated for a further 1 hour at 37°C. siRNAs (20µM) were stored at -20°C until transfection. HL cells were diluted with media to a density of 2.5 x10^5 cells per 250µl in 24 well plates. A mixture containing 3µl of the RiboJuice (Novagen, Germany) transfection reagent, 47µl of serum-free medium and siRNAs (final concentration 2µM), was added to each well. Plates were left overnight at 37°C, in 5% CO₂; samples were then centrifuged and resuspended in 1ml of culture medium. Bmi-1 expression was knocked down either by nucleofection of HL cell lines with a pSUPER-retro vector expressing a Bmi-1-specific short hairpin (sh) RNA (target sequence gta ttg tcc tat ttg tga t - gift of Maarten van Lohuizen, The Netherlands Cancer Institute, Amsterdam), or with an exogenous oligonucleotide (5’CCAGACCACUACUGAAUAU3’) as described above.

**Trypan blue cell viability assay**

Cell viability following siRNA transfection was assessed in triplicate using the trypan blue reagent (Sigma-Aldrich, UK). 100µl of cell suspension were removed from plates and mixed with 100µl of trypan blue reagent for 2 minutes. Viability was determined by direct counting of unstained cells in a haemocytometer.
**Immunohistochemistry**

Paraffin-embedded tissues were cut at 5µm on to adhesive-coated slides (Vectabond reagent, Novocastra Laboratories Ltd). After de-waxing of paraffin sections, endogenous peroxidase activity was blocked by incubating all slides for 10 minutes in 3% hydrogen peroxide in methanol. Antigens were retrieved by incubation overnight in EDTA (1mmol/l pH 8.0) /Tween 20 (0.1%) buffer on a hot-plate stirrer at 65°C. Primary antibodies used were: Bmi-1 (Upstate Laboratories, dilution 1:50); LMP1 (CS1-4, dilution 1:50) [24]; and HK2 (Santa Cruz, dilution 1:50). Detection of bound primary antibody was performed using the Envision IHC Select kit (DAKO).

**Gene expression analysis**

For analysis of gene expression following Bmi-1 knockdown, L428 cells were transfected with either a pSUPER retro vector expressing Bmi-1-specific shRNA or control vector as described above. Successful knockdown of Bmi-1 was confirmed using RT-PCR and immunoblotting. Knockdown was performed in triplicate and pooled RNA from each transfection used to prepare biotinylated RNA which was hybridised to Affymetrix HG-U133 Plus 2.0 arrays (Affymetrix Inc. Santa Clara, California, USA). Differentially expressed probe sets were identified using the change algorithm of Affymetrix GCOS with the default settings; only those with “Increase” or “Decrease” calls were included.
RESULTS

LMP1 induces Bmi-1 expression in HL cells

First, we investigated the expression of Bmi-1 in HL cell lines. Figure 1A shows that Bmi-1 was expressed in both EBV-positive (L591) and EBV-negative (KMH2, L428) cell lines. We examined the influence of LMP1 on Bmi-1 expression by comparing its expression in EBV-positive and EBV-negative L591 HL cells; loss of EBV was associated with the downregulation of Bmi-1 expression (Figure 1A). Next we investigated if LMP1 regulated Bmi-1 expression: knockdown of LMP1 in L591 cells by specific siRNA led to the downregulation of Bmi-1 expression (Figure 1B); whereas the ectopic expression of LMP1 in EBV-negative L428 cells led to its upregulation (Figure 1C). We conclude that LMP1 can induce the expression of Bmi-1 in HL cells. Ectopic expression of LMP1 in EBV-negative Burkitt’s lymphoma cell lines did not result in the upregulation of Bmi-1 expression. However, LMP1 was able to upregulate the expression of TRAF1, an established LMP1 target in BL cells (Figure 1S). These data suggest that among B cells the upregulation of Bmi-1 by LMP1 might be restricted to certain B cell lineages.”

LMP1 induction of Bmi-1 is NF-κB dependent

A number of approaches were used to determine if NF-κB mediates the upregulation of Bmi-1 by LMP1. First, EBV-positive L591 cells were treated with the broad spectrum NF-κB inhibitor, TLCK; this led to the downregulation of Bmi-1 (data not shown). Next, L591 cells were transfected with a mutant form of IkBa, which inhibits NF-κB activity
[27]; this also downregulated Bmi-1 expression (Figure 2A). Finally, we showed that whereas the ectopic expression of wtLMP1 in EBV-negative L428 cells resulted in Bmi-1 upregulation, the expression of a mutant LMP1 (AxAxA386stop LMP1) which is incapable of activating NF-κB, did not (Figure 2B). In keeping with these observations we were able to demonstrate that loss of the EBV genome, and therefore also LMP1 expression, from L591 cells results in significantly decreased NF-κB activity (data not shown). We conclude that LMP1 induces Bmi-1 expression by activating the NF-κB pathway.

_Bmi-1 expression in EBV-negative HL cells is also NF-κB dependent_

We found that Bmi-1 expression in primary HRS cells did not vary with EBV status. (Figure 3A, Table 1). Therefore, we examined the influence of NF-κB activity on Bmi-1 expression in EBV-negative HL cells. Bmi-1 was downregulated in EBV-negative L428 and KMH2 cells after treatment with the NF-κB inhibitor, TLCK (Figure 3B), and following expression of the mutant IκBα (Figure 3C). We conclude that Bmi-1 expression in HL cells is regulated by NF-κB in both EBV-positive and in EBV-negative HL cells.

_Bmi-1 promotes the survival of HL cells_

Given that Bmi-1 is an NF-κB target, and that NF-κB activation has been shown to be critical for the survival of HL cells [3], we investigated the contribution of Bmi-1 to HL cell survival. Knockdown of Bmi-1 led to a fall in the viability of L591 cells and of L428 cells (Figure 4). In order to confirm the specificity of these effects we also knocked down
Bmi-1 in L591 and L428 cells using an exogenous siRNA directed to a different region of Bmi-1. Compared to cells transfected with a scrambled siRNA, the Bmi-1-specific exogenous siRNA reduced the viability of these HL cells (Figure 2S). We conclude that in HL cells, Bmi-1 may mediate the pro-survival effects of NF-κB activation.

*Gene expression profiling reveals Bmi-1-regulated genes in HL cells*

Gene expression profiling of L428 cells following Bmi-1 knockdown revealed the upregulation of 771 probe sets, and the downregulation of 383 (including Bmi-1). The complete list of differentially expressed genes is available as supplemental data (Table 1S). Bmi-1 downregulated a large number of genes associated with differentiation (Table 2S), including a number of B cell lineage markers (e.g. CD20/MS4A1, BLK, LY9).

A number of genes induced by Bmi-1 are known to be overexpressed in HL [28-30]: these include STAT1 and c-MET; both are known transcriptional targets of LMP1 [31-33]. We confirmed the changes in the expression of several Bmi-1-induced genes, including hexokinase 2 (HK2), Bcl-2/adenovirus E1B 19 kDa interacting protein-3 (BNIP3) and prolyl 4-hydroxylase alpha subunit (P4HA1), (Figure 5); these genes were also shown to be induced by LMP1. We were also able to show that ectopic expression of LMP1 in L428 cells led to upregulation of HK2 (Figure 6A). Although HK2 was overexpressed in primary HRS cells (Figure 6B), this was unrelated to EBV status.

*Bmi-1 and LMP1 downregulate the ATM tumor suppressor in HL cells*

Bmi-1 repressed genes included several tumor suppressor genes, which are epigenetically inactivated in other cancers (Table 2). As we have previously shown that one of these
genes, ATM, is not expressed at the protein level in HRS cells [26], this gene was selected for further study. We confirmed that ATM gene expression is induced following Bmi-1 knockdown in both the L428 and KM-H2 cell lines using either the endogenous knockdown (Figure 7A) or exogenously supplied Bmi-1-specific siRNA which targets a different region of Bmi-1 (Figure 2S). This exogenous siRNA also downregulated the expression of HK2 (data not shown). Next we showed that ATM expression is lower in EBV-positive L591 cells compared with EBV-negative L591 cells; and that knockdown of LMP1 in EBV-positive L591 cells upregulated ATM expression (Figure 7B). Finally we showed that ATM was also upregulated following inhibition of NF-κB in L428 cells (Figure 7C). We conclude that the downregulation of ATM by NF-κB may be mediated by Bmi-1.
DISCUSSION

Here we demonstrate that LMP1, the major transforming protein of EBV, can upregulate the Bmi-1 oncogene, and that the upregulation of Bmi-1 in both EBV-positive and EBV-negative HL cells, is mediated by NF-κB. Our observations directly link the overexpression of Bmi-1 in HRS cells to the aberrant NF-κB signalling which is characteristic of this tumor. LMP1 may be the major regulator of Bmi-1 expression in EBV-positive HL, whereas other activators of NF-κB, for example, IκBα mutations, may be responsible for Bmi-1 upregulation in EBV-negative disease [34, 35]. Given that Bmi-1 is overexpressed in a wide range of cancers, and that NF-κB deregulation is strongly associated with oncogenesis [36], a similar relationship between NF-κB and Bmi-1 should be explored in other cancers.

We found that the viability of HL cells was decreased following Bmi-1 knockdown. Constitutive NF-κB activation is important for the survival of HL cells [3, 37, 38] and therefore the influence of NF-κB on HL cell survival may in part be mediated by the ability of NF-κB to induce Bmi-1 expression. Our findings are consistent with those of a recent report that showed that Bmi-1 is required for the short-term survival of cancer cells [39].

A microarray analysis following the knockdown of Bmi-1 in L428 cells, revealed for the first time, the impact of Bmi-1 on the cellular transcriptional programme in a transformed cell. Consistent with a recent study in which human embryonic fibroblasts were depleted of PRC1 and PRC2 proteins [40], we observed that Bmi-1 downregulated a large number of differentiation-related genes; several of these were B cell lineage markers (e.g. CD20/MS4A1, BLK, LY9) that have previously been shown to be downregulated in HRS.
cells [41]. Thus, Bmi-1 may contribute to the loss of B cell identity which is characteristic of HL.

Bmi-1 upregulated a number of genes, including STAT1 and c-MET, which have previously been reported to be overexpressed in HL [28-30], and which are also known transcriptional targets of LMP1 [31-33]. MET is the receptor tyrosine kinase for hepatocyte growth factor that has recently been shown to induce ERK and PI3K activation and to contribute to oncogenesis in other lymphomas [42]. Furthermore, we have shown that other Bmi-1-induced genes were also upregulated by LMP1; one of these, HK2, was shown to be overexpressed in HRS cells; this gene is overexpressed in other cancers, where it is essential for the maintenance of high glycolytic activity [43].

Bmi-1 also downregulated a number of tumor suppressor genes which have previously been shown to be epigenetically silenced in cancer. These include IGSF4, which has been shown to directly bind the PRC1 and PRC2 complexes [40] and which we have recently shown to be methylated in the majority of cases of primary HL [44]. The tumor suppressor gene, ATM, the biallelic inactivation of which increases susceptibility to lymphomas, was also downregulated by Bmi-1 and LMP1. We have shown previously that expression of the ATM protein is lost in the majority of primary HL cases [26]. We show here that NF-κB downregulates ATM. While mutation and promoter hypermethylation of ATM have been shown to be responsible for the inactivation of this gene in haematopoietic and solid malignancies, we have been unable to detect any evidence for either of these mechanisms in HL [Bose et al., manuscript in preparation]. Although our data suggest that the upregulation of Bmi-1 may be responsible for the loss of ATM expression in HRS cells, the mechanism for the transcriptional downregulation
of ATM has yet to be identified; the observation that Bmi-1 has H2A-K119 ubiquitin E3 ligase activity merits further investigation [45].

In conclusion, our data suggest that Bmi-1 contributes to LMP1-induced oncogenesis in HL, and provide additional insights into how this PcG protein might contribute to oncogenesis at other sites of cancer.
REFERENCES


Table 1: Bmi-1 expression in primary HL
Immunohistochemistry was used to study Bmi-1 expression in HRS cells, which was recorded as negative, positive (Bmi-1 expression weaker than or as strong as that observed in surrounding lymphocytes) or strong positive (Bmi-1 expression stronger than surrounding lymphocytes).

<table>
<thead>
<tr>
<th>EBV Status</th>
<th>Bmi-1 expression</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td>Strong</td>
</tr>
<tr>
<td>EBV positive</td>
<td>1/14 (7%)</td>
<td>5/14 (36%)</td>
<td>8/14 (57%)</td>
</tr>
<tr>
<td>EBV negative</td>
<td>2/46 (4%)</td>
<td>21/46 (46%)</td>
<td>23/46 (50%)</td>
</tr>
<tr>
<td>Total</td>
<td>3/60 (5%)</td>
<td>26/60 (43%)</td>
<td>31/60 (52%)</td>
</tr>
</tbody>
</table>

Table 2: Tumor suppressor genes epigenetically inactivated in other cancers that were upregulated by Bmi-1 knockdown in L428 cells

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene ID</th>
<th>Fold change following Bmi-1 knockdown</th>
<th>Gene Title/ Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLC1</td>
<td>NM024767</td>
<td>2.0</td>
<td>Deleted in liver cancer 1</td>
</tr>
<tr>
<td>ATM</td>
<td>BG623786</td>
<td>2.3</td>
<td>ataxia telangiectasia mutated gene</td>
</tr>
<tr>
<td>CASP8</td>
<td>A1830471</td>
<td>2.5</td>
<td>Caspase 8, apoptosis-related cysteine peptidase</td>
</tr>
<tr>
<td>ID4</td>
<td>U16153</td>
<td>2.4</td>
<td>Inhibitor of DNA binding 4, dominant negative helix-loop-helix</td>
</tr>
<tr>
<td>IGSF4</td>
<td>AU146373</td>
<td>4.6</td>
<td>Immunoglobulin superfamily, member 4 / TSCL1</td>
</tr>
<tr>
<td>TUSC3</td>
<td>A1760262</td>
<td>4.6</td>
<td>Tumor suppressor candidate 3</td>
</tr>
<tr>
<td>GLIPR1</td>
<td>AK024153</td>
<td>5.2</td>
<td>GLI pathogenesis-related 1</td>
</tr>
<tr>
<td>FAM45A</td>
<td>AK025354</td>
<td>6.06</td>
<td>Family with sequence similarity 45, member A</td>
</tr>
<tr>
<td>SERPINB5</td>
<td>NM002639</td>
<td>7.5</td>
<td>Serpin peptidase inhibitor, clade B (ovalbumin), member 5</td>
</tr>
<tr>
<td>LOX</td>
<td>BE503425</td>
<td>21.1</td>
<td>Lysyl oxidase</td>
</tr>
<tr>
<td>ST18</td>
<td>BC025662</td>
<td>29.8</td>
<td>Suppression of tumorigenicity 18</td>
</tr>
<tr>
<td>MEG3</td>
<td>BC036602</td>
<td>36.7</td>
<td>Maternally expressed 3</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1: LMP1 induces Bmi-1 expression in HL cells
(A) Expression of Bmi-1 in EBV-negative HL cell lines L428 and KMH2 and in the EBV-positive (LMP1-positive) L591 HL cell line compared with the EBV-negative variant, L591-SD3. Although Bmi-1 is highly expressed in all parental lines, irrespective of EBV status, loss of the EBV episome from L591 cells resulted in its downregulation (for all figures mRNA and protein are shown in upper and lower panels, respectively). (B) Knockdown of LMP1 expression (L591+LMP1 siRNA) downregulates Bmi-1 expression in EBV-positive L591 cells compared to cells treated with RiboJuice transfection reagent alone. (C) Ectopic expression of LMP1 in EBV-negative L428 cells upregulates Bmi-1 expression.

Figure 2: LMP1 induction of Bmi-1 is NF-κB dependent
(A) L591 cells transfected with a plasmid expressing an IκBα mutant that inhibits NF-κB activation. Expression of the IκBα mutant resulted in the downregulation of Bmi-1 expression compared with cells transfected with control vector. (B) L428 cells transfected with control plasmid (empty vector), wild type (wt) LMP1 or a mutant LMP1 incapable of inducing NFκB (AxAxA378stop). WtLMP1, but not AxAxA378stopLMP1, induces Bmi-1 expression.

Figure 3: Bmi-1 expression in EBV-negative HL cells is also NF-κB dependent
(A) Immunohistochemistry for Bmi-1 in primary HL. Upper panel shows typical nuclear staining of HRS cells (arrowed) observed in majority of cases. Only 3/60 cases lacked Bmi-1 expression. Middle panel shows typical case lacking HRS cell expression of Bmi-1 (arrowed). Lower panel shows that mantle zone B cells (M) and centrocytes (CC) were Bmi-1 positive in control tonsil whereas centroblasts (CB) were negative. (B) Treatment of L428 cells with the NF-κB inhibitor, TLCK, resulted in the downregulation of Bmi-1 expression. Changes in Bmi-1 mRNA and protein are shown in the upper and lower panels, respectively. Similar results were obtained with EBV-negative KMH2 cells (data not shown). (C) KM-H2 cells transfected with a plasmid expressing an IκBα mutant that inhibits NF-κB activation. Expression of the IκBα mutant resulted in the downregulation of Bmi-1 expression compared with cells transfected with control vector.

Figure 4: Bmi-1 promotes the survival of HL cells
(A) Knockdown of Bmi-1 expression in L591 cells (upper panel) and in L428 cells (lower panel). mRNA and protein are shown in the left and right panels, respectively. (B) Knockdown of Bmi-1 led to a marked reduction in the viability of L591 cells (upper panel) both at 24hrs and 48hrs post-transfection when compared to control cells (GFP only vector). Knockdown of Bmi-1 in L428 cells had a significant but less marked effect on cell viability (lower panel).

Figure 5: Validation of Bmi-1 target genes identified by microarray analysis
Knockdown of Bmi-1 expression in L428 cells resulted in the downregulation of HK2, c-MET, BNIP3 and P4HA1 mRNA (left panel). Changes in the protein levels of HK2, c-MET and BNIP3 were also demonstrated (right panel). P4HA1 could not be confirmed at the protein level due to the lack of a suitable antibody.

**Figure 6: HK2 is a transcriptional target of LMP1 and is overexpressed in HRS cells**  
(A) Ectopic expression of LMP1 in L428 cells upregulated the expression of HK2 (mRNA and protein are shown in left and right panels, respectively). (B) Immunohistochemistry was used to study the expression of HK2 in primary HL. Upper panel shows low-level HK2 expression in germinal centre (GC) and mantle zone (MZ) B cells of normal tonsil. Middle and lower panels show strong staining of HK2 in HRS cells (arrowed).

**Figure 7: Bmi-1 and LMP1 downregulate expression of the ATM tumor suppressor in HL cells**  
(A) Knockdown of Bmi-1 expression in L428 cells resulted in the upregulation of ATM expression (left panel shows changes in mRNA, and right panel changes in protein). Knockdown of Bmi-1 in the KM-H2 cell line also produced similar effects on ATM expression (data not shown). (B) ATM expression was higher in EBV-negative L591 cells compared to EBV-positive L591 cells (left panel). Knockdown of LMP1 in EBV-positive L591 cells led to the upregulation of ATM transcription (right panel). These data show that ATM expression is suppressed by LMP1 in EBV-positive HL cells. (C) Inhibition of NF-κB in L428 and cells upregulated ATM expression. Similar effects were also observed in KM-H2 cells (data not shown).
Figure 1

A

B

C

K04.02

1A5

EDV

EDV pos. L51

EDV neg. L51 + S3

Bmi-1

LMP1

GAP-DH

Bmi-1

LMP1

GAP-DH

Bmi-1

LMP1

Actin

Bmi-1

LMP1

Actin

Bmi-1

LMP1

Actin
Figure 3

---

**A**

---

**B**

---

**C**

---

For personal use only.
Figure 4

A

Bmi-1

GAP-DH

Actin

Control positive

Bmi-1

GAP-DH

Actin

Control positive

Bmi-1

GAP-DH

Actin

B

% Agrein u.e. + FBS

Days

Time (hours)

Control

go Rb: Bmi-1

Control

go Rb: Bmi-1

24hr

48hr

2hr

10hr
Figure 7

A

<table>
<thead>
<tr>
<th>Control / siRNA</th>
<th>Bmi-1</th>
<th>ATM</th>
<th>GAP-DH</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA Bmi-1</td>
<td><img src="image" alt="Bmi-1" /></td>
<td><img src="image" alt="ATM" /></td>
<td><img src="image" alt="GAP-DH" /></td>
</tr>
<tr>
<td>siRNA control</td>
<td><img src="image" alt="Bmi-1" /></td>
<td><img src="image" alt="ATM" /></td>
<td><img src="image" alt="GAP-DH" /></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>EBV+/p055/L</th>
<th>LMP1</th>
<th>ATM</th>
<th>GAP-DH</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV-/p055/L</td>
<td><img src="image" alt="LMP1" /></td>
<td><img src="image" alt="ATM" /></td>
<td><img src="image" alt="GAP-DH" /></td>
</tr>
<tr>
<td>XBA + p055</td>
<td><img src="image" alt="LMP1" /></td>
<td><img src="image" alt="ATM" /></td>
<td><img src="image" alt="GAP-DH" /></td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Control / LTCX</th>
<th>ATM</th>
<th>GAP-DH</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTCX</td>
<td><img src="image" alt="ATM" /></td>
<td><img src="image" alt="GAP-DH" /></td>
</tr>
</tbody>
</table>
BMI-1 is induced by the Epstein-Barr virus oncogene LMP1, and regulates the expression of viral target genes in Hodgkin’s lymphoma cells

Amanda Dutton, Ciaran B Woodman, Marilyn B Chukwuma, James IK Last, Wenbin Wei, Martina Vockerodt, Karl RN Baumforth, Joanne R Flavell, Martin Rowe, A. Malcolm R Taylor, Lawrence S Young and Paul G Murray