

## Research Article

# Up-regulation of Lamin A/C Expression in Epstein-Barr Virus Immortalized B Cells and Burkitt Lymphoma Cell Lines of Activated B Cell Phenotype

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**Abstract:** Lamin A, B and C, the nuclear intermediate-filament proteins, play a role in epigenetic regulation. While Lamin B is expressed in all nucleated cells studied, Lamin A/C are transcribed in most somatic cell types except mature B lymphocytes. Since Epstein-Barr virus (EBV), a human gammaherpesvirus, is associated with tumorigenic processes and is known to alter the epigenotype of its host cells, we studied the expression of the *LMNA* gene and its epigenetic marks in EBV-carrying human lymphoid cell lines. We observed a high lamin A/C mRNA and protein expression in EBV-immortalized lymphoblastoid cell lines (LCLs) and in group III Burkitt lymphoma (BL) lines where hypomethylated first exons were observed with activating histone marks. In most cell lines with low promoter activity a highly methylated first exon could be detected. Our data showed that methylation of the first exon of *LMNA* was associated with the downregulation of *LMNA* expression whereas euchromatic histone marks were enriched at active *LMNA* promoters in EBV-immortalized LCLs. These data suggest a role for viral latency products to activate *LMNA* in EBV-infected latency type III B cells *in vitro*. Expression of lamin A/C may contribute to the establishment of activated B cell phenotype that needs further explorations.

**Keywords:** Lamin, EBV latency, transformation, epigenetic regulation, activated B cell

## 1. Introduction

Lamins are intermediate-filament proteins forming a network called nuclear lamina between the inner nuclear membrane and the nucleoplasm that protects the chromatin from physical damage. They play a role in chromatin organization, too, and act as epigenetic regulators affecting the activity of

promoters located to the lamin-associated domains (LADs) of the genome [1-7]. In addition, lamins may affect the temporal pattern of replication origin firing [8].

There are two types of lamins: A and B. The *LMNA* gene codes for the widely co-expressed major isoforms, lamin A and lamin C (lamin A/C) generated by alternative splicing [9]. Human B type lamins are expressed from *LMNB1* and *LMNB2*. At least one type B lamin was detected in all nucleated metazoan cells studied so far. In vertebrates lamin A/C is expressed predominantly in differentiated somatic cells; only a low level was found in pluripotent mouse embryonic stem cells [10-12]. Murine and human B cells don't express or express only low levels of lamin A/C [13-15].

Epstein-Barr virus (EBV), a human gammaherpesvirus, is regularly associated with human lymphomas of B cell origin and immortalizes human B cells with a high efficiency *in vitro* [16]. The expression of latent EBV genomes is highly restricted in Burkitt lymphoma (BL) cells that phenotypically resemble resting B cells: only EBNA1, an EBV-encoded nuclear antigen, and a set of nontranslated viral RNAs can be detected [17]. The restricted EBV gene expression pattern characteristic of BLs *in vivo* and BL-derived cell lines that maintain the BL biopsy phenotype *in vitro* is called latency type I.

A less stringent gene expression pattern (latency type II) was described in nasopharyngeal carcinomas (NPCs): they typically express EBV-encoded latent membrane proteins (LMP1, LMP2A, and LMP2B) in addition to the viral latency products detected in BLs. We note, however, that LMP1 is not expressed in a subset of NPCs [18].

LMPs are also expressed in latency type III, characteristic of post-transplant lymphoproliferative disease (PTLDs) developing in immunosuppressed patients and their *in vitro* counterparts, the *in vitro* immortalized B lymphoblastoid cell lines (LCLs). In addition to LMPs, however, six nuclear antigens (EBNAs) and three BHRF1 microRNAs are also expressed in latency type III [17,19,20]. It is worthy to note, that type III latency is also a characteristic of BL cells that acquired an activated B cell phenotype during *in vitro* cultivation.

Although it has been well documented that the host cell phenotype-dependent expression of viral oncoproteins could switch on or epigenetically silence a series of host cell promoters in EBV-positive lymphomas and carcinomas [21,22], the expression of *LMNA* gene in B lymphoid cells in response to EBV transformation has not been studied systematically. Although *LMNA* expression levels of several B cell lines and NPCs included into microarray experiments are accessible in the GEO database (GEO Profiles Reference numbers: GSE12452, GSE4525, GSE13597, GSE1880), it is difficult to compare the data collected under variable experimental conditions. Here we analyzed the expression of *LMNA* mRNA and of lamin A/C proteins in well-characterized B lymphoid cell lines carrying latent EBV genomes. Unexpectedly, we observed a high level of *LMNA* transcription and expression of lamin A/C proteins in LCLs and the majority of group III BL lines characterized by an activated B cell phenotype, but not in latency type I or EBV-negative BL cells. The 5' *LMNA* promoter flanking sequences were hypomethylated in most of the cell lines analyzed and their methylation pattern did not correlate with promoter activity. We observed, however, that the first *LMNA* exon that partly overlapped with a CpG island was highly methylated, with one exception, at silent *LMNA* promoters, suggesting a role for first exon methylation in the regulation of *LMNA*. Active *LMNA* promoters were enriched in euchromatic histone marks.

## 2. Materials and Methods

### 2.1. Cell lines and culture conditions

Well-characterized EBV-negative BL lines, EBV-positive BL cell lines or clones, EBV-immortalized LCLs and carcinoma cell lines were maintained as described earlier [23,24] (Table 1). Cell lines were regularly tested for latency type-associated mRNA transcripts of LMP1 (EBV latency type II and III) and ENBA2 (EBV latency type III) using reverse transcription and real-time PCR to verify their originally described characteristics.

**Table 1.** Characteristics of the cell lines included in the study

Designation	Origin / EBV latency type	Note	Reference
DG75	Sporadic BL <sup>1</sup> / EBV-negative		[23]
BJAB	Sporadic BL / EBV-negative		[23]
BL41	Sporadic BL / EBV-negative		[23]
Rael	Endemic BL / latency type I		[25]
Mutu-BL-I-CI-216	Endemic BL / latency type I	Subclone of the BL line Mutu	[26]
BL41-E95B	<i>In vitro</i> EBV-converted BL III	BL41 cells were converted with the B95-8 EBV strain	[27]
Mutu-BL-III-CI-99	Endemic BL / latency type III	Subclone of the BL line Mutu	[26]
Raji	Endemic BL / latency type III		[28]
Jijoye p79	Endemic BL / latency type III		[23]
CB-M1-Ral-STO	LCL <sup>2</sup> / latency type III	Immortalized by the Rael EBV strain	[25]
LCL-721	LCL / latency type III	Immortalized by the B95-8 EBV strain	[29]
KR4	LCL / latency type III		[23]
C666-1	NPC <sup>3</sup> / latency type I	Established from an undifferentiated NPC	[30-32]

<sup>1</sup>BL: Burkitt lymphoma; <sup>2</sup>LCL: lymphoblastoid cell line; <sup>3</sup>NPC: Nasopharyngeal Carcinoma cell line.

## 2.2. Real-time quantitative PCR

Total RNA was isolated from the cells using TRI Reagent (Sigma), followed by DNase I treatment of 1 µg RNA and reverse transcription with Transcriptor High Fidelity cDNA Synthesis Kit (Roche) using lamin A/C (LMN-RT) and GAPDH-specific (GAPDH-RT) oligonucleotides. The relative levels of transcripts initiated at *LMNA*<sub>p</sub> (the *LMNA* promoter) were determined with real-time polymerase chain reaction (LightCycler 480; Roche) using LightCycler FastStart DNA Master SYBR Green I Kit (Roche) with primers complementary to lamin A/C and GAPDH coding sequences (Supplementary materials, Table S1). The relative expression of lamin A/C mRNA was normalized to the level of GAPDH mRNA. To present reliable expression data we normalized Lamin A/C mRNA level to 18s rRNA and β-actin mRNA amounts (not presented) as well, giving nearly identical results.

## 2.3. Western blot analysis

Western blotting was performed as described earlier [33] using an antibody reacting with both lamin A and lamin C (Santa Cruz Biotechnology, sc-6215) and beta-actin proteins (Cell Signaling, #4967).

## 2.4. CpG island search

We used the Methyl Primer Express v1.0 Software (Applied Biosystems), with the following parameters: minimum length of island, 400; maximum length of island, 2000; C+Gs/total bases > 60%; CpG observed/CpG expected > 0.65.

### 2.5. Sequencing of control and sodium bisulfite-modified DNA samples

Control DNA sequences of the 5' regulatory region and part of the first exon of the *LMNA* gene were determined using the primers listed in Table 2. GenBank accession numbers: BL41: KF791131; BJAB: KF791133; DG75: KF791134; Jijoye p79: KF791135; Rael: KF791136; LCL-721: KF791137; CB-M1-Ral-STO: KF791138; Raji: KF791139; Mutu-BL-I-CI-216: KF791142; Mutu-BL-III-CI-99: KF791143; C666-1: KF791144.

For bisulfite sequencing, we used the method of Frommer et al. [34] adapted for an automated DNA sequencer [30,35] using primers listed in Table 2. The degree of cytosine methylation was estimated as described earlier [23,35].

### 2.6. Chromatin immunoprecipitation (ChIP)

ChIP [36] was performed according to Weinmann and Farnham (2002) [36] with some adaptations as described earlier [23,32,37] using specific antibodies against Acetylated Histone 3 (Upstate, 06-599), Acetylated Histone 4 (Upstate, 06-598) and dimethylated lysine 4 of Histone 3 (Upstate, 07-030) or normal rabbit IgG (Santa-Cruz Biotechnology, sc-2027). Obtained DNA fractions were analyzed using primers specific for the 5' regulatory region and exon 1 of *LMNA* (Table 2).

### 2.7. Trichostatin A and 5-azacytidine treatment

The histone deacetylase-inhibitor trichostatin A (TSA; Sigma) was added to  $5 \times 10^6$  DG75 or BJAB cells in 150 nM end concentration. After treatment, *LMNA* transcripts were quantified as described above. The demethylating agent 5-azacytidine (AzaC; Sigma) was added to  $5 \times 10^6$  DG75, BJAB or Rael cells in 10 or 4  $\mu$ M end concentration for 0, 1, 2, or 3 days, followed by lamin A/C mRNA analysis. The efficiency of AzaC treatment was monitored by assessing the level of the viral LMP1 mRNA that is known to be upregulated by AzaC in Rael cells [27,32,38].

### 2.8. Statistical analysis

All variables were tested for normal distribution using F probe to select the appropriate parametric or non-parametric statistical procedure. As a result, Mann-Whitney U test was used for the statistical evaluation of mRNA expression levels between groups of cell lines. Statistical significance was accepted at a p value of  $< 0.01$ .

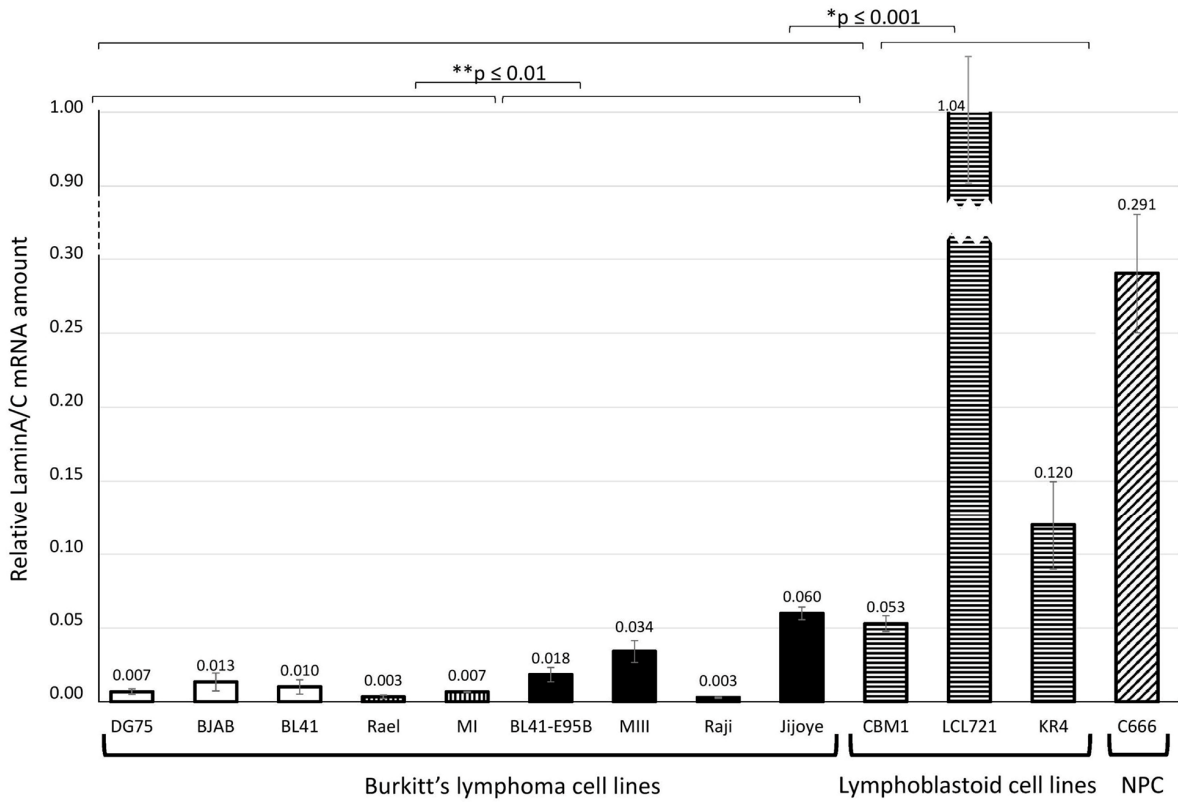
## 3. Results

### 3.1. Analysis of lamin A/C expression

The level of *LMNA* transcripts was high in the LCLs: CB-M1-Ral-STO, LCL-721 and KR4 and in C666-1 (an NPC line) and in latency type III BL cell line: Jijoye p79, moderate in latency type III BL cells (Mutu-BL-III-CI-99 and BL41-E95B), (Figure 1A). A low level of *LMNA* transcripts was detected in EBV latency type I BL cells (Rael, Mutu-BL-I-CI-216), as well as in the latency type III Raji and the EBV-negative BL lines DG75, BJAB and BL41. The difference in *LMNA* mRNA levels between LCLs and other B cells was highly significant ( $p < 0.001$ ). In addition the expression was significantly higher in BLs carrying latency type III EBV genomes compared to other Burkitt lymphoma cells ( $p < 0.01$ ).

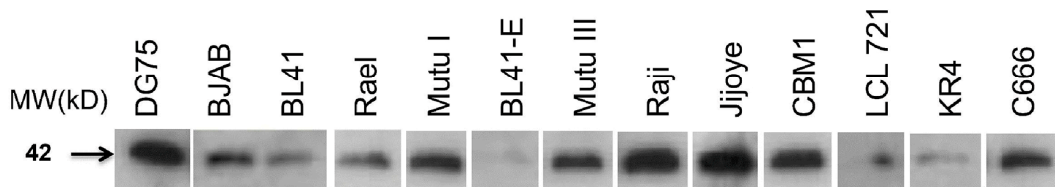
By Western blotting we found a high level of lamin A and lamin C protein in the LCLs: CB-M1-Ral-STO and LCL-721 and in a third EBV-positive LCL, KR4 (Figure 1B). BL cells showed a variable lamin A/C expression: Jijoye p79 cells expressed high levels of lamin A and C proteins, whereas a moderate expression was detected in Mutu-BL-III-CI-99. In contrast, lamin A/C proteins could not be detected in Rael cells carrying the same EBV strain as the LCL CB-MI-Ral-STO and Mutu-BL-I-CI-216 carrying the same EBV strain as Mutu-BL-III-CI-99. Raji, a latency type III BL line carrying EBV genomes with deletions in EBNA3C (EBNA6) and LMP2A coding sequences, was also lamin A/C-negative. The carcinoma cell line expressed lamin A and C proteins at a high level. In contrast, lamin A/C was barely detectable in EBV-negative BL lines (Figure 1B).

A



B

**$\beta$ -actin**



**Lamin A/C**

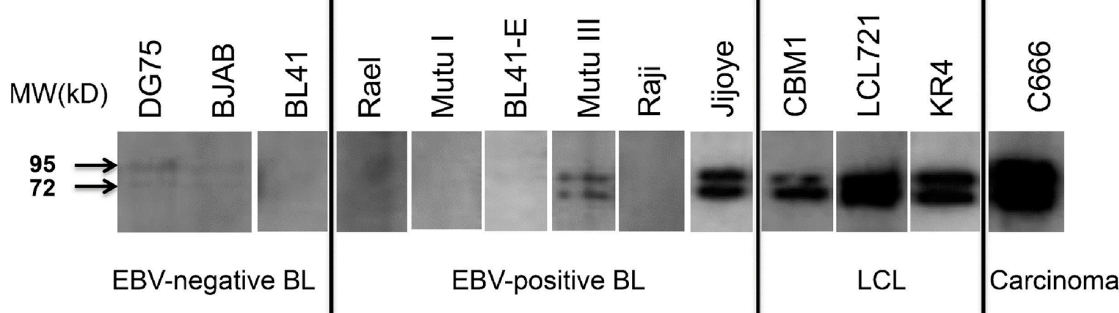


Figure 1. Expression of lamin A/C in B lymphoid and carcinoma cell lines.



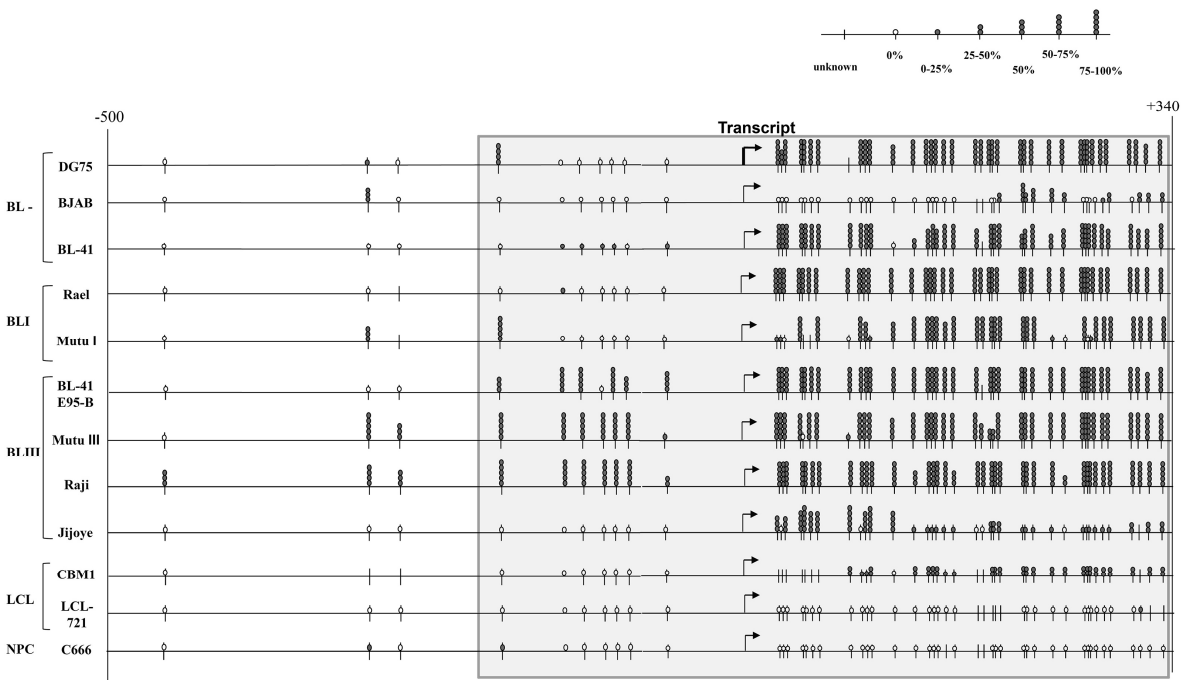
In panel A, Relative lamin A/C mRNA expression was quantified by real-time RT-PCR normalized to GAPDH mRNA level. Results are average of three biological replicates. In panel B, the Western blots were probed with human  $\beta$ -Actin and lamin A/C-specific antibodies. Abbreviations: CBM1: CB-M1-Ral-STO; MI and Mutu I: Mutu-BL-I-CI-216; MIII and Mutu III: Mutu-BL-III-CI-99, C666: C666-1; BL41-E: BL41-E95-B BL: Burkitt lymphoma; LCL: lymphoblastoid cell line; NPC: nasopharyngeal carcinoma cell line. The p value between groups of BL derived cells versus LCLs is indicated above the chart; similarly, the p value between a group of cell lines consisting of EBV-negative BLs and EBV-positive latency type I BLs versus EBV-positive latency type III BLs is also indicated.

3.2. CpG methylation map of the LMNA promoter

Control sequencing of the 5' and 3' flanking sequences of the *LMNA* promoter revealed a C/A heterozygosity at position 4774 in Mutu-BL-I-CI-216 and Mutu-BL-III-CI-99 and a C-to-A change at position 5282 in C666-1 cells, compared with the reference sequence NG\_008692.1.

Using the Methyl Primer Express Software v1.0 (Applied Biosystems) we identified a 739 bp long CpG island encompassing the 3' end of the regulatory region and the 5' end of the first exon of *LMNA* (nucleotides -244 to +495) (see Figure 2. grey box).

In order to correlate lamin A/C expression with the CpG methylation pattern of *LMNA*, we performed bisulfite genomic sequencing (Figure 2). Independently of *LMNA* transcription level, the 5' regulatory region was hypomethylated with the exception of BL41-E95B, Mutu-BL-III-CI-99 and Raji, where highly or moderately methylated CpGs were detected. The situation was quite different at the 3' flanking sequences of the promoter: we found highly methylated CpG dinucleotides within the first *LMNA* exon in cell lines showing a low level of *LMNA* mRNA expression, except for BJAB that was hypomethylated in this region. In contrast, in LCLs and C666-1 nasopharyngeal carcinoma cells that expressed high levels of *LMNA* mRNA, exon 1 was either unmethylated or hypomethylated.



**Figure 2.** CpG methylation map of the 5' regulatory sequence and exon 1 of *LMNA*.

The localization of the CpG island in the analyzed region is indicated by a grey box. Symbols: rightward arrow, transcription start site; thin vertical lines, positions of CpG dinucleotides. Cytosine methylation levels in cell lines and clones are indicated as follows: stick only, undetected; empty dot, 0%; full dot, 0%–25%; two dots, 25%–50%; three dots, 50%–75%; four dots, 75%–100%. Abbreviations: BL, EBV-negative Burkitt lymphoma; BLI: EBV-positive Burkitt lymphoma, latency I; BLIII: EBV-positive Burkitt lymphoma, latency

III; LCL: lymphoblastoid cell line; NPC: nasopharyngeal carcinoma cell line; Mutu I: Mutu-BL-I-Cl-216; Mutu III: Mutu-BL-III-Cl-99; CBM1: CB-M1-Ral-STO; C666: C666-1 nasopharyngeal carcinoma line.

### 3.3. Analysis of euchromatic histone marks at the *LMNA* promoter

We analyzed the euchromatic, activating histone marks at *LMNA* using ChIP. Both the 5' regulatory region and exon 1 of *LMNA* were enriched in acetylated Histone 3 (AcH3) in all three LCLs (KR4, CB-M1-Ral-STO, and LCL-721) expressing lamin A/C. In the lamin A/C-positive C666-1 cells, AcH3 was detected in high abundance only within the first *LMNA* exon (Figure 3A).

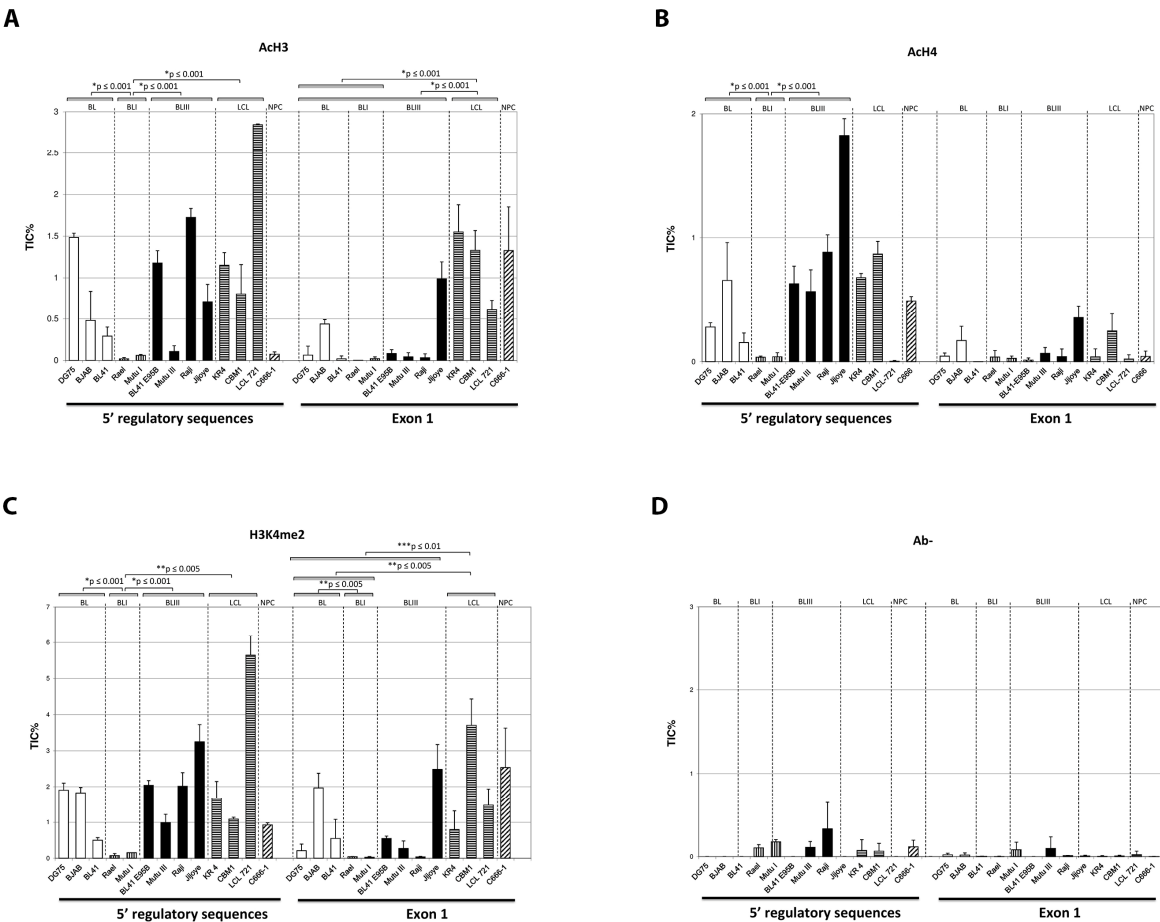
In BL cells, the level of AcH3 was highly variable in the 5' regulatory sequences of *LMNA* (Figure 3A): it was hardly detectable in Rael and Mutu-BL-I-Cl-216 (low promoter activity), and in Mutu-BL-III-Cl-99 (moderate promoter activity), and highly enriched in cell lines both with low (DG75, Raji) or moderate (Jijoye p79) promoter activity. In exon 1, AcH3 was nearly undetectable in BL cells except BJAB and Jijoye p79.

Acetylated Histone 4 (AcH4) was hardly detectable in the *LMNA* 5' regulatory region in Rael and Mutu-BL-I-Cl-216, but it was abundant in all latency type III cells independently of promoter activity, with the exception of LCL-721 (Figure 3B). C666-1 cells were enriched in AcH4 in the *LMNA* 5' regulatory region. Exon 1 of *LMNA* was poor in AcH4 in all cell lines except BJAB, Jijoye p79, and CBM1-Ral-STO (Figure 3B).

Histone H3 dimethylated at lysine 4 (H3K4me2) was hardly detectable at the *LMNA* 5' regulatory region in Rael and Mutu-BL-I-Cl-216 but it was moderately or highly enriched in all other cell lines examined. Exon 1 of *LMNA* showed an elevated H3K4me2 level in all latency type III cell lines with moderate or high *LMNA* promoter activity, except Mutu-BL-III-Cl-99 (Figure 3C).

The abundance of all three histone modifications on the 5' regulatory sequence was significantly higher in latency type III BLs and LCLs compared to group I BL cell lines.

Immunoprecipitation with control normal rabbit IgG showed no significant enrichment in any cell lines (Figure 3D).



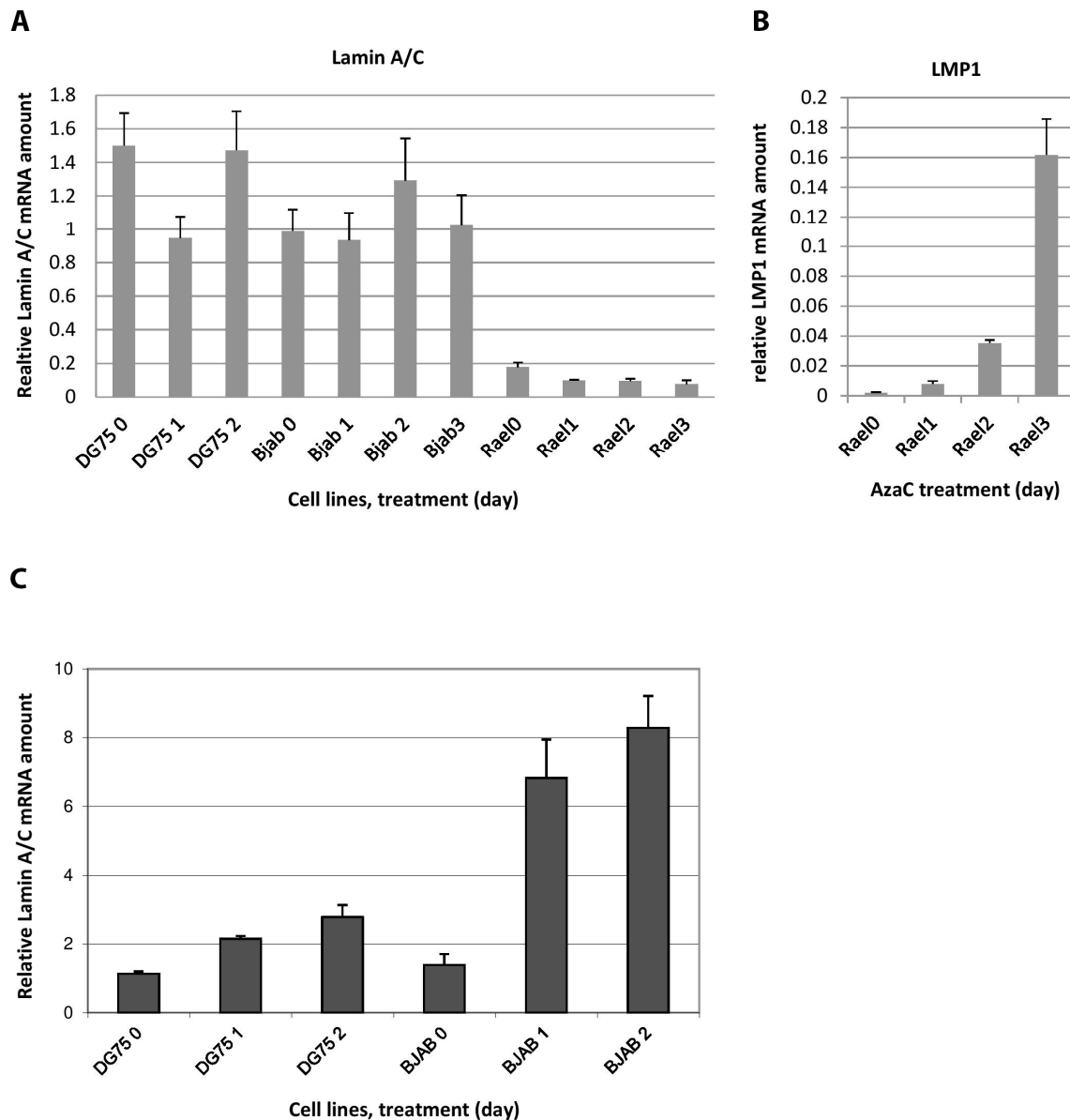
**Figure 3.** Levels of euchromatic histone modifications at the *LMNA* promoter. Chromatin immunoprecipitation (ChIP analysis) of AcH3 (A), AcH4 (B) and H3K4me2 (C) levels. In D normal rabbit IgG was used. Results are average of three biological replicates expressed as the percentage of input DNA (TIC, total input chromatin). Antibodies specifically recognizing diacetylated histone H3 (AcH3), tetraacetylated histone H3 (AcH4) and histone H3 dimethylated at lysine 4 (K4), respectively, were used for ChIP analysis as described earlier [37]. Vertical dashed lines separate groups of cell lines as indicated on the top of the chart. Grey, thick horizontal lines represent groups that were analyzed for statistical significance. The p values between groups analyzed for significance are indicated on the top of the charts. Abbreviations: BL, EBV-negative Burkitt lymphoma; BLI: EBV-positive Burkitt lymphoma, latency I; BLIII: EBV-positive Burkitt lymphoma, latency III; LCL: lymphoblastoid cell line; NPC: nasopharyngeal carcinoma cell line; Mutu I: Mutu-BL-I-CI-216; Mutu III: Mutu-BL-III-CI-99; CBM1: CB-M1-Ral-STO; C666: C666-1 nasopharyngeal carcinoma line.

3.4. Effect of AzaC and TSA treatment on *LMNA* transcription

The DNA hypomethylating agent 5-azacytidine did not affect the expression of *LMNA* in DG75, BJAB and Rael cells (Fig. 4A), although it upregulated the expression of the methylated viral *LMP1* gene in Rael cells (Figure 4B), as observed earlier [27,32,38], indicating that the demethylation was effective.

To test whether increased histone acetylation indeed can activate *LMNA*, we treated EBV-negative BL lines with the histone deacetylase-inhibitor TSA. The *LMNA* 5' regulatory sequences were unmethylated in both cell lines, but they differed as to the methylation pattern of exon 1 that was methylated in DG75 cells but unmethylated in BJAB cells (Figure 2). TSA induced a moderate elevation in *LMNA* gene expression in DG75 cells and a much stronger increase in *LMNA* promoter activity in BJAB cells (Figure 4C).





**Figure 4.** Effect of 5-azacytidine and trichostatin A (TSA) treatment on *LMNA* transcription

A) DG75, BJAB and Rael cells were treated with 10 or 4  $\mu$ M 5-azacytidine for 0, 1, 2, or 3 days and analyzed for relative lamin A/C mRNA expression. B) Rael cells treated with 4  $\mu$ M 5-azacytidine for 0, 1, 2, or 3 days were analyzed for the expression of the methylation-sensitive EBV latency gene *LMP1*. C) DG75 and BJAB cells were treated with 150 nM TSA for 0, 1 or 2 days, respectively and analyzed for relative lamin A/C mRNA expression. Relative lamin A/C mRNA expression was quantified by real-time RT-PCR. Results are average of three biological replicates.

#### 4. Discussion

Type B lamins are widely expressed in metazoan cells [39,40]. Although both type A/C and B lamins are present in most murine and human somatic cells, hematopoietic cells express lamin B only [15,41,42]. Accordingly, B lymphoid cells (centrocytes and centroblasts) expressed lamin B1, whereas mantle zone lymphocytes were lamin B1 and B2 positive [14]. Cell lines derived from human neoplasms usually mirrored the lamin expression profile of the cell type they derived from [43-46].

Because EBV can alter the epigenotype and gene expression pattern of its target cells [16], and lamins are epigenetic regulators themselves [1], we did a systematic analysis of lamin A/C expression in EBV-positive and EBV-negative B lymphoid cell lines and analyzed the epigenetic marks of *LMNA*.

Unexpectedly, we observed that similarly to the carcinoma cell line of epithelial origin, there was a high level of *LMNA* transcription and a high level of lamin A/C protein expression in LCLs and in the majority of group III BL lines characterized by an activated B cell phenotype. These cells express typically six nuclear antigens (EBNAs) and three latent membrane proteins (LMPs) encoded by the virus (EBV latency type III) [16]. In contrast, lamin A/C protein was undetectable or present only at a low level in EBV-negative BL lines and in EBV latency type I BL cell lines that expressed only EBNA1.

We noticed that there was a difference in lamin A/C protein expression between two clones of the BL line Mutu: Mutu BL-I-CI-216 (latency I) did not express A-type lamins, whereas Mutu-BL-III-CI-99 (latency III), was lamin A/C positive (Figure 1B). Because these clones carry the same EBV strain, one may speculate that one of the EBV-encoded oncoproteins or microRNAs expressed in type III latency may contribute to the activation of *LMNA*p. Based on microarray data uploaded by Maier *et al.* (2006) [47] (<https://www.ncbi.nlm.nih.gov/geo/profiles/24088892>) EBNA-2 seems not to be the candidate EBV protein responsible for *LMNA*p activation, as the expression of EBNA-2 protein in EBV-negative BLs did not result in altered *LMNA* activity. Besides, BL41-E95B is reported to express lower level of LMP1 compared to other EBV converted strains [27] and showed less active *LMNA*p suggesting a role for LMP1 in Lamin activation, but this needs further examinations. Similarly, to Mutu clones, Rael cells and CB-M1-Ral-STO cells carry the same EBV strain but differ in latency type and expression of A-type lamins: in Rael cells (latency type I, EBNA1 only), lamin A and C could not be detected, whereas they were present in high levels in CB-M1-Ral-STO cells expressing six EBNAs and three LMPs (Figure 1). This suggest a role for latent EBV gene products in the regulation of *LMNA* expression.

A very low level of lamin A/C protein expression or a moderate level of *LMNA* mRNA, but not protein, was also observed in EBV-negative BL cells (BJAB, DG75; BL41), indicating that cellular factors may also support type A lamin expression to some extent.

Using bisulfite sequencing, we analyzed the CpG methylation pattern of *LMNA*p located to a CpG island (Figure 2). A shorter stretch of the very same CpG island was found to be unmethylated in nodal diffuse large B cell lymphomas actively using the *LMNA* promoter [43]. The CpG methylation pattern of the *LMNA* 5' regulatory region did not correlate with the activity of the *LMNA* promoter. Exon 1 was regularly hypermethylated, however, in the vicinity of low-activity *LMNA* promoters with the exception of BJAB, where it was hypomethylated (Figure 2). These data suggested a role for first exon methylation in the down-regulation of *LMNA* transcription. It is interesting to note that Brenet *et al.* found a linkage between densely methylated first exon sequences and transcriptional silencing in a human acute myelogenous leukemia-derived cell line [48]. Similarly, Yan *et al.* found that DNA methylation progressively spread from the first exon to the promoter of *RASSF1A*, a tumor suppressor gene silenced in breast cancer [49]. The *LMNA* CpG island may certainly correspond to such a densely methylated first exon in cell lines with low *LMNA*p activity. In cell lines with highly active *LMNA* promoters exon 1 was unmethylated or only partially methylated. However, in Mutu-BL-III-CI-99 and Jijoye that expressed moderate levels of *LMNA* mRNA, longer or shorter stretches of highly methylated CpGs of exon 1 sequences flanked the promoter. These data indicated that in addition to DNA methylation, other regulatory mechanisms also influence *LMNA*p activity.

We observed that in EBV-immortalized LCLs (latency III) and an NPC cell line the sequences flanking the active *LMNA* promoter were enriched in euchromatic histone marks (Figure 3). Activating histone marks were absent, however, from low activity *LMNA* promoters in latency type I BLs. In addition, the histone deacetylase inhibitor TSA could upregulate the activity of *LMNA*p in two EBV-negative BL lines, whereas the hypomethylating agent 5-azacytidine was ineffective (Figures 4). TSA treatment was apparently more effective in BJAB cells where the *LMNA*-associated CpG island was hypomethylated, than in DG75 cells, where the flanking sequences were highly methylated (Figure 4). Thus, in lymphoid cells, both CpG methylation and histone modifications seems to affect *LMNA*p activity.

In latency type I BL lines and LCLs, there was a clear correlation between the abundance of euchromatic histone modifications and *LMNA*p activity. We found, however, that in the EBV-negative BL lines DG75 and BJAB enrichment of activating histone modifications at *LMNA*p was insufficient for

high promoter activity (Figures 1 and 3). Thus, the presence of euchromatic histone marks at the promoter-flanking sequences could not upregulate *LMNA* transcription in EBV-negative BL cells, possibly due to the lack of a key viral or cellular activator.

Similarly to LCLs, latency type III BL cells (Mutu-BL-III-CI-99, Jijoye p79) also expressed lamin A/C proteins, except Raji (Figure 1). Raji cells don't express, however, the full set of EBV latent proteins: due to deletions in the viral genome [28], they lack EBNA3C (EBNA6) and LMP2A. The activity of *LMNA*<sub>p</sub> was low in Raji cells, although its flanking sequences were enriched in euchromatic histone modifications (Figures 1 and 3). Further studies may clarify the potential role of EBNA3C (EBNA6) or LMP2A in the regulation of *LMNA*<sub>p</sub>.

## 5. Conclusions

Our data showed that methylation of the first exon of *LMNA* was associated with the downregulation of *LMNA* expression whereas euchromatic histone marks were enriched at active *LMNA* promoters in EBV-immortalized LCLs. These data suggest that viral latency products or their combination may activate *LMNA*<sub>p</sub> in EBV infected latency type III B cells *in vitro*. Expression of lamin A and lamin C may contribute to the establishment of activated B cell phenotype by providing new attachment sites for chromatin loops and thereby affecting the activity of promoters located to lamin-associated domains (LADs) of the genome. Further studies may reveal the contribution of lamin A and C upregulation to the epigenetic reprogramming of LCLs immortalized *in vitro* and PTLs, their *in vivo* counterparts.

## Supplementary Materials

Table S1. List of primers used in the experiments

Primer name	Primer sequences	Primer positions on the reference sequences	Method; NCBI Accession numbers
LPR-F1	5'-gag tat ggt gct ggc ctt tgc-3'	3890	DNA control sequencing; NG_008692.1
LPR-F2	5'-cac tga gat gca gct ctg aat gg-3'	4514	
LPR-F3	5'-ttg agc ctc acg cag tta gg-3'	4850	
LPR-R1	5'-gat cat tga gct cct gca ggt c-3'	5370	
LPR-R1b	5'-aga ctg ctc gga gtc gga gtg-3'	5166	
LPR-R2	5'-tca ccc ttc ttc cac cac tgg-3'	4619	
LPR-R2b	5'-aag gac cgt tgg ggt gtg g-3'	4919	
LPR-mod-K1	5'-ctc aaa ttc caa aac ttt act cc-3'	4448	Bisulfite sequencing; NG_008692.1
LPR-mod-K2	5'-gtt aag yga tta ttg agt ttt tg-3'	5377	
LPR-mod-UF1	5'- <i>M13 univ seq</i> <sup>1</sup> - att cct tct cca ccc cac tc-3'	4488	
LPR-mod-UF2	5'- <i>M13 univ seq</i> - cta ata aac acc aac ctc aac c-3'	4627	
LPR-mod-UF3	5'- <i>M13 univ seq</i> - tcc tta act cta ccc tct aac-3'	4754	
LPR-mod-BF3	5'-Biotin-tcc tta act cta ccc tct aac-3'	4754	
LPR-mod-UR1	5'- <i>M13 univ seq</i> - agy gat tat tga gtt ttt gta gg-3'	5373	
LPR-mod-UR2	5'- <i>M13 univ seq</i> - ggg tyg aag gat aga gat tgt-3'	5190	
LPR-mod-UR3	5'- <i>M13 univ seq</i> - ggg taa agg ttt taa tag gt-3'	5026	
LPR-mod-BR4	5'-Biotin-gaa gga tyg ttg ggg tgt g-3'	4920	
LMN-RT1	5'-gag ttg atg aga gcc gta c-3'	23052	Reverse transcription; NG_008692.1 NG_007073
GAPDH-RT	5'-ctt gat ttt gga ggg atc-3'	7451	
LMP1-RT	5'-tgt aac gca act aag tca tag-3'	168897	
LMN-rlt1	5'-gtc caa tga g /splice/ ga cca gtc cat g-3'	21758→21767/22252→22263	Real-time quantitative PCR in expression study and AzaC treatment; NG_008692.1 NG_007073 V01555
LMN-rlt2	5'-tgt tct gtg cct tcc aca cca g-3'	22510	
GAPDH-up	5'-gga agg tga agg tcg gag tca-3'	5347	
GAPDH-low	5'-atg ggt gga atc ata ttg gaa ca-3'	7223	
LMP1 codL	5'-gag cag gag ggt ga /splice/ t cat cag-3'	(169119–169128 /169207–169213)	
LMP1 codU	5'-cgc tac tgt ttt ggc tgt aca tc-3'	(169371–169349)	
LPR-498F	5'-cac tcc tgg cac act gag atg ca-3'	4503	
LPR-R2up	5'-tca ccc ttc ttc cac cac tgg-3'	4619	Real-time quantitative PCR in ChIP experiments; NG_008692.1
LPR+335F	5'-tgc agg aga agg agg acc tg-3'	5335	
LPR+484R	5'-cct tga tgc cgg aca cct c-3'	5484	

<sup>1</sup>*M13 univ. seq.*: 5'-gta aaa cga cgg cca gt-3'

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**Conflicts of Interest:** The authors declare no conflict of interest.

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