

Epstein - Barr virus Encoded EBER RNAs

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Abstract

Epstein-Barr virus (EBV) establishes a lifelong latent infection in 95% of the world's population and is associated with several human cancers. Some of the most abundant viral products in latent infection are the functional EBV-encoded RNAs EBER1 and EBER2. The aim of this thesis was the identification of distinct functions of EBER1 and EBER2 during EBV infection.

Epstein-Barr virus bacterial artificial chromosomes with deletion of either EBER1 or EBER2 and corresponding revertant viral genomes were constructed to analyse broad range effects of EBER1 or EBER2 on host cell gene expression. The resulting recombinant viruses were used to infect primary B lymphocytes and establish lymphoblastoid cell lines (LCLs). Microarray expression profiling revealed clear changes in host cell gene expression correlating with EBER expression and significant differences between gene sets regulated with EBER1 and EBER2. Functions of EBER target genes include membrane signalling, regulation of apoptosis and the interferon response.

In additional studies, the interaction of EBER1 with ribosomal protein L22 (RPL22), a component of the large ribosomal subunit, was demonstrated in LCL extracts. Using recombinant viruses and EBER expression vectors, the nuclear redistribution of RPL22 by EBER1 was investigated. The delocalisation of RPL22 from nucleoli into the nucleoplasm upon EBV infection was demonstrated in HEK 293, nasopharyngeal carcinoma and gastric carcinoma-derived cell lines. EBER1 was identified as the only viral component necessary for the delocalisation of RPL22. In contrast to the cancer-derived cell lines, LCLs showed a predominantly cytoplasmic expression of

RPL22, which was not significantly changed by EBER1. Subsequently, a possible role of RPL22 and EBER1 in p53-dependent stress responses was explored.

The data presented in this thesis provide further understanding of the role of EBERs in EBV infection and several of the EBER-regulated genes might be used as markers to elucidate the mechanism of EBER action.

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Declaration

All the work presented in this thesis is the result of my own work (except where indicated) and in no way forms part of any other thesis. This work was carried out at the Section of Virology, Faculty of Medicine, Imperial College London under the supervision of Professor Paul J Farrell.

Rachel Bosshard

In all things of nature there is something of the marvellous.

Aristotle

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Abbreviations

Abbreviation	Denotation
°C	degree Celsius
ABCB1	ATP-binding cassette sub-family B member 1
ADARB1	RNA-specific adenosine deaminase B1
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	analysis of variance
APS	ammonium persulfate
ARHGEF4	Rho guanine nucleotide exchange factor 4
ASS	argininosuccinate synthetase
ATF2	activating transcription factor 2
BAC	bacterial artificial chromosome
BARTs	BamHI rightward transcripts
Bax	Bcl-associated X protein
bp	base pairs
BCL-2	B cell lymphoma 2
BCR	B cell receptor
BL	Burkitt's lymphoma
BSA	bovine serum albumin
cam	chloramphenicol
CCDC74B	coiled-coil domain containing 74B
CD	cluster of differentiation molecule

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Abbreviation	Denotation
CDC42BPA	CDC42 binding protein kinase alpha
cDNA	complementary DNA
CHL1	close homologue of L1
CHST6	carbohydrate sulfotransferase 6
CMV	Cytomegalovirus
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C motif) receptor
DAVID	Database for Annotation, Visualization and Integrated Discovery
DEPC	diethylpyrocarbonate
dH ₂ O	distilled water
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
DTT	dithiothreitol
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence reagent
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
eIF2 α	eukaryotic initiation factor 2 α
EHV	Equine Herpes virus
EPDR1	ependymin related protein 1
ER	endoplasmic reticulum
Exp5	exportin 5

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Abbreviation	Denotation
FADD	Fas associated death domain
FBS	foetal bovine serum
FDR	false discovery rate
FITC	fluorescein isothiocyanate
g	gram
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	gastric carcinoma
GFP	green-fluorescent protein
gp	glycoprotein
GPR	G-protein coupled receptor
GRU	green raji units
HEK	human embryonic kidney cell
HHV	Human herpesvirus
HHEX	Homeobox hematopoietically expressed
HLA	human leukocyte antigen
HRP	horse-radish peroxidase
HSUR	Herpesvirus saimiri U-rich non-coding RNA
HSV	Herpes simplex virus
hTERT	human telomerase reverse transcriptase
hTR	human telomerase RNA
HTR2C	human serotonin receptor 2C
HVP	Herpesvirus papio
hygro	hygromycin
IF	immunofluorescence
IFN	interferon
IFNA	interferon alpha

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Abbreviation	Denotation
Ig	immunoglobulin
IGF1	insulin like growth factor 1
IGSF4	immunoglobulin superfamily member 4
IL	interleukin
IL1R2	interleukin 1 receptor type 2
IL12RB2	interleukin 12 receptor beta 2
ILR6	interleukin 6 receptor
IPTG	isopropylthio- β -D-galactoside
IR	internal direct repeats
IRES	internal ribosomal entry site
IRF	interferon regulatory factor
JNK	jun N-terminal kinase
kan	kanamycin
kb	kilobases
kDa	kilodalton
KPB	potassium phosphate buffer
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Luria Bertani
LCL	lymphoblastoid cell line
LCV	Lymphocryptovirus
LFA	lymphocyte-function associated antigen
lincRNA	large intergenic non-coding RNA
LMP	latent membrane protein
lncRNA	long non-coding RNA
M	mol/liter
μ F	microfarad

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Abbreviation	Denotation
μg	microgram
μl	microliter
μM	micromolar
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
MEF	mouse embryonic fibroblast
mg	milligram
min	minutes
miRNA	microRNA
ml	milliliter
mM	millimolar
MBP	maltose-binding protein
MHV68	Murine gammaherpesvirus 68
mRNA	messenger RNA
MUM1L1	mutated melanoma-associated antigen 1 like protein
ncRNA	non-coding RNA
NEB	New England Biolabs
NF- κB	nuclear factor kappa B
NK	natural killer cell
Noxa	NADPH oxidase activator 1
NPC	nasopharyngeal carcinoma
nt	nucleotide
NTRK2	neurotrophic tyrosine kinase receptor type 2
Ω	ohm
o/n	overnight
OAS	oligoadenylate synthetase

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Abbreviation	Denotation
ORF	open reading frame
oriP	origin of latent replication
PACT	protein activator of PKR
PAGE	polyacrylamide gelelectrophoresis
PARP	poly(ADP)ribose polymerase
PASR	promoter associated small RNA
PBS	phosphate buffer saline
PCA	principal component analysis
PCR	polymerase chain reaction
PFA	paraformaldehyde
PFGE	pulsed field gel electrophoresis
piRNA	PIWI-interacting RNA
PKR	protein kinase R
PMA	phorbol myristate acetate
PMSF	phenylmethysulphonyl fluoride
poly(I:C)	polyriboinosinic polyribocytidylic acid
PROMPT	promoter upstream transcript
pen/strep	penicillin and streptomycin
PTLD	Post-transplant lymphoproliferative disease
PUMA	p53 upregulated modulator of apoptosis
puro	puromycin
PVDF	polyvinylidene fluoride
RDV	Rhadinovirus
RIG-I	retinoic acid-inducible gene I
RISC	RNA-induced silencing complex
RNA	ribonucleic acid

Continued on next page

Abbreviation	Denotation
RNF43	ring finger protein 43
RNP	ribonucleoprotein particles
RPL22	ribosomal protein L22
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
RUNX	Runt-related protein
s	seconds
SASH1	SAM and SH3 domain containing 1
scaRNAs	Cajal body-specific RNAs
SCB	Santa Cruz Biotechnology
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SELEX	systematic evolution of ligands by exponential enrichment
siRNA	small interfering RNA
SLC7A11	solute carrier family 7
snoRNA	small nucleolar RNA
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
TBE	Tris Borate EDTA
TBS	Tris buffered saline
TCEAL3	transcription elongation factor A protein like 3
TE	Tris-EDTA
TEMED	tetramethylethylenediamine

Continued on next page

Abbreviation	Denotation
tet	tetracycline
TFIII	transcription factor for polymerase III
tiRNA	transcription initiation RNA
TLR3	Toll-like receptor 3
TNF	tumour necrosis factor
TR	terminal repeat
TRRAP	transformation/transcription domain-associated protein
tRNA	transfer RNA
TSS	transcription start site
TSSa-RNA	TSS-associated RNA
UL	unique long region
US	unique short region
UTR	untranslated region
UV	ultraviolet
V	volt
VA RNA	adenovirus-associated RNA
v/v	volume per volume
VZV	Varicella-zoster virus
WB	western blot
WT	wild type
w/v	weight per volume
WWC3	WWC family member 3
XA	ExAlpha Biologicals
XBP	x-box binding protein
ZEB	zinc finger E-box binding homeobox

1 Introduction

1.1 The Epstein-Barr virus

1.1.1 The Epstein-Barr virus

Epstein-Barr virus (EBV) was first discovered in 1964 by Epstein, Achong and Barr by electron microscopy due to its association with Burkitt's lymphoma (BL) ^[1]. The malignant lymphoma had previously been described by Denis Burkitt in 1958 ^[2] and the association of EBV with the malignancy made EBV the first candidate human tumour virus. Even though BL is particularly prevalent in young children in equatorial Africa and New Guinea (endemic BL) with cofactors being malaria and AIDS, BL is not restricted to this geographical region and lower incidences are found all over the world (sporadic BL) ^[3]. Similarly, EBV is not limited to the region of its first discovery and has since been characterised as a ubiquitous gammaherpesvirus that infects over 90% of the world's population.

1.1.2 The *Herpesviridae* family

The *Herpesviridae* family is part of the recently established order of the *Herpesvirales*, which also comprises the families of the *Alloherpesviridae* and the *Malacoherpesviridae*. While *Herpesviridae* infect mammals, birds and reptiles, the relatively new *Alloherpesviridae* and *Malacoherpesviridae* consist of fish and frog viruses or a bivalve virus, respectively ^[4].

The *Herpesviridae* family is further divided into the three subfamilies

Alphaherpesvirinae, *Betaherpesvirinae* and *Gammapherpesvirinae*, according to the biological properties and DNA sequences of the viruses (figure 1.1).

Characteristic for *Alphaherpesvirinae* is a short reproductive cycle, a rapid spread in cell culture and a variable host range. Examples of human alphaherpesviruses are Herpes simplex virus 1 and 2 (HSV-1 and HSV-2) and Varicella-zoster virus (VZV). In contrast, *Betaherpesvirinae* are defined by a host range that is restricted to mammalian hosts, a long reproductive cycle and a slow progression in culture. Members of *Betaherpesvirinae* infecting humans are Cytomegalovirus (CMV), Human herpesvirus 6 (HHV-6) and HHV-7. The *Gammapherpesvirinae* subfamily is characterised by a host range that is limited to the family or order to which the natural host belongs and *Gammapherpesvirinae* replicate mainly in lymphoblastoid cells. This subfamily is further classified into four genera: *Lymphocryptovirus* (LCV), *Rhadinovirus* (RDV), *Macavirus* and *Percavirus*. EBV, formally designated Human herpesvirus 4 (HHV-4), belongs to the LCV and is the only human pathogen known in this genus, whereas Kaposi's sarcoma-associated herpesvirus (KSHV) is the only human RDV [5, 6].

The *Herpesviridae* family contains 138 herpesviruses [7], but only 8 infect humans (figure 1.1). Most herpesviruses display a narrow host specificity as a result of co-evolution of the viruses with their host and some herpesviruses are even asymptomatic in their natural host [8].

1.1.3 The EBV particle

The EBV particle shares its overall structure with all other herpesviruses. The linear, double-stranded DNA (dsDNA) genome surrounds a protein core and is enclosed by an icosahedral capsid of approximately 100-110 nm in diameter formed by 12 pentavalent and 150 hexavalent capsomeres. A proteinaceous tegument composed of several viral proteins and cellular proteins, such as actin, tubulin, cofilin and heat shock proteins, surrounds the nucleocapsid [6]. The virus particle is completed by a lipid envelope displaying several viral glycoproteins (gp) on the surface, including gp350/220, gH

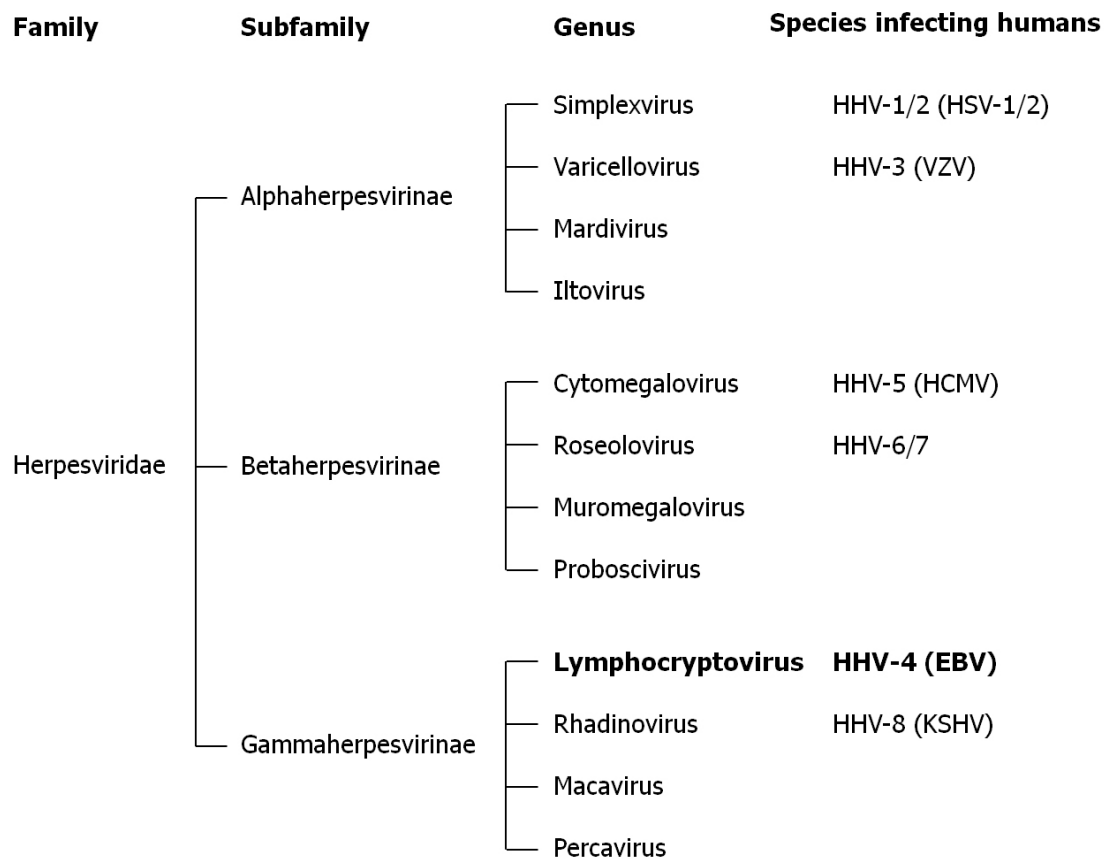


Figure 1.1: *Herpesviridae* taxonomy.

(gp85), gB (gp110), gp42 and gp150, which are important for cell tropism and receptor recognition ^[9].

Herpesviruses have large DNA genomes of 125–290 kbp ^[6]. The genome of EBV is 172 kbp and encodes approximately 85 proteins ^[10] plus multiple functional RNAs, including Epstein-Barr virus encoded RNA 1 and 2 (EBER1 and EBER2) and microRNAs (miRNAs). The EBV genome is present in a linear form in the viral capsid but circularises into an episome during infection of B cells via the terminal repeat (TR) sequences ^[11]. The TR are 0.5 kb tandem direct repeats located at both termini of the EBV genome and are a typical feature of gammaherpesviruses. Further structural features which EBV shares with most other LCVs are a single overall format and gene arrangement, 6 to 12 short internal direct repeats (IR) of 3 kb and largely unique long and short regions (UL and US). The UL and US sequences contain the majority of the coding capacity of the genome ^[5].

The B95-8 strain of EBV was the first herpesvirus genome to be cloned and subsequently the first herpesvirus genome to be sequenced completely ^[10]. B95-8 EBV contains a 13.6 kb deletion of parts of the BamHI rightward transcripts (BART) miRNAs. This section of the EBV genome was later sequenced in the Raji strain ^[12] and additional minor corrections were reported more recently ^[13]. Sets of overlapping fragments resulting from BamHI and EcoRI digestion of the genome were used for the sequencing of the B95-8 strain. The cloned BamHI fragments were labeled alphabetically in order of their size, with A being the largest fragment. Open reading frames (ORF) were designated by their order in a rightward or leftward direction within each BamHI fragment. For example, BARFI is the first rightward ORF starting in the largest BamHI fragment, A.

EBERs are encoded close to one another in rightward direction on the EcoRI J fragment in B95-8 EBV and are located adjacent to the latent origin of replication (oriP) ^[13, 14].

1.1.4 The EBV life cycle

1.1.4.1 Primary infection, attachment and cell entry

Infection with EBV usually occurs in childhood and leads to a lifelong, asymptomatic latent infection in which the virus persists within the B lymphocyte pool in 1 out of 10^5 to 10^6 cells. Persistent infection is, however, not entirely limited to B cells; some epithelial, T and natural killer (NK) cells can also be infected by the virus. Despite a generally sub-clinical infection, EBV is the causative agent of infectious mononucleosis ^[15], a self limiting lymphoproliferative disease as the result of a strong T cell response to the virus that occurs frequently if EBV infection is delayed until adolescence or adulthood ^[16].

EBV is transmitted primarily via saliva but can also be acquired from blood and transplanted hematopoietic cells or organs ^[17]. EBV infects naive B lymphocytes in the oropharyngeal lymphoid organs of the new host and establishes latent infection. The roles of the epithelium of the Waldeyer's ring during the primary infection process, especially with respect to the initial target cell of EBV infection, and viral shedding are still a matter of debate. Primary infection of B cells might be facilitated by replication in

epithelial cells in the oropharynx ^[18].

Alternatively, EBV could directly infect B cells infiltrating the reticular epithelium of the tonsillar crypts in the Waldeyer's ring without an active involvement of epithelial cells ^[19]. In favour of this model are the facts that infection of epithelial cells with EBV is inefficient *in vitro* and that EBV is only rarely detected in tonsillar epithelial tissue of infectious mononucleosis and persistently infected but otherwise healthy patients ^[20–22].

B cells on the other hand are essential for a productive infection and EBV is not able to establish a successful infection in patients with X-linked agammaglobulinaemia which lack mature B cells. Furthermore, B cells can dramatically enhance infection of epithelial cells *in vitro*. Thereby, virions bound to the B cell surface infect epithelial cells without requirement for gp42-mediated entry into B cells. Consistently, lytic replication in the B cells is not essential for the infection of epithelial cells, as was shown by using BZLF1 knockout virus. EBV remains attached to the B cell surface for up to 2 days and can infect epithelial cells within 10 minutes by forming B cell-epithelial conjugates. This transfer infection is dependent on EBV gH and gB, which are involved in epithelial fusion and penetration ^[23] and is restricted to the basolateral surface of the epithelial cells ^[24].

Changes in the glycoprotein content of the viral envelope according to the cell type in which EBV replicates support an interplay of lymphocytes and epithelial cells during EBV infection in a dual tropism model ^[18]. EBV released from B cells infects epithelial cells more efficiently, while virus shed from epithelial cells infects lymphocytes more efficiently.

Although the role of the epithelium in primary EBV infection remains to be clarified, EBV is clearly important in certain epithelial malignancies and EBV is consistently detected in malignant epithelial cells of nasopharyngeal carcinoma (NPC) ^[25] and gastric carcinoma (GC) ^[26].

The very efficient infection of B cells with EBV is initiated by attachment of the major EBV envelope glycoprotein gp350/220 to the CD21 receptor, also known as complement receptor CR2 ^[27, 28], followed by endocytosis of the virus into smooth vesicles ^[29]. CD21

is a receptor for complement C3d, which has some sequence similarity to gp350.

Internalisation of the virus is triggered by the interaction of the essential co-receptor for B cell infection human leukocyte antigen (HLA) class II with the EBV envelope glycoprotein gp42 [30]. The core fusion complex comprising gH (gp85), gL (gp25) and gp42 is essential for penetration of the virus [31, 32] with a possible role for gB [33]. EBV capsids are released into the cytoplasm [34] and the viral genome subsequently translocated into the nucleus where it is maintained as an episome after circularisation [11].

In contrast to B cells, epithelial cells do not normally express CD21 receptor and HLA class II. Complexes comprising gH and gL in the absence of gp42 are essential for the infection of epithelial cells [35]. EBV virions express three-part gH-gL-gp42 complexes or two-part gH-gL complexes, whereby the ratio of the two different complexes changes depending on the cell type from which the virion was released. EBV particles originating from epithelial cells express more three-part complexes containing gp42, favouring B cell infection, while EBV from B cells preferentially expresses gH-gL complexes, enabling epithelial cell infection. This modification of glycoprotein ratios in turn explains the switch in EBV tropism from B cells to epithelial cells and the other way around [18].

1.1.4.2 Lytic infection

In common with all herpesviruses, EBV persists in a latent infection and reactivates to replicate during lytic infection. Induction of the lytic cycle allows the production of new infectious virus particles, spread of EBV from one host to the next and completion of the viral life cycle. Lytic cycle induction is coupled to normal B cell maturation of memory cells into plasma cells upon antigen recognition and EBV has been found to undergo reactivation into the lytic cycle in antibody-secreting plasma cells in the tonsil epithelium [36, 37]. The location of these terminally differentiated plasma cells allows easy access of new virions into the saliva and thereby supports transmission of EBV to new hosts [38]. Virus released from B cells might also infect epithelial cells and resulting plaques of EBV-infected epithelial cells could explain a continuous shedding of virus from some

healthy carriers [39].

Genes expressed during the lytic cycle are classified into immediate early genes, early genes and late genes. The master regulator of lytic cycle induction is BZLF1, which can alone initiate the switch from latency to the lytic cycle [40]. BZLF1 is the only viral gene which responds directly to B cell antigen receptor (BCR) signal transduction [41], which triggers the lytic cycle *in vitro*. BZLF1 is a transcription factor with some sequence similarity to c-FOS and transactivates several viral and cellular gene promoters as well as its own expression [42, 43]. Additionally, BZLF1 activates the expression of transcription factor BRLF1, the second major regulator of lytic cycle initiation [44].

1.1.4.3 Latent infection *in vitro*

The transforming abilities of EBV are highlighted by the unique capability of EBV to transform primary B cells into latently infected, proliferating lymphoblastoid cell lines (LCLs). LCLs are characterised by the expression of the six EBV nuclear antigens EBNA1, EBNA2, EBNA-LP, EBNA3A, EBNA3B, EBNA3C and the three latent membrane proteins LMP1, LMP2A and LMP2B [45, 46]. Additionally, EBV expresses EBER1 and EBER2, BART and BHRF1 miRNAs in LCLs [5, 47].

EBV circularises its genome in the host cell nucleus 12-16 h post-infection [5]. Then the first latent gene products to be expressed are EBNA2 and EBNA-LP, whose mRNAs are differentially spliced from similar transcripts controlled by the Wp promoter. These two proteins drive expression from viral and cellular promoters [48]. EBNA2 reaches its steady state expression levels 24-36 h post-infection and upregulates expression of EBNA1, EBNA3A, EBNA3B, EBNA3C, LMP1 and LMP2s in cooperation with EBNA-LP. Expression of EBERs is delayed compared to other latency genes and begins only 36 h post-infection to reach constant EBER levels around 70 hours after infection [47].

EBNA2 is crucial for B cell transformation by EBV and induces expression of many cellular genes important for cell proliferation by mimicking the cellular Notch pathway [49, 50]. Examples of genes induced by EBNA2 are proto-oncogene c-MYC, transcription factor RUNX3 and activation markers CD23 and CD21 [51-55].

Additional B cell activation markers such as CD30, CD39, CD70, cell adhesion molecules lymphocyte-function associated antigen 1 (LFA-1) and LFA-3 and intercellular cell adhesion molecules are highly expressed on LCLs [5, 56]. These markers are commonly increased by antigenic and mitogenic stimuli in physiological B cell proliferation and explain the clustering phenotype of LCLs in tissue culture.

Besides EBNA2, several further EBV proteins are absolutely essential for the transformation of primary B cells and the continuous growth of LCLs. These include LMP1 and EBNA3C [57–61]. Further, EBNA3A improves the efficiency of B cell transformation and the proliferation rate of established LCLs [62, 63]. The transformation of B cells is also increased by EBNA-LP [57] and possibly LMP2 [60]. EBERs are not essential for B cell transformation, as will be discussed in section 1.2.5 [64–66]. The BHRF1 miRNAs significantly enhance the efficiency of B cell transformation by EBV [67], while mutation of the miR-BART cluster does not reduce the transformation rate of primary B cells [68]. EBNA1 is necessary to maintain the viral genome as an episome in proliferating cells as EBNA1 binds to the plasmid origin of replication oriP and chromosomes and enables the transmission of the EBV episome to progeny cells during mitosis [69].

As described above, expression of EBNA1, EBNA2, EBNA-LP, EBNA3A, EBNA3B, EBNA3C, LMP1, LMP2A and LMP2B is typical for LCLs. Expression of this specific set of latent genes is known as the growth programme or latency III expression pattern.

More restricted forms of EBV viral gene expression patterns are found during latent infection of other cells according to host cell type and differentiation stage. These programmes are distinct in the proteins and levels of miRNAs expressed and encode a limited number of proteins that are important for growth transformation and evasion of the immune system. Strikingly, all latency expression patterns share the presence of high levels of both EBER1 and EBER2.

Type I latency gene expression is found in BL cells and its hallmark is the expression of Epstein-Barr nuclear antigen EBNA1. GC cells commonly maintain latency I gene

expression with addition of expression of LMP2A in approximately 50% of the cases.

Latency II, which is also referred to as the default programme, is prevalent in Reed-Sternberg cells in Hodgkin's disease, NPC and T cell lymphomas. Latency II is defined by the presence of EBNA1 and the latent membrane proteins LMP1 and LMP2s.

The association of EBV with the cancers mentioned above will be described in section 1.1.5.

1.1.4.4 Latent infection *in vivo*

In contrast to the transforming abilities of EBV in B cells *in vitro*, EBV persists in a quiescent state in approximately 1 per 10^5 to 10^6 resting memory B cells circulating in the peripheral blood *in vivo*. Thorley-Lawson proposed a model to address this paradox [70], in which EBV is thought to follow the normal differentiation route of B cells (figure 1.2).

Naive B cells encountering antigen normally migrate to lymph follicles and undergo strong proliferation, class switch recombination, hypermutation of the variable immunoglobulin (Ig) chain and clonal selection in germinal centres. As a result, selected B cells producing high affinity antibodies differentiate into plasma cells and memory B cells. Signals through BCR in response to antigen binding and through CD40 from T helper cell CD154 are essential for survival of the cells in this pathway [72]. EBV mimics this B cell differentiation path to gain access into the memory B cell pool.

Infection of naive B cells with EBV leads to the expression of the growth programme (latency III) in the cells and consequently to activation of the B cells, resembling natural antigen activation in terms of surface markers and morphological characteristics of the B cells [73]. Expression of the growth programme allows the activated B cells to proliferate and EBV to expand the number of infected cells. This expansion time is only short, as the virus must establish latent infection to avoid a strong cytotoxic T cell response. The activated B blasts migrate to germinal centres, where the virus switches to the default expression programme, which includes LMP1 and LMP2.

Consistent with physiological B cell maturation of centroblasts to centrocytes,

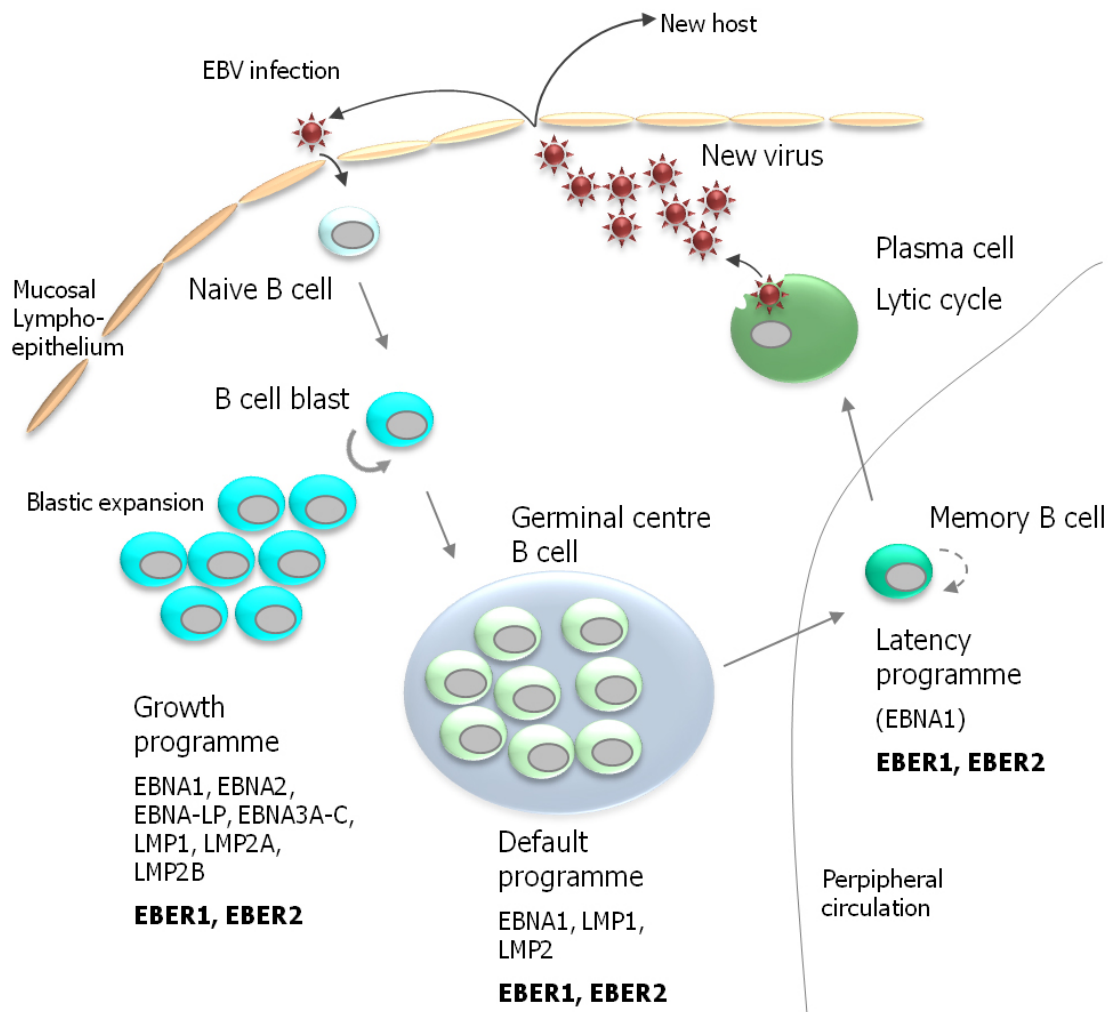


Figure 1.2: EBV infection of B cells *in vivo*. EBV infection of B cells *in vivo* follows the physiological differentiation route of B cells. EBV enters lymphoepithelial structures of a new host via saliva, crosses the epithelial barrier and infects naive B cells in the Waldeyer's ring. Expression of the growth programme leads to cell differentiation into B cell blasts and strong proliferation. The B cell blasts migrate to germinal centres in lymph node follicles. Subsequent expression of the default programme in germinal centre B cells results in a differentiation of the infected cells into memory B cells which circulate in the blood. Resting memory B cells express no viral proteins (latency 0) with exception of EBNA1 during the rare memory B cells divisions (latency I). However, EBER1 and EBER2 are abundantly expressed in memory B cells. Memory B cells can reenter the tonsil and differentiate into plasma cells in order to initiate the lytic cycle. New virions are shed into saliva and can be transmitted to new hosts. Adapted from Thorley-Lawson ^[71].

EBV-infected B cells undergo somatic hyper-mutation, Ig genes class switch and affinity maturation [74, 75]. To ensure survival and differentiation of the B blasts into memory B cells, LMP1 substitutes for CD40 signalling from T helper cells and LMP2A substitutes for survival signals usually provided by the BCR [61, 76, 77]. Thus LMP1 and LMP2 provide the necessary survival signals to rescue the B cells from apoptosis and allow progression of differentiation into memory B cells.

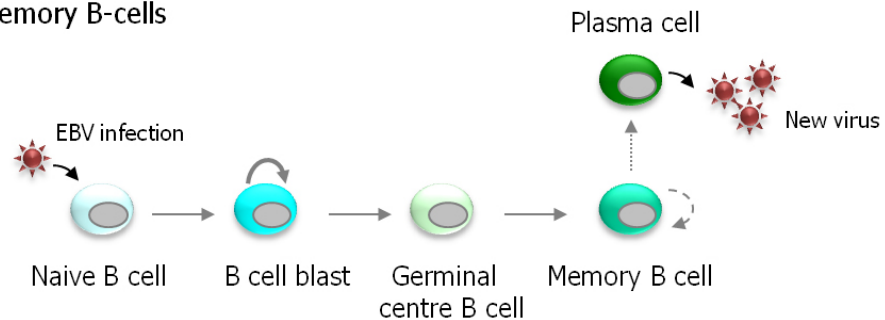
Differentiated memory B cells exhibit the latency programme, in which EBNA1 is the only viral protein expressed during the infrequent memory B cell divisions to allow EBV DNA replication [71, 78, 79]. It is important to remember that during the latency programme, even if no viral proteins are expressed, the latent EBV genome is not transcriptionally silent and EBV expresses multiple viral RNA transcripts, characteristically BARTs miRNAs, EBER1 and EBER2 in the memory B cells [80, 81]. The memory B cells represent a good niche for EBV persistence as they are long-lived and the limited expression of viral protein reduces detection by the immune system.

In contrast to the peripheral blood, the default programme is expressed in EBV-positive memory B cells in the tonsils [82]. The local expression of LMP1 and LMP2 is believed to supply the necessary signals for long term survival of memory B cells. This idea is in line with findings showing that healthy memory B cells require survival signals provided by a functional BCR to survive [83]. EBV-infected memory B cells are true memory B cells as they display the hallmarks of antigen selection such as expression of Ig genes, isotype class switching and somatic hyper-mutation [79]. Occasionally, EBV is reactivated by differentiation of the host cell into a plasma cell in the Waldeyer's ring to shed new viral particles into the saliva and complete the viral life cycle (section 1.1.4.2).

Infection of B cells *in vitro* resembles the early stages of *in vivo* B cell infection. LCLs are comparable to B cell blasts in that they both express the growth programme and undergo strong proliferation. Additionally, LCLs express high levels of B cell activation markers including CD23, CD30, CD39, CD70 and cell adhesion molecules typical for activated B cells [5, 56]. LCLs are therefore a very useful model for the investigation of the initial stages of B cell infection and transformation (figure 1.3).

In vivo:

Persistence in memory B-cells



In vitro:

Transformation of B-cells

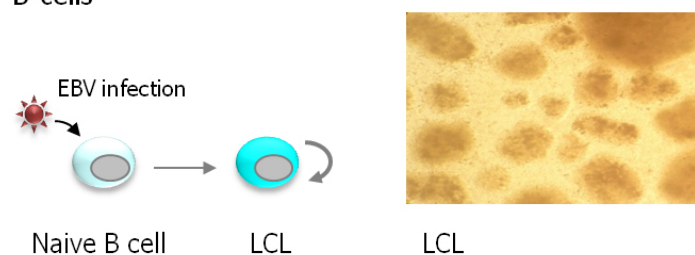


Figure 1.3: Comparison of EBV infection of B cells *in vivo* and *in vitro*. EBV infection of B cells *in vivo* (top) follows the normal differentiation route of B cells. EBV infection of B cells *in vitro* (bottom) results in the establishment of strongly proliferating LCLs, which express the EBV growth programme and resemble B cell blasts. A phase contrast microscopy image of an LCL is shown.

1.1.5 The role of EBV in human cancers

In vitro, resting B cells are transformed into indefinitely proliferating LCLs by EBV infection [84]. Consistent with these growth transforming properties, EBV is associated with approximately 1% of all human cancer cases and contributes to the development of several epithelial and lymphoid malignancies.

In addition to BL (section 1.1.1), these include Hodgkin's lymphoma, post-transplant lymphoproliferative disease (PTLD), AIDS-associated lymphomas and X-linked lymphoproliferative disease as well as nasal T/NK lymphoma, peripheral T cell lymphoma and epithelial malignancies such as GC and NPC [5, 85].

In some parts of the world, an extremely high percentage of BL tumours carry EBV, with up to 98% in endemic areas [86], but the role of EBV in the development of BL has remained unclear as the latency I programme is expressed in BL cells which lacks

expression of the common growth promoting latent proteins. A chromosomal translocation of c-MYC proto-oncogene in the juxtaposition of an Ig heavy or light chain enhancer that results in a constitutive activation of c-MYC is typically found in BL cells [87, 88] and contributes to the high level of proliferation in BL cells [89]. BL cells have an Ig gene hyper-mutation similar to memory B cells but have a cellular phenotype of germinal centre cells [90, 91]. Additionally, BL cells frequently contain mutations of the p53 pathway [92]. EBV might simply enhance proliferation of B cells with a c-MYC translocation acquired independent of the infection process. Additionally, EBV might protect BL cells from apoptosis induced by deregulated c-MYC. EBERs, which are expressed in all forms of latent infection, confer resistance to apoptosis in BL cell lines [93] (section 1.2.5).

About 40% of all Hodgkin's lymphomas are EBV-positive. EBV expresses the latency II programme, corresponding to latently infected germinal centre B cells, in Reed-Sternberg cells which have hyper-mutated Ig genes [94]. Infectious mononucleosis is a risk factor for Hodgkin's disease and mutations of germinal centre B cells that block B cell differentiation together with the growth signals provided by LMP1 and LMP2 could explain the development of the tumour [78].

PTLD arises in immunosuppressed patients after transplantation. 90% of post-transplant lymphomas contain EBV and resemble LCLs by expressing the EBV growth programme. PTLD might arise when EBV infects a naive B cell in the Waldeyer's ring and causes it to proliferate under the influence of the growth programme [95]. In the immunosuppressed patients, the impaired cytotoxic T cell response could allow an uninhibited growth of the B cells. Moreover, the EBV-infected cell must remain proliferating and not differentiate into a resting memory cell. To this end, the proliferating cell must acquire additional mutations. Alternatively, it is suggested that non-naive bystander B cells could be the source of PTLD [78, 96]. In summary, B cell derived lymphomas can arise at different stages of EBV B cell infection and give rise to a wide variety of tumours.

EBV is further associated with epithelial malignancies and is present in virtually all NPC cases. LMP1 was detected in almost 80% of NPC cases, supporting a crucial role for

EBV-mediated transformation in the pathogenesis of NPC [97]. Recent studies expressing LMP1 and LMP2 specifically in epithelial cells in transgenic mice showed increased formation of papillomas and carcinomas in response to LMP1, whereas LMP2 did not initiate malignant growth but still contributed to tumour progression [98]. Despite a considerable variation in the level of expression of LMPs between individual tumours [99], anti-apoptotic B cell lymphoma 2 (BCL-2), which is upregulated by LMP1, is consistently overexpressed in malignant NPC cells [97]. Interestingly, EBERs can also upregulate BCL-2 in BL and NPC cells and might therefore be responsible for induction of the oncoprotein in NPC tumours [100, 101]. EBV microRNAs were also found to be strongly expressed and implicated in tumorigenicity in NPC tumours [102, 103].

Moreover, EBV is present in 5-10% of all GC cases. Very little is known about the contribution of EBV to these cancers, but the induction of the autocrine growth factor insulin like growth factor 1 (IGF1) is consistently detected in EBV-positive biopsies in contrast to EBV-negative samples and might support the outgrowth of the tumours [104].

EBV-associated malignancies such as BL and NPC have distinct geographical frequencies of incidence, which suggests that environmental and genetic factors contribute to the formation of these tumours in addition to EBV. BL is endemic in equatorial Africa and New Guinea, whereas NPC has an especially high incidence in Taiwan and Southern China [5].

1.2 Epstein-Barr virus encoded RNAs

1.2.1 Functional RNAs

The majority of the genome of mammals is transcribed into non-coding RNAs (ncRNA), many of which are spliced or processed to control a vast amount of complex physiological processes such as epigenetic features, transcription, RNA splicing, RNA editing and translation ^[105]. The protein coding exons of eukaryotic genes account for only 1.5% of the genome but up to 90% of the genome is transcribed. In fact, approximately 98% of all human transcripts are ncRNAs located in introns of protein-coding genes or exons and introns of non-protein-coding genome regions. Additionally, ncRNAs can be transcribed antisense to protein-coding genes or overlap coding regions. As a result, ncRNAs drastically increase the genomic output and add a level of complexity to the regulation of gene expression and cellular processes which might potentially contribute to the variety of cell types in complex organisms ^[106]. Consistent with this idea, the number of ncRNAs grows with the complexity of the organism. Phenotype variations between species and individuals are more likely to be the result of the strong differences between the non-coding regions of genome sequences rather than the relatively conserved protein-coding sequences. Between humans, less than 1% of sequence variation is in protein-coding regions, suggesting that individual phenotypes might be accounted for by differences in regulatory non-coding domains of the genome ^[107].

Novel roles of ncRNAs in cellular metabolism, infection and disease are constantly emerging with the growing understanding of the mechanisms of action of ncRNAs and the discovery of new RNA members of existing ncRNA classes as well as completely new classes of ncRNAs. The many described functional ncRNAs are roughly divided into small ncRNAs and long ncRNAs. Long ncRNAs are generally considered to be longer than 200 nucleotides in length, but there is currently no official classification system for ncRNAs. The most important and best understood classes of ncRNAs will be introduced below to highlight the growing importance of regulatory RNAs in the

regulation of development and disease.

It is important to note that classical non-translated RNAs such as tRNAs and rRNAs are also part of ncRNAs. Furthermore, it is to be expected that many more functions of ncRNAs will be explored in coming years and that whole new classes of functional RNAs are still waiting for their discovery.

Cellular ncRNAs are possible intervention points for viruses to adapt cellular processes for their benefits or prevent cellular responses to the viral infection. Moreover, viral ncRNAs are important regulatory components for viruses not only to allow the control of viral and cellular gene expression but also to avoid immune responses by the host organism due to the low levels of immunogenicity and antigenicity of RNAs.

1.2.1.1 Small non-coding RNAs

The major classes of small ncRNAs are miRNA, small interfering RNA (siRNA), small nucleolar RNA (snoRNA) and PIWI-interacting RNA (piRNA) ^[108].

Micro RNAs

miRNAs are by far the best understood ncRNAs and are key regulatory elements in essential physiological processes, including cell proliferation, development and apoptosis. miRNAs are short, single-stranded RNAs (ssRNA) of 21-23 nucleotides, which inhibit protein translation or promote mRNA degradation by at least partial complementary base pairing, usually with the 3' untranslated region (UTR) of their target RNA ^[109]. miRNAs are derived from endogenous short hairpin precursors generally transcribed by RNA polymerase II or rarely by RNA polymerase III ^[110]. These mostly capped, polyadenylated pri-miRNAs are processed to 65-75 nucleotide pre-miRNAs by RNase III endonuclease Drosha ^[111]. The pre-miRNAs are subsequently exported from the nucleus by exportin 5 ^[112] and are processed into imperfect, approximately 22 bp duplexes by RNase III endonuclease Dicer in the cytoplasm ^[109, 113].

One strand of the miRNA duplex is then incorporated into the ribonucleoprotein

RNA-induced silencing complex (RISC), which contains an Argonaute protein that can have catalytic endonuclease activity to cleave target RNAs. The miRNA component guides the RISC complex to specific, complementary target RNA. Generally, perfect complementarity promotes the degradation of the mRNA by the Argonaute protein, while complementarity limited to the seed-sequence induces the reduction of protein translation without altering mRNA levels or by inducing deadenylation-dependent mRNA decay. Interestingly, some miRNAs can alternatively induce or repress translation of their targets depending on cell cycle progression ^[114]. Many miRNAs regulate individual target genes, but some miRNAs are master regulators of whole gene networks and control the expression levels of hundreds of genes. It is estimated that the expression of more than 60% of protein-coding genes is regulated by miRNAs and more than 1400 miRNAs have been described in the human genome so far ^[105, 108].

Expression levels of numerous miRNAs are significantly altered in tumours and crucial roles for many miRNAs have been identified in the development of cancer, whereby several miRNAs were found to function as classical tumour suppressors or oncogenes ^[108]. For example, miR-21 acts as oncogene in pre-B-cell lymphoma ^[115], the miR-200 family regulates epithelial-to-mesenchymal transition through epigenetic changes and miR-34 is a component of the p53 network ^[116]. These miRNAs are also known to be deregulated in EBV-infected cells, as will be elucidated in section 1.2.2.

Small interfering RNAs

Small interfering RNAs (siRNA) are similar to miRNAs in length, function and mechanism of action. siRNA are produced by DICER by processing of complementary dsRNA duplexes or long hairpin precursors, often of exogenous origin. siRNAs usually target homologous sequences for gene silencing. siRNA and miRNA are produced by the same pathway and perform similar functions using identical mechanisms ^[117].

Small nucleolar RNAs

Small nucleolar RNAs (snoRNAs) are functional RNAs of 60-300 nucleotides in length and are most commonly found in the nucleus. Over 300 snoRNAs have been described

in humans ^[117]. The best understood roles of snoRNAs are ribosomal, spliceosomal and messenger RNA modifications ^[105] and snoRNAs are likely to be crucial for the production of functional ribosomes ^[118]. snoRNA hybridise with specific RNA target sequences and recruit protein partners to form small nucleolar ribonucleoprotein complexes which then modify the target RNA. There are two main subsets of snoRNAs which differ significantly in their overall structure to allow the binding of specific proteins which perform different RNA modifications. C/D box snoRNAs guide the methylation of RNAs, while H/ACA box snoRNAs define the target sites for pseudouridylation ^[119].

Most snoRNAs are located in intron regions of both protein coding and non-coding genes. This position allows the simultaneous regulation of the expression of the snoRNAs together with genes which are modified by the snoRNAs or which are important in the processes influenced by the snoRNA modifications. On the other hand, this common location of snoRNA complicates the differentiation of effects of the snoRNA and the encoded protein in knockout studies.

Recent experiments suggest functions of snoRNAs in oncogenesis, development and disease in addition to the well described functions in ribosomal housekeeping ^[119]. C/D box snoRNA SNORD115 is processed to a shorter ncRNA which hybridises with human serotonin receptor 2C (HTR2C) pre-mRNA and guides correct splicing of the mRNA to produce an accurately functioning serotonin receptor ^[120]. Inhibition of correct HTR2C pre-mRNA splicing is associated with Prader–Willi syndrome with typical symptoms being developmental delay, restricted growth and excessive weight gain ^[121]. Furthermore, snoRNAs might play important roles in the development of cancer and have been found to promote as well as inhibit the development of specific tumours. U50 deregulation was reported in prostate cancer ^[122] and breast cancer ^[123], whereas SNORA42 is frequently overexpressed in non-small-cell lung cancer ^[124, 125].

Some snoRNAs are currently being identified as precursors of miRNAs ^[126]. H/ACA box ACA45 snoRNA-derived miRNA associates with Argonaute and regulates the translation of specific target mRNA such as cyclin-dependent kinase 11 ^[127]. It is speculated that

snoRNAs might be the evolutionary precursors of the first miRNAs.

Consistently with a possible involvement of snoRNAs in tumour development, H/ACA box snoRNA-derived miR-605 is transcriptionally activated by p53. Under stress, miR-605 represses the translation of MDM2, which results in the rapid accumulation of p53, inhibition of cell cycle progression and induction of apoptosis. A deregulation of miR-605 might be relevant in cancer biology ^[128].

Cajal body-specific RNAs

Cajal body-specific RNAs (scaRNAs) are similar to snoRNAs in structure, size and function but contain Cajal body localisation signals ^[129]. Consequently, scaRNAs accumulate in Cajal bodies in the nucleus and guide post-transcriptional modification of small nuclear RNAs (snRNA). Cajal bodies are membraneless subnuclear organelles of 0.1–2 μm involved in the modification of snRNAs and snoRNAs and the assembly of ribonucleoprotein particles (RNP) and telomerase ^[130].

PIWI-interacting RNAs

PIWI-interacting RNAs (piRNAs) are approximately 24–30 nucleotides in length and interact with the PIWI subfamily of Argonaute family proteins. piRNAs hybridise to transcripts of transposable elements which are consequently cleaved by the PIWI proteins, as observed in *Drosophila* ^[131]. Alternatively, piRNAs can control heterochromatin-mediated gene silencing ^[132]. Both mechanisms result in reduced expression of transposable elements and increase genome stability.

Additional small ncRNAs

Multiple additional classes of ncRNA associated with gene promoters and transcription start sites (TSSs) have been identified and are reviewed here briefly. The exact functions of these ncRNAs are still a matter of speculation. Most likely, these ncRNAs are involved in transcriptional regulation but as yet unidentified further roles of these ncRNAs can not be excluded.

Transcription initiation RNAs (tiRNAs) are evolutionarily conserved, 17-18 nucleotide

RNAs located within -60 to +120 nucleotides of the TSSs and are preferentially associated with highly expressed transcripts and G/C-rich promoters [133].

Promoter associated small RNAs (PASRs) are 22-200 nucleotides in length and cluster at the 5' end of genes. PASRs were identified by RNA mapping and sequence conservation. Expression levels of PASRs usually correlate with expression levels of the overlapping gene [134]. These transcripts are extremely common and more than 10,000 PASRs have been identified in the human genome.

TSS-associated RNAs (TSSa-RNAs) flank active promoters with peaks of antisense RNAs 250 nucleotides upstream and sense RNAs 50 nucleotides downstream of TSSs [135]. TSSa-RNAs include RNAs of 20-90 nucleotides in length and more than 10,000 were predicted in humans, similar to PASRs [136]

Finally, promoter upstream transcripts (PROMPTs) are highly unstable RNAs located 0.5 to 2.5 kb upstream of TSSs and their expression usually correlates with gene activity [136]. Several further classes of ncRNAs such as X-inactivation RNAs, Sno-derived RNAs, microRNA-offset RNAs, vault RNA, tRNA-derived RNAs, MSY2-associated RNAs, telomere small RNAs and centromere-associated RNAs were described and their functions are the subject of ongoing investigations. These unexplored ncRNAs are not discussed here but were recently reviewed by Taft *et al* [137].

1.2.1.2 Long non-coding RNAs

In addition to the relatively well investigated short RNAs, an increasing number of long non-polyadenylated, ncRNAs are described that act functionally as RNAs. Long ncRNAs are generally considered to be more than 200 nucleotides in length, but snoRNAs, commonly classified as small ncRNA, can be up to 300 nucleotides in length. lncRNA are very variable in their length, ranging up to 100 kb and usually function without processing.

More than 80% of all cellular transcripts are estimated to be long ncRNAs (lncRNA) [134]. Expression of many lncRNAs is restricted to specific developmental stages in mice [138] and distinctive subcellular localisations are displayed by lncRNAs in the brain [139],

indicating that lncRNAs are not transcriptional noise due to low polymerase fidelity. In addition, expression of lncRNA is regulated by conventional transcription factors^[140] and recent data suggest high evolutionary conservation for many lncRNAs^[141, 142].

lncRNA are involved in the regulation of crucial cellular functions such as epigenetic changes through chromatin modification by recruiting chromatin remodeling complexes, for example Polycomb Repressive Complex 2^[143]. In particular, a recently described subclass of evolutionarily conserved lncRNAs, large intergenic ncRNA (lincRNA) is associated with chromatin complexes and histone modifications which result in altered expression of specific genes^[141, 144]. Moreover, lncRNAs are engaged in post-transcriptional regulation including splicing^[145], translation and RNA degradation^[146, 147]. In addition, some lncRNAs act directly on transcriptional regulation by interaction with the initiation complex, RNA Polymerase II or by recruiting transcription factors, for example to downregulate cyclin D1 in response to DNA damage^[148]. Recently, lncRNAs have been further implicated in the integrity of nuclear architecture of membraneless subnuclear compartments and particularly in the assembly of paraspeckles^[130].

Considering the suggested regulation of protein-coding genes by lncRNAs in normal development both on transcriptional and post-transcriptional levels, important impacts of lncRNAs mutations and deletions can be expected. Accordingly, a deregulation of lncRNAs is observed in many human diseases and malignancies, including colon cancer^[149], breast cancer^[150] and Alzheimer's disease^[151]. lincRNA HOTAIR upregulation in breast cancer alters histone H3 lysine 27 methylation via its interaction with Polycomb Repressive Complex 2. The subsequent genome-wide changes in gene expression promote metastasis and cancer progression^[152].

The consequence of lncRNA deregulation in the carcinogenesis of leukemia and carcinomas^[153] is in some cases explained by antisense lncRNA controlling their protein-coding partner epigenetically^[137]. A well studied example of a protein regulated by this mechanism is tumour suppressor p15 which is implicated in the genesis of leukaemia^[154]. In mouse embryonic stem cells, increased expression of antisense p15

lncRNA causes p15 silencing in a Dicer independent manner and results in enhanced cell proliferation. *In vivo*, p15 lncRNA expression might promote the development of leukemia. Bidirectional transcription is surprisingly common and 40-50% of protein coding genes have antisense ncRNAs transcribed from their opposite strand [155, 156].

The limited understanding of many classes of ncRNA leaves room for speculation of possible regulatory roles in many physiological processes and future experiments might reveal additional functions of the previously underestimated functional RNAs. The growing understanding of ncRNAs will also allow an investigation of effects of EBV or other viral infections on expression and function of ncRNAs in the host. Moreover, the discovery of new ncRNAs in viruses could reveal previously unknown mechanisms for EBV to establish latent infection, avoid immune surveillance or contribute to oncogenesis in EBV-associated cancers. Overall, the massive number and variety of ncRNAs highlight the importance of functional RNAs as an essential aspect of normal physiological cell functions and consequently for host-virus interactions.

1.2.2 The importance of functional RNAs in EBV infection

EBV is an exceptional example of a virus that uses functional ncRNAs and expresses snoRNA, numerous miRNAs and the two non-coding EBV-encoded RNAs EBER1 and EBER2 during persistent infection and immune evasion [157]. In fact, the most abundant viral transcripts in latent EBV infection are functional ncRNAs [158].

EBV v-snoRNA1 was the first and so far only snoRNA to be discovered in a viral genome and a computationally predicted homolog exists only in the rhesus lymphocryptovirus genome. v-snoRNA1 is expressed during latent infection in LCLs but is 30-fold upregulated during the lytic cycle. The C/D box v-snoRNA1 was found to bind to common snoRNA interaction partners fibrillarin, RNA methyltransferases Nop56 and Nop58 by co-immunoprecipitation. The function of v-snoRNA1 during EBV infection is not yet clear. EBV infection rates, cell transformation, viral gene expression

and virus replication were not altered in v-snoRNA1 knockout EBV compared to wild type (wt) virus. However, a shorter version of v-snoRNA1, v-snoRNA1(24pp) was identified, which is complementary to the 3'UTR of viral DNA polymerase BALF5 mRNA and might modulate BALF5 expression as a miRNA [159].

The best understood ncRNAs in viral infections are miRNAs. EBV both represses and activates the expression of cellular miRNAs but also uses its own viral miRNAs to inhibit cellular immune responses and to promote cell transformation. In fact, EBV was the first virus found to employ miRNAs. EBV encodes at least 25 pre-miRNAs according to the Sanger database (<http://www.mirbase.org>) and some studies suggest up to 44 mature miRNAs [160]. 4 of the EBV miRNAs are located in introns of the BHRF1 gene while the remaining EBV miRNAs are located in the BART cluster [161, 162]. It is of note that B95-8 EBV expresses only 13 listed miRNAs as a part of the BART cluster is deleted from the viral genome. Expression profiles of EBV miRNAs differ significantly between different EBV-infected cell types and EBV-associated tumours. The BART miRNAs are highly expressed in latently infected epithelial cells while BHRF1 miRNAs are mainly found in latency III [163–165].

EBV uses its extensive repertoire of miRNAs to regulate viral genes to successfully establish latent infection and avoid immune surveillance [166, 167]. The expression of both LMP1 [168] and LMP2A [169] is reduced by EBV BART miRNAs. Inhibition of LMP1 expression might prevent toxic effects of LMP1 in infected cells and regulate growth properties of infected B cells. Reduction of the expression of immunogenic proteins limits the exposure of EBV to the immune system and allows the virus to circumvent immune surveillance. Accordingly, immunogenic EBNA2 is downregulated by miR-BART6 [170]. Regulation of EBV proteins by miRNAs might also promote transition into specific latency programmes in B cells; both lytic cycle EBV DNA polymerase BALF5 and lytic cycle inducer BZLF1 are inhibited by miR-BART2 or miR-BART6 [170, 171].

Furthermore, EBV miRNAs modulate the expression of numerous cell genes relevant to

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antiviral immune response and apoptosis [160, 167]. MICB, a natural killer (NK) cell ligand induced by viral infections and tumour transformation is a cell target of EBV miRNAs. Downregulation of MICB helps the virus to escape recognition and consequent elimination by NK cells [172]. MICB is also targeted by KSHV, indicating a general function of the ligand in the antiviral defense against herpesviruses. Additionally, EBV miRNAs lead to downregulation of interferon (IFN) inducible T cell attracting chemokine CXCL11/I-TAC in AIDS-related diffuse large B cell lymphoma as a further way to circumvent the host immune system [173]. Furthermore, miR-BART5 reduces sensitivity to apoptotic stimuli and so promotes host cell survival by downregulation of p53 upregulated modulator of apoptosis (PUMA) in NPC [174]. Consistently, apoptosis is also counteracted by miR-BART cluster 1 by targeting the 3'UTR of the pro-apoptotic BIM [175]. The targetome of EBV miRNAs was established in latently infected B cells by immunoprecipitation of Argonaute-containing RISC complexes using anti-Argonaute antibodies and subsequent microarray analysis of the target RNAs (RIP-Chip). 44 targets were identified for EBV miRNAs; confirmation and further investigations of the regulation of these targets and their roles during EBV infection will certainly reveal new interesting insights in EBV miRNA biology [176].

Additionally, EBV exploits the existing cellular miRNA network to regulate cell transformation and modulate innate immunity [177–179]. Important examples of EBV downregulated cellular miRNAs are members of the let 7 tumour suppressor miRNA family, namely let 7b, which targets cyclin and cyclin dependent kinases in various cancers, and let 7d, which is able to revert c-MYC-induced growth in BL cells [177]. At the same time, oncogenic miR-155 which causes tumours in mice is upregulated by EBV infection [180]. LMP1 activates miR-155 transcription through the nuclear factor kappa B (NF- κ B) pathway, allowing miR-155 to influence the oncogenic potential of EBV and crucially contribute to LCL transformation [181, 182]. KSHV similarly regulates miR-155 targets by expressing the viral orthologue miR-K11 [183]. This emphasises the importance of miR-155 for human tumour viruses. Multiple additional miRNAs associated with cancer are deregulated by EBV. The previously mentioned,

cancer-associated miRNAs miR-21 and miR-200 are deregulated in EBV-associated microdissected Reed-Sternberg cells in Hodgkin's lymphoma ^[184]. miR-200a and miR-200b were also found to be decreased in EBV-associated GC compared with EBV-negative carcinoma by quantitative reverse transcription-PCR analysis. Transfection of EBV-negative cells with BARF0, EBNA1 and LMP2A resulted likewise in a reduction of the miR-200 family and consequently in an upregulation of the E-cadherin repressors zinc finger E-box binding homeobox ZEB1 and ZEB2. The resulting decline of E-cadherin expression in turn changes cell adhesion and morphology and promotes cell migration and invasion ^[185]. Additionally, overexpressed ZEB1 and ZEB2 repress transcription of BZLF1 promoter and significantly reduce lytic reactivation in fibroblasts and B cells, thus providing a mechanism for the maintenance of latent infection ^[186].

Expression levels of cellular miRNA are also influenced by EBV to alter the host immune response. LMP1 modulates the expression of 35 cellular miRNAs with the strongest effect on miR146a, a cellular miRNA involved in IFN signalling ^[187]. Deregulation of miR146a might allow EBV to regulate the intensity or duration of an antiviral IFN response.

Strikingly, EBV miR-BART6 targets Dicer mRNA in latently infected Daudi Burkitt lymphoma and NPC C666-1 cells, which allows control of the processing of the entire cellular and viral miRNA network ^[170]. As a result, repression of many host miRNAs is observed after transient expression of miR-BART6 in HeLa cells and in freshly EBV-infected primary B cells ^[177]. However, the latter study used B95-8 EBV, which contains a partial deletion of miR-BART6. A reduction in cellular miRNA expression could therefore be mediated by additional viral factors or be part of the cellular response to infection.

Recently, 146 viral miRNAs were listed in miRNA databases ^[188]. Impressively, 139 of these are encoded by members of the herpesvirus family such as CMV, KSHV and EBV, suggesting an important role for miRNAs in the herpesvirus life cycle. Even though evolutionary conservation of gammaherpesvirus miRNAs is generally rare, the exception is formed by 7 miRNA hairpins known to be shared by EBV and the closely related rhesus

lymphocryptovirus, hence emphasising a special role of functional RNA for EBV [189]. So far, viral miRNAs have only been identified in DNA viruses; polyomaviruses and adenoviruses have recently joined herpesviruses on the limited list of virus families that use miRNAs [179].

Infection with EBV or KSHV specifically stimulates the expression of novel vault RNA, an essential part of the ribonucleoprotein vault complex, in B lymphocytes [190]. The large, hollow vault complex is potentially involved in resistance to apoptosis, innate immune signalling and multidrug resistance [191]. The observed increase of vault RNA after EBV infection could be a physiological response to the viral infection or part of a viral strategy to manipulate the immune system.

The examples of ncRNAs in EBV infection described above clearly illustrate an extraordinary role for ncRNAs in the EBV life cycle. EBV additionally expresses the highly abundant 167 and 172 nucleotides EBV-encoded RNAs EBER1 and EBER2, the topic of this thesis, discussed in detail in the following section 1.2.3. EBERs are some of the most abundant viral transcripts during latent infection and are constantly expressed in all EBV-infected cell types and differentiation states during latency. EBER RNAs are not expressed in the lytic cycle observed in hairy leukoplakia in AIDS patients, which implies a special importance of EBERs in persistent infection.

1.2.3 Epstein-Barr virus encoded RNAs

1.2.3.1 Structure, regulation and expression of EBERs during EBV infection

EBER1 and EBER2 are expressed at very high levels of up to 5×10^6 copies per cell in virtually all EBV-infected cell lines and EBER expression is a hallmark of the latency programme in memory B cells *in vivo*, where no EBV proteins are expressed. EBER1 and EBER2 are non-translated, non-polyadenylated RNAs of 167 and 172 nucleotides, respectively [192].

Both EBER genes are located on the EBV EcoRI J fragment of EBV B95-8, close to the

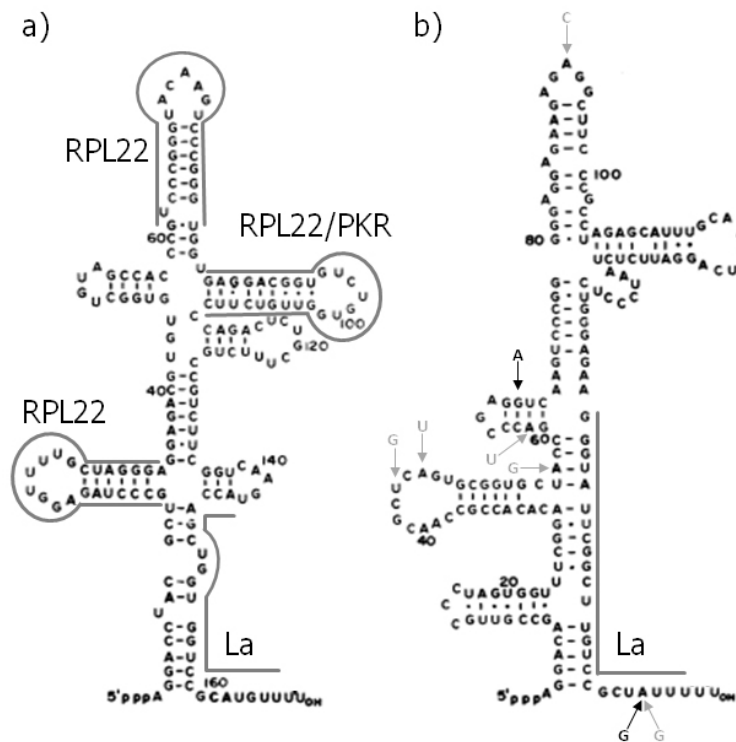


Figure 1.4: EBER RNA structure. a) EBER1 and b) EBER2 are highly structured single-stranded RNAs of 167 and 172 nucleotides, respectively. The binding sites of RPL22, PKR and La are indicated schematically. The EBER2 sequence of type I EBV is shown and the nature and location of the two type II EBV specific polymorphisms ^[193] (black arrows) and consensus mutations frequently found in NPC ^[194] (grey arrows) in the EBER2 coding region are indicated. Adapted from Rosa *et al* ^[14].

origin of replication of the EBV episome and at a distance of 161 bp from each other ^[10]. The primary sequence similarity is only 54% between EBER1 and EBER2 and is mainly present at their 3' and 5' termini ^[93]. However, both EBERs show comparable secondary structures and form several stable stem loops, predicted by nuclease digests of purified EBERs and structure predictions based on optimal minimisation of free energy (figure 1.4) ^[14].

EBERs are very strongly conserved ^[193, 195] but some EBER region variation is present in accordance with the two EBV families type I and type II, which are defined by variations in EBNA2, EBNA3A, EBNA3B and EBNA3C ^[196]. Type I and type II EBV have only 10 polymorphisms within the 1 kb EBER region. In detail, the EBER1 sequence is completely conserved, 2 differences are located in the EBER2 coding region and 8 differences are present in the region between EBER1 and EBER2. These EBER

variations were identified in type I EBV B95-8 or type II EBV AG876 and P3HR-1, respectively. Recent investigations comparing EBER polymorphism in NPC and GC by PCR identified up to 6 further consensus mutations in the EBER2 coding region and mutations in the intergenic region which define three additional EBER variants but there is no evidence at present for functional consequences of these variations [194]. The EBER1 coding region was again completely conserved, implying a crucial importance of EBER1 during EBV infection. The EBER2 region appears more flexible and some of the observed EBER2 mutations in NPC are predicted to be located in EBER2 loops (figure 1.4). Protein interaction partners commonly bind to RNA stem loop regions and mutations in these structures might therefore change the interaction of EBER2 with any protein partners. Mutations in the EBER2 sequence might also result in changes in the secondary structure of the RNA. Type I and type II EBV were heterogenous in respect to the three new EBER variants and only one EBER type was found per sample, indicating that EBER sequence is not defined by EBV type and is stable during infection. Interestingly, the new EBER variants were preferentially found in NPC compared to healthy donors and GC [194]. EBER2 polymorphism might therefore be associated with neoplastic transformation in NPC. Further detailed investigations of EBER2 sequences in EBV-associated tumours could reveal significant correlations between EBER2 and tumour formation.

The main fraction of EBERs in the host cell is concentrated in the nucleoplasm [197, 198]. However, EBERs are present in the cytoplasm of interphase Raji cells, where EBERs locate to the rough endoplasmic reticulum (ER) and the Golgi apparatus [199]. The dual localisation of the EBERs is in agreement with the described interaction of EBERs with cellular protein partners present in the nucleus or the cytoplasm (section 1.2.4).

EBERs are transcribed by RNA polymerase III, but regulatory elements of RNA polymerase II are also present [14]. Typical for RNA polymerase III transcribed genes, the EBERs rely on intragenic promoter elements, downstream of their transcriptional start sites and contain promoter regions Box A (+10) and Box B (+50). The intragenic

promoter elements are required for normal EBER expression. Mutation of Box A reduces EBER expression by approximately 20% and mutation of Box B by 97% compared to wildtype EBER expression [200]. Interestingly, the intragenic promoter regions are not sufficient to achieve the high EBER expression levels observed in latently infected cells and EBERs require three upstream promoter elements typical for RNA polymerase II transcripts. These are an Sp1 motif, an ATF motif and a TGTA motif in the EBER1 upstream promoter or a classical TATA box motif in the EBER2 upstream promoter, respectively. The combination of RNA polymerase II and III promoter elements is very unusual and only a few cellular RNA polymerase III genes share this promoter arrangement with the EBERs, as for example 7SL and vault ncRNAs [201, 202]. Other cellular RNA polymerase III genes either rely exclusively on intragenic promoter regions (tRNA) or on upstream promoter elements (U6 and 7SK ncRNAs) [203, 204]. Both EBERs terminate in a poly-thymidine region, the common termination signal for RNA polymerase III. EBER1 contains two additional upstream enhancer boxes [205]. The promoter regions of EBER1 and EBER2 are outlined in figure 1.5, which also illustrates the high similarity of the regulatory arrangements of EBER1 and EBER2.

The special promoter arrangement might contribute to the substantial expression of EBERs by RNA polymerase III and it is not known if EBER1 and EBER2 are additionally transcribed by RNA polymerase II *in vivo*. EBERs depend on the host cell for their expression and the high abundance of the EBERs is the result of cellular transcription factors, induced by EBV, which activate EBER expression. Activating transcription factor 2 (ATF2) is phosphorylated in EBV-infected cells to interact with the EBER promoter. Additionally, all five subunits of the Transcription Factor for polymerase III C (TFIIIC) and BDP1, a subunit of the polymerase III-specific transcription factor TFIIIB are overexpressed in EBV-infected cells and contribute to an increase in EBER expression [206]. Moreover, these transcription factors strengthen the expression of RNA polymerase III transcripts in general, which results in enhanced tRNA and 5S rRNA levels in EBV-infected cells. Increased levels of ncRNAs essential for translation could be beneficial for the increased protein synthesis required for the

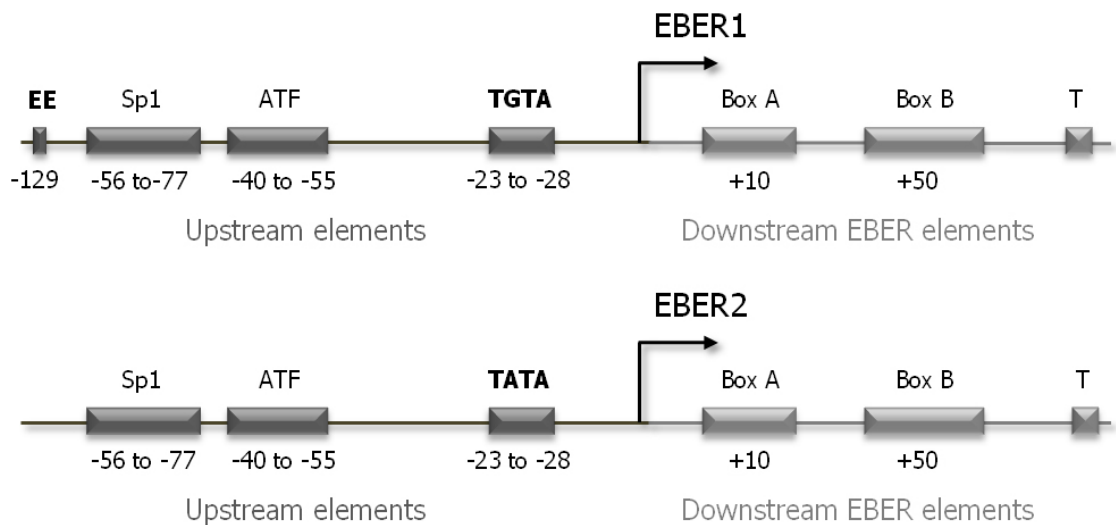


Figure 1.5: Promoter structure of EBER1 and EBER2. The EBER1 and EBER2 genes contain intragenic polymerase III control regions Box A and Box B and upstream RNA polymerase II Sp1, ATF and TGTA or TATA motifs. Typical for RNA polymerase III transcripts, EBER1 and EBER2 terminate with a poly-thymidine region (T). The promoter regions of EBER1 and EBER2 are almost identical but EBER1 comprises a TGTA motif in contrast to a TATA box in EBER2 and contains two upstream enhancer boxes (EE).

strong proliferation of B cell blasts and LCLs. Upregulation of TFIIC and ATF2 is mediated by EBNA1 which also increases c-MYC [207]. The proto-oncogene is additionally induced by EBNA2 [53] and enhances EBER1 expression by binding to the two E boxes (CAGATG and CACGTG) in the EBER1 promoter [205]. c-MYC can also activate expression of many RNA polymerase III transcripts, with the exception of EBER2, through its interaction with TFIIB [208]. Additionally, CpG-methylation of the EBER promoters and chromatin structure could regulate EBER transcription [209, 210]. The region around oriP and the EBERs was found to be exceptionally sensitive to micrococcal nuclease which suggests an unusual nucleosomal structure [209].

EBER2 is transcribed at higher rates than EBER1, with a ratio of EBER2 to EBER1 transcription of 1-5:1 in Daudi cells [211]. However, the level of EBER1 in the cells is at least ten fold greater than that of EBER2. This is due to the much shorter half-life of EBER2 of only 45 minutes compared to a half-life of more than 9 h for EBER1 [211]. The reasons for the difference in the stability of the two RNAs has not been explored but implies independent roles for EBER1 and EBER2 during infection.

Expression of both EBERs is delayed compared to all other latency genes and does not begin until 36 h post-infection. The characteristic, high EBER expression levels are reached around 70 h post-infection ^[47]. EBERs are expressed consistently throughout latent infection but EBER expression is downregulated early after induction of the lytic cycle ^[212]. Interestingly, the transcription of EBER1 is reduced to a larger extent than the transcription of EBER2, again indicating diverse roles for the two RNAs. A reduction of the EBERs was also demonstrated in mutant viruses defective for a complete, productive lytic replication, but expressing BZLF1 and BRLF1. This suggests that EBER expression is downregulated by cellular transcription factors, potentially different ones for EBER1 and EBER2. Despite a clear reduction in EBER transcription, EBER levels in the cells remain constant for up to 72 hours after induction of the lytic cycle. It is so far not clear what mechanism maintains the EBERs stable over such a long time nor what the function of the EBERs during these early stages of lytic replication might be.

Only very few gene products beside EBERs are expressed in latent infection ^[71, 78]. Together with the high evolutionary conservation of EBERs and the fact that EBERs are highly expressed in persistence but not during lytic cycle, this suggests a fundamentally important role of the EBERs in EBV latent infection and a possible function of the ncRNAs in sustaining latency.

EBERs contain a sequence also found in the splicing site of the plasma cell specific transcription factor X-box binding protein 1 (XBP1, figure 1.6) ^[213]. In plasma cell lines, XBP1 binds and transactivates promoter Zp of BZLF1, the master regulator of lytic cycle activation in EBV ^[214]. XBP1 exists in an unspliced inactivated form and is converted into an active transcription factor by cytoplasmic splicing as a part of the unfolded protein response ^[215]. EBERs might theoretically be able to prevent induction of the lytic cycle by competing for splicing enzymes with XBP1. Other viruses, such as Hepatitis C virus, are also known to prevent IRE1 driven splicing of XBP1 ^[216].

EBERs are similar in size and secondary structure to two small adenovirus-associated RNAs, VAI and VAII which are essential for the efficient initiation of translation. Similar to EBERs, VA RNAs are transcribed by RNA polymerase III ^[217]. EBERs are

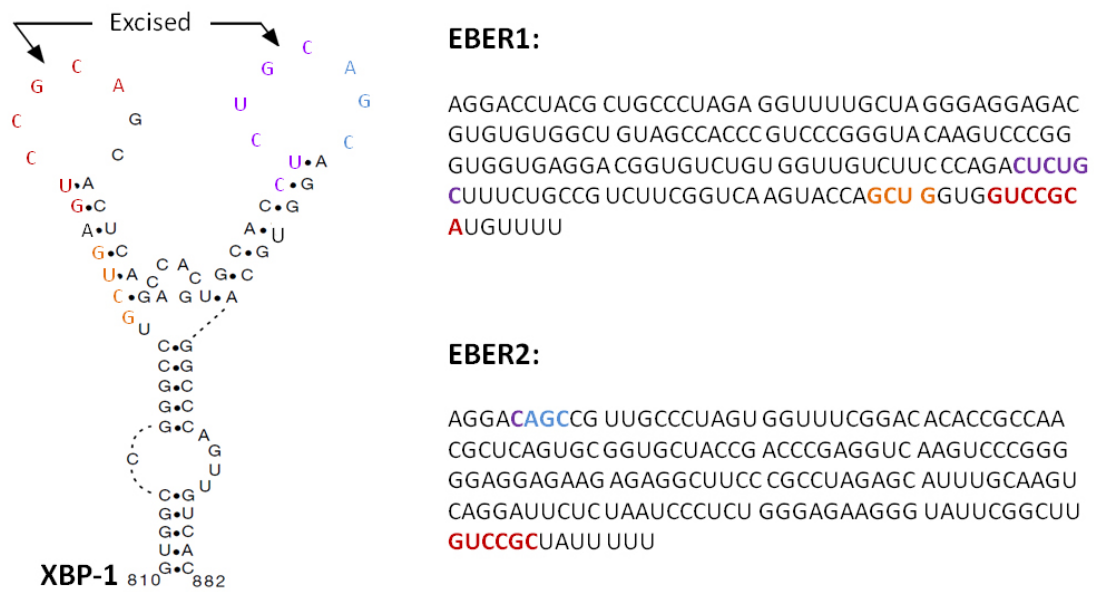


Figure 1.6: Comparison of XBP1 RNA and EBER RNA sequences.

XBP1 splice site sequences (left) are present in EBER1 and EBER2 sequences (right) as indicated in colour. Adapted from Calfon *et al* [213].

able to partially substitute for VA function and to complement VA-deletion [218, 219]. This strongly suggests a similar role of the RNAs during infection. It was recently proposed to use this functional complementation to attack tumour cells by selectively replicating VAI-deleted adenovirus in EBV-positive tumour cells [220].

Further viral RNA polymerase III transcripts similar to EBERs are Herpesvirus papio (HVP) RNA 1 and 2 [221]. Herpesvirus papio can transform marmoset lymphocytes into LCLs. EBER1 has a sequence similarity of 83% with HVP RNA 1, except for a 22 nucleotides insert in the HVP RNA and EBER2 is 65% identical to HVP RNA 2. Additionally, the upstream promoter regions between EBERs and HVP RNAs are highly conserved (60% and 50%) and the predicted secondary structures are remarkably similar.

The exact role of the EBERs during EBV infection has so far remained unclear and investigations on similar functional RNAs in viral infections have only revealed very limited information. However, several EBER interaction partners have been described and the functions of these proteins in non-infected cells or antiviral defense represent an effective starting point for the investigations of EBER1 and EBER2 during EBV infection and cell transformation.

1.2.4 Cellular EBER interaction partners

EBER1 and EBER2 have been reported to form complexes with cellular La autoantigen (section 1.2.4.2) and the dsRNA-binding proteins interferon inducible serine/threonine protein kinase R (PKR, section 1.2.4.3), retinoic acid-inducible gene I (RIG-I, section 1.2.4.4) and 2'-5'-oligo-adenylate synthetase (2'-5'-OAS, section 1.2.4.5). EBER1 alone interacts directly with ribosomal protein L22 (section 1.2.4.1). The distinct interaction partner for EBER1 and EBER2 implies independent roles for the two RNAs once more. The interaction with cellular components could present a way for EBV to influence the host cell to support a successful infection by suppressing antiviral responses and employing existing cellular pathways in the viral life cycle. Only five EBER interaction partners have been discovered so far. It is plausible that further interaction partners exist for both EBER1 and EBER2. Identifying all EBER-binding partners would crucially advance our understanding of EBER function and complement our knowledge of cell responses to viral RNA and EBV specifically. Devising a strategy to reliably identify cellular factors that interact with EBER1 or EBER2 is one objective of the work presented here (section 1.3).

1.2.4.1 Ribosomal protein L22

EBER1 interacts strongly with ribosomal protein L22 (RPL22), a constituent of the large ribosomal subunit. RPL22 is also known as EBER-associated protein or heparin binding protein. RPL22 is a ubiquitously expressed, small 14.8 kDa (128 amino acids) highly hydrophilic protein with an acidic C-terminal end. No similarities between RPL22 and other RNA-binding proteins were described. RPL22 is strongly conserved during evolution and rat and human RPL22 are identical in amino acid sequence [222].

EBER1 interacts strongly with RPL22

RPL22 binds EBER1 directly in B cells and is associated with EBER2 in ribonucleoprotein complexes including La protein [223, 224]. However, EBER2 does not interact with RPL22 directly. The interaction of EBER1 with RPL22 is frequent and

EBER1 is capable of binding three RPL22 proteins simultaneously on binding sites in stem loops 1, 3 and 4 in a sequence-specific manner (figure 1.4) [225–227]. The general RNA binding motif of RPL22 on EBER1 was established by systematic evolution of ligands by exponential enrichment (SELEX) and was confirmed by electrophoretic mobility-shift assays using wt and mutated EBER1 in addition to non-binding RNAs containing EBER1 stem loop inserts [226]. The *in vitro* interactions of RPL22 with EBER1 were further validated in HEK 293 cells by UV cross-linking experiments [227].

As a consequence of EBER1-RP22 interaction, 95% of EBER1 is predicted to be bound by RPL22. In infected cells, approximately 30-50% of RPL22 is bound by EBER1 while the rest of the protein is embedded in ribosomes. EBER1 itself is not directly associated with ribosomes [224, 227].

Strikingly, RPL22 is delocalised from the nucleoli and the cytoplasm into the nucleoplasm in the presence of EBV [224] and EBER1 specifically [228]. RNA binding by RPL22 is achieved through an acidic amino acid cluster binding site ranging from amino acids 80 to 93 [229], which interacts with a conserved RNA stem loop sequence present in 28S and 18S ribosomal RNA and in EBER1. The RNA binding site is crucial for the assembly of RPL22 into the 60S ribosomal subunit. To be incorporated into the ribosome, RPL22 is assumed to be co-transported into nucleoli by binding to 28S RNA, whereas nuclear import of the protein is dependent on a classic nuclear localisation signal of four lysines at positions 13-16 [226]. Competition of EBER1 with 28S for binding to RPL22 might retain RPL22 in the nucleoplasm and inhibit co-transport into nucleoli, especially because binding of RPL22 to EBER1 occurs with higher affinity than binding to 28S RNA. RPL22 is present at normal levels in ribosomes in EBV-positive Raji cells [224] but more recent investigation shows a depletion of RPL22 from ribosomes upon EBV infection in Akata cells [228]. To clarify this contradiction, the incorporation of RPL22 into ribosomes in EBV-infected cells was reanalysed in this thesis (section 1.3).

Understanding the function of RPL22 in non-infected cells might reveal crucial hints for the role of EBER1 interaction with RPL22 during EBV infection. The role of RPL22

in uninfected cells remains to be established since RPL22 is not essential for translation [230, 231]. Nevertheless, RPL22 might play a regulatory role in ribosome assembly or protein synthesis, potentially dependent on cell type, developmental status or extracellular signals. The importance of RPL22 for the translation of individual proteins has not been analysed so far.

In general, the establishment of high-resolution structures of eukaryotic 80S ribosomes and the mapping of eukaryotic ribosomal proteins on translating ribosomes might advance our comprehension of the function of individual ribosomal proteins [232, 233]. Also, extrachromosomal functions of ribosomal proteins are relatively common [234] and RPL22 is involved in a variety of cellular processes which might be altered crucially during EBV infection.

RPL22 plays a role in p53-dependent T cell death in mice

Surprisingly, RPL22 is not essential for survival or overall development of RPL22 knockout mice, even though a germline disruption of many other ribosomal proteins is lethal. However, deletion of RPL22 leads to a selective induction of apoptosis in $\alpha\beta$ -T lymphocyte precursors at the β -selection checkpoint of T cell development, resulting in a drastic reduction of $\alpha\beta$ -T cells to only 1% of the level in wt control mice [231]. The death of $\alpha\beta$ -T cells is induced by increased p53 translation and deletion of p53 rescues $\alpha\beta$ -T cells from apoptosis in RPL22-deficient mice. Deletion of RPL22 leads to the induction of p53 specifically in $\alpha\beta$ -T cells, while $\gamma\delta$ -T cell precursors are spared and develop as normal [231]. It is remarkable that the ubiquitously expressed RPL22 mediates such a profound effect limited to a specific cell type and stage in development. Several p53 targets are activated in Δ RPL22 $\alpha\beta$ -T cells, including miR-34a, p21^{waf}, PUMA, BCL-associated X protein (Bax) and NADPH oxidase activator 1 (Noxa). In addition, the levels of pro-apoptotic Bim are augmented in Δ RPL22 $\alpha\beta$ -T cells. The main mediators of $\alpha\beta$ -T cell depletion in Δ RPL22 mice are Bim and PUMA, as the development of the $\alpha\beta$ -T cells is only completely rescued by elimination of both Bim and PUMA simultaneously [235].

These results clearly indicate a role of RPL22 in a p53-mediated developmental checkpoint in T lymphocytes. Whether RPL22 is also involved p53-mediated stress responses in EBV-infected cells was investigated in this thesis (section 1.3).

Interestingly, other ribosomal proteins including RPL5, RPL11, RPL23, RPL26, RPS7, RPS19 and RPS20 can induce cell cycle arrest or apoptosis via p53 signalling in conditions which inhibit ribosomal biogenesis and lead to the release of nucleolar proteins, such as serum starvation, low concentrations of actinomycin D or overexpression of a dominant-negative mutant of rRNA processing factor Bop1 [236–240]. Indeed, ribosomopathies emerge as a result of malfunctioning developmental processes through unscheduled activation of p53 [241].

Other functions of RPL22

RPL22 interacts directly with human telomerase RNA [242], the essential RNA component of the mammalian telomerase ribonucleoprotein which serves as internal template to synthesise telomeric repeat sequences on chromosome ends. Telomerase activity and telomere length are associated with both cellular senescence and cancer. Additionally, telomerase activity is increased in many human tumours and during immortalisation of cells in tissue culture [243]. It is not known if RPL22 affects telomerase activity in healthy cells or if telomerase activity can be modified by the interaction of EBER1 with the protein.

Furthermore, RPL22 is involved in transcriptional regulation in *Drosophila* by interacting with linker Histone H1. RPL22 and H1 colocalise on condensed chromatin and are excluded from active chromatin regions with high levels of histone H3K4 methylation and H4K8 acetylation. The association of RPL22 with chromatin is dependent on H1. Overexpression of H1 or RPL22 leads to a deregulation of a similar set of more than 1000 genes with approximately 70% of the genes being downregulated [244]. The molecular mechanism of RPL22-mediated gene regulation is still unclear. Overexpression of H1 represses histone modifications typical for active chromatin and could at least explain downregulation of its target genes. In contrast, an increase in

RPL22 does not significantly influence histone modifications, suggesting a different pathway for RPL22 to regulate transcription.

Recently, RPL22 was identified as an interaction partner of AKT1 protein kinase. The function of the association was not investigated and it is therefore not known if RPL22 is phosphorylated by the kinase or influences AKT1 activity ^[245].

EBV is not the only virus that interacts with RPL22. RPL22 can interact with Herpesvirus papio RNA 1 due to the previously described similarities in the sequences of EBER1 and HVP RNA 1 ^[225]. The consequences of the interaction have not been elucidated. RPL22 localises to nuclear structures in HSV-1-infected cells and interacts with infected cell proteins ICP4 and ICP22, thereby disrupting the interaction of ICP4 with its cognate promoter binding sites and inhibiting its function as transactivator or repressor ^[246, 247]. RPL22 also binds to the conserved 98 nt 3'X UTR of Hepatitis C virus and can stimulate internal ribosomal entry site (IRES)-mediated translation ^[248]. Additionally, RPL22 interacts with Equine Herpes virus 1 (EHV) immediately-early gene protein IE that activates transcription of viral early and late promoters. Similar to EBV infection, RPL22 is delocalised upon EHV infection, in this case from the nucleoli to the cytoplasm ^[249].

These various examples of interactions of viral proteins with RPL22 suggest a common mechanism of the viruses to influence their hosts or imply a general role of RPL22 in the cellular response to viral infections, perhaps different from its function as a ribosomal protein.

The function of the interaction of EBER1 with RPL22 and the consequences of the delocalisation of RPL22 are still largely unknown and are one of the main focuses of this thesis (section 1.3). One proposed function of the interaction is competition of RPL22 with PKR for binding to EBER1 to prevent inhibition of PKR activation by EBER1 ^[250]. This will be discussed further in section 1.2.4.3.

1.2.4.2 La

EBER1 and EBER2 are assembled into RNPs with La [192, 198, 251], a protein generally involved in biogenesis and maturation of polymerase III transcripts. La interacts with the 3' uridylate stretch of EBERs and nascent RNA polymerase III transcripts in general. The interaction of La with EBERs might stabilise the secondary structure of the EBERs and support the interaction with other binding partners. However, more active functions of La in physiological processes and viral infection are now being discovered and the interaction of EBERs with La might not be limited to structural roles.

Cap-independent viral translation using IRES [252, 253] and translational repression of HIV [254] are strongly influenced by La. La can also bind and sequester dsRNA from PKR and thereby reduce activation and autophosphorylation of PKR as well as phosphorylation of PKR target eukaryotic initiation factor 2 α (eIF2 α) [255–257]. Moreover, La is able to convert bound dsRNA to ssRNA and thereby reduces the amount of PKR activator. The helicase activity of La is not well understood. La further promotes the release of cleaved mRNA from the RISC complex by separating the siRNA-target mRNA duplex. This enhances turnover of the RISC complex and RNA interference [258].

The location of the interaction of La with EBERs may also be an issue, as EBERs were demonstrated to be retained in the nucleoplasm while La shuttles from the nucleus to the cytoplasm [198]. On the other hand, the described secretion of EBER1 in complex with La from LCLs indicates that EBER1-La complexes at least transmigrate through the cytoplasm (section 1.2.5) [259].

1.2.4.3 PKR

Interferon inducible serine/threonine kinase PKR, a key mediator of antiviral effects, can be bound by EBER1 and EBER2 [260, 261]. PKR undergoes conformational changes in response to binding of dsRNA, typically produced by viruses, which subsequently ends autoinhibition of the PKR kinase domain by its dsRNA-binding domain and allows homodimerisation and autophosphorylation of the protein [262, 263].

As a main antiviral effect, activated PKR phosphorylates its key substrate eIF2 α to inhibit translation initiation and promote apoptosis [264–266]. Moreover, PKR induces the expression of proinflammatory proteins, such as IFN- β , by activation of NF- κ B, interferon regulatory factor 3 (IRF3) and p38 mitogen-activated protein kinase (p38 MAPK) [267]. PKR is not only crucial for cellular antiviral responses but also regulates cell responses to a variety of physiological signals by blocking translation and performing pro- or possibly anti-apoptotic functions independent of translational arrest. There is a mechanism for the activation of PKR independent of dsRNA via protein activator of PKR (PACT) [268], which can be activated by bacterial products and cellular stress [269] and PKR has been implicated in p53 activity [270].

PKR is found both in the cytoplasm and the nucleoli. Cytoplasmic PKR localises frequently to the rough ER, similar to EBERs [271]. EBER1 and EBER2 bind to PKR with comparable affinity and a K_d of approximately 0.3 nM [261]. Remarkably, high EBER concentrations reduce PKR phosphorylation and activation *in vitro* and prevent PKR-mediated abrogation of protein translation [261, 272, 273] and IFN-induced apoptosis [274].

EBERs bind only the outer of the two PKR dsRNA binding domains, which is not sufficient to prevent autoinhibition of the PKR kinase domain and thus leaves PKR in an inactive state. Moreover, EBERs remain associated to PKR and obstruct binding of other dsRNA to PKR [275, 276]. Consequently, EBERs not only prevent activation of PKR-mediated antiviral effects and abrogation of translation by themselves but also by other dsRNA such as EBV RNA transcripts from the W major internal repeat expressed during latent infection [277]. Expression of EBER1 in EBV-negative cells can also reduce translational arrest in response to physiological stress [278] and overexpression of EBERs reduces PKR activation by polyriboinosinic polyribocytidylic acid (poly(I:C)) in NPC cells [101]. These data suggest that EBERs can prevent the PKR antiviral response and rescue their host cells from translational arrest caused by external stress signals. It is, however, interesting to note that EBER1 is able to partially protect cells from stress-induced translational arrest in PKR knockout cells [278]. Thus, EBER1 can

enhance protein synthesis or prevent inhibition of translation in an additional, PKR-independent manner. EBER1 counteracts PKR activation more efficiently than EBER2 and together with the higher abundance of EBER1 *in vivo* this could suggest EBER1 as the key EBER in this process [261].

PKR binds EBER1 on stem loop 4 [279] and thus directly competes with RPL22 for binding to EBER1 (figure 1.4) [250]. In fact, RPL22 interferes with the binding of EBER1 to PKR and thereby averts the inhibitory effects of EBER1 on PKR. The sequestration of EBER1 from PKR could be an antiviral function of RPL22, but considering the previously described functions of RPL22 (section 1.2.4.1), it seems unlikely that this is the only role of RPL22. Interestingly, RPL22 can serve as a substrate for PKR phosphorylation and EBER1 reduces interaction of RPL22 with PKR, which also contains three ribosome interaction sites [277, 280]. RPL22 is likely to prevent EBER1 from binding to PKR to allow the activation of antiviral responses but alternatively EBER1 could prevent RPL22 from binding to PKR to attenuate physiological roles of RPL22 and support EBV infection.

Many viruses have evolved strategies to counteract PKR as a consequence of its major role in antiviral responses and cell survival. Adenovirus VA1 prevents PKR-mediated translational arrest [261, 281] and the ability of EBERs to substitute for VA function in VA-deletion adenoviruses could be the result of a similar effect of VAs and EBERs on PKR [218, 219].

In general, the high sensitivity of cells to dsRNA makes the investigation of innate immune responses to RNA challenging, as synthesis of small RNAs by *in vitro* transcription can produce dsRNA which can potentially obscure the effect of the RNA of interest [282, 283].

1.2.4.4 RIG-I

Both EBERs can interact with retinoic acid-inducible gene I (RIG-I) [284]. RIG-I is a cytosolic protein that detects the 5'-triphosphate moiety of viral RNA [285, 286], which is also present in the EBERs. RIG-I activates NF- κ B and IRF3 to initiate the production of

type I IFNs and inflammatory cytokines. EBERs induce expression of the anti-inflammatory cytokine interleukin 10 (IL-10) at the transcriptional level in BL-derived cell lines [287]. IL-10 acts as an autocrine growth factor and supports the growth of BL cells. Induction of IL-10 depends on IRF3 signalling by RIG-I, but not on NF- κ B, as shown using expression of dominant-negative RIG-I and siRNA knockdown of IRF3 or RIG-I [288]. EBER-induced expression of IL-10 might be a crucial factor for the development of BL.

1.2.4.5 2'-5'-oligoadenylate synthetase

EBER1 and VAI activate the dsRNA-dependent 2'-5'-oligoadenylate synthetase (2'-5'-OAS), which is involved in antiviral effects of IFN [289]. EBER2 is at least able to bind 2'-5'-OAS. Induction and regulation of the resulting RNase L-mediated RNA degradation during EBV infection has not been studied in detail and might suppress the expression of antiviral genes or even viral genes to sustain latency. In general, RNase L can have proapoptotic effects and might act as a tumour suppressor in prostate cancer [290].

1.2.5 Possible roles for EBERs during EBV infection

1.2.5.1 EBERs are not essential for infection and B cell transformation *in vitro*

EBERs are not essential for primary infection, viral replication and B cell immortalisation in EBER1/EBER2 double deletion mutants in an EBV P3HR-1 background *in vitro* [64]. Wt P3HR-1 EBV is unable to transform B cells as the virus lacks EBNA2, which is essential for B cell immortalisation (section 1.1.4.3). The B95-8 sequence spanning the EBER locus to EBNA2 including EBERs or a deletion of the EBERs was introduced into P3HR-1 by homologous recombination to create a transformation-competent virus. Deletion of both EBERs from the B95-8 fragment did not alter the transformation of B lymphocytes into LCLs or subsequent proliferation rates of established LCLs. Furthermore, latent EBV protein expression was not altered by the absence of the EBERs and neither was the amount of sporadic, spontaneous lytic

cycle replication evaluated by measuring BHRF1 expression ^[64].

Subsequent studies using the Akata system confirmed that EBERs are nonessential for B cell transformation into LCLs, but identified a specific contribution of EBER2, but not EBER1, to the efficiency of B lymphocyte transformation and the growth potential of LCLs, especially at low cell density ^[65, 291]. The observed 50% transforming dose of EBER2 deletion EBV was amplified 100-fold compared to wt EBV.

Our laboratory recently revisited the role of EBER1 and EBER2 in the transformation of primary B cells using EBER1 or EBER2 deletion virus constructed in the B95-8 EBV strain. In agreement with Swaminathan *et al* ^[64] no significant effects of EBER deletion on transformation efficiency or growth of LCLs at different cell densities were detected ^[66]. Unidentified variations in the experimental procedures or the use of different EBV strains might explain the conflicting results. The B95-8 strain is the most frequently used prototype for type I EBV and harbours a high intrinsic transformation ability that could potentially mask transformation-enhancing roles of EBER2. B95-8 also lacks part of the miR-BART sequence which is present in the Akata strain. However, all miR-BARTs are present in P3HR-1 EBV ^[292] and effects of these miRNAs can therefore not explain the difference between the role of EBERs in B95-8 and Akata EBV in the establishment and growth of LCLs.

Nevertheless, it would certainly be extremely interesting to investigate whether EBERs regulate the expression of EBV miRNAs or perform cooperative functions with miRNAs during latent infection (section 3.2.11). On balance, results suggest that EBERs do not contribute to the immortalisation of primary B cells and the proliferation of LCLs *in vitro*. Of course, the data do not exclude possible crucial roles of EBERs for the establishment of B cell infection or malignant transformation *in vivo*.

1.2.5.2 EBERs potentially enhance oncogenesis

Comparison of EBV-positive and EBV-negative BL-derived Akata cell lines expressing latency I indicates a role for EBV in the malignant phenotype of the cells. EBV was reported to support growth of Akata cells in low serum and soft agar and enhance

tumorigenicity in nude mice [293]. Moreover, EBV was found to confer an increased resistance to apoptosis in BL cells in response to apoptotic stimuli such as cycloheximide or UV light by increasing the anti-apoptotic BCL-2 protein and decreasing c-MYC [294, 295]. A chromosomal translocation of c-MYC resulting in a constitutive expression of c-MYC is characteristically found in BL cells [88]. Enhanced expression of anti-apoptotic genes such as BCL-2 or repression of pro-apoptotic BIM is believed to counteract pro-apoptotic effects of c-MYC while maintaining the strong c-MYC driven cell proliferation. BCL-2 and BIM regulate mitochondrial outer membrane permeabilisation, cytochrome c release from mitochondria and activation of caspases. Due to its role in cell survival, BCL-2 is frequently deregulated in cancer [296]. Remarkably, expression of EBERs alone in EBV-negative Akata or BJAB cells restored the malignant phenotypes of BL cells in an EBER concentration-dependent manner. EBERs were found to enable growth in soft agar, support tumourigenicity in severe combined immune disorder (SCID) mice and confer resistance to apoptotic inducers such as cycloheximide, glucocorticoid and hypoxic stress [297]. Moreover, proliferation of cells under normal conditions and serum starvation was augmented by EBER expression in BJAB cells [298]. The role of EBER1 in tumorigenesis is further supported by the fact that transgenic mice expressing EBER1 in the lymphoid cells develop lymphoid hyperplasia and tumours of B cell origin more frequently than normal mice [299]. It is remarkable that expression of EBERs alone might mediate such dramatic oncogenic effects.

Transfection of cells with EBER vectors was reported to lead to an upregulation of BCL-2 oncoprotein in BL and NPC cells which can explain the advanced resistance to apoptosis [100, 101]. Expression of BCL-2 in EBV-negative cells increased resistance to apoptosis comparable to EBV-positive cells, but not tumorigenicity. This suggests that EBERs modify additional cellular signalling pathways which may result in enhanced tumorigenicity. In line with this idea, experiments by Ruf *et al* confirmed an improved tumourigenic potential of EBER-positive Akata cells in immunodeficient SCID mice in the absence of BCL-2 upregulation. As a consequence of the normal BCL-2 levels,

EBER expression could not improve cell proliferation and resistance to apoptosis in serum-deprived conditions in these cells [300]. The inconsistent upregulation of BCL-2 by EBERs could potentially be explained by different EBER expression levels achieved from the EBER vectors in the different studies or might be the result of inherent differences between EBV-negative and EBV-positive Akata clones employed. However, the many observations taken together clearly support the concept that EBERs mediate an increased resistance to apoptotic stimuli at least partly by increasing BCL-2 and additionally enhance tumorigenicity in an BCL-2-independent manner. The results further imply that the anti-apoptotic effects of EBERs could be highly dependent on cell type and stress stimuli.

The tumourigenic potential of the EBERs might also be dependent on the host cell type. NPC-derived NP69 cells expressing EBERs from plasmids were not able to grow in soft agar and are non-tumorigenic in nude mice, but EBERs still increased the growth rate of the NPC cells [101]. Further investigations in mouse epithelial cells observed severe changes in cell morphology as a result of expression of EBERs. This indicates a stronger transformation of the epithelial cells, even though EBERs did not alter growth rate or confining cell density [278].

EBERs were subsequently found to enhance IL-10 expression through RIG-I mediated signalling in Akata cells [287, 288]. IL-10 acts as an autocrine growth factor in BL cells. The importance of IL-10 in BL is reinforced by the fact that EBV-positive BL biopsies typically express IL-10. Moreover, the growth of Akata cells can be inhibited by anti-IL-10 antibodies and increased by addition of recombinant IL-10 under low serum conditions.

Consistent with these growth promoting effects of EBERs in BL-derived cells, EBERs induce cellular cytokines that act as autocrine growth factors in additional host cell types. Transfection of EBERs into the GC cell line NU-GC-3 enhances expression of IGF1, which promotes epithelial cell growth and is regularly increased in EBV-positive GC biopsies but not in EBV-negative tumours [104]. The induction of IGF1 as an autocrine

growth factor by EBERs was also observed in three NPC-derived cell lines [301].

Additionally, EBER-induced IL-9 was found to promote growth and tolerance to high cell density in T cells [302]. The physiological importance of this finding is emphasised by a consistent upregulation of IL-9 in 75% of nasal NK/T cell lymphoma biopsies.

Furthermore, EBV induces IL-6 which promotes cell growth and malignant transformation in LCLs as well as tumorigenesis in SCID mice transplanted with human peripheral blood leukocytes [303]. Additionally, IL-6 was shown to support the pathogenesis of PTLD [304]. IL-6 was found to be induced by EBER2 in LCLs and reduces the number of apoptotic cells in LCLs infected with EBV Akata strain [65].

Unfortunately, most of the above studies treated EBER1 and EBER2 as one single entity and the contribution of the individual EBERs to oncogenesis can not be attributed in detail. Exceptions are the induction of IL-6 and IL-10, which is mainly induced by EBER2 or EBER1, respectively [65, 287]. Nevertheless, the ability of EBERs to promote growth by inducing a variety of cytokines that act as autocrine growth factors in the corresponding cells is remarkable. An increase in cell proliferation combined with an enhanced tolerance to apoptotic signals could significantly contribute to the development of EBV-associated malignancies.

The mechanism for the induction of growth promoting genes by the EBERs is not clear and many additional autocrine growth factors are conceivably activated during infection. Transient expression of EBER1 results in increased expression of several co-transfected reporter proteins, independent of the specific gene [278]. This effect of EBER1 on overall gene expression was also observed in cells lacking PKR and is therefore unlikely to be the result of EBER-mediated reduction of inhibition of translation by PKR. However, other effect of EBERs on host cell translation cannot be ruled out. Alternatively, the effect of EBER1 or EBER2 on the basal transcription levels or the transcriptional regulation of specific host cell genes has not been investigated so far. Analysis of host cell gene expression depending on EBER1 or EBER2 could clarify autocrine growth factor induction and promotion of cell proliferation by the two RNAs and determine

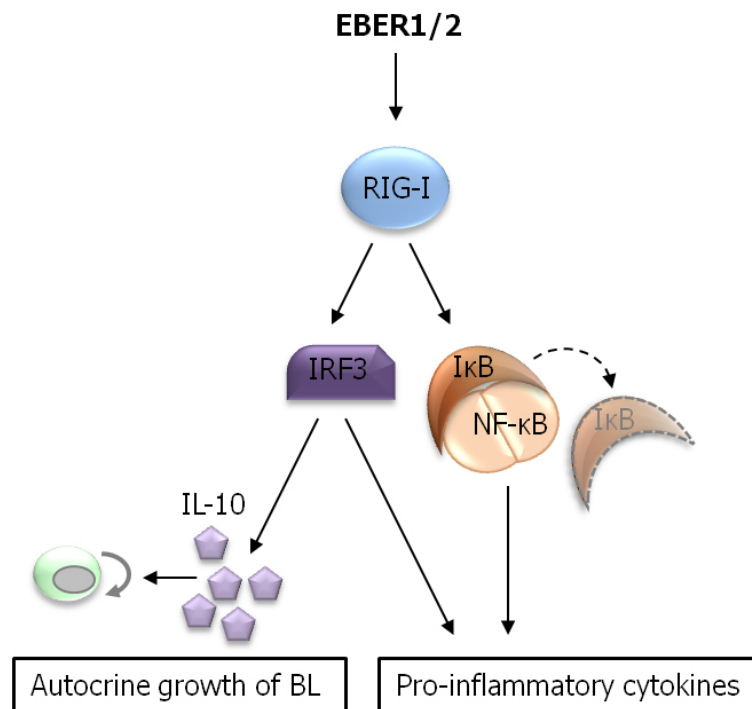


Figure 1.7: EBER interaction with RIG-I. RIG-I activation leads to expression of pro-inflammatory cytokines via IRF3 and NF- κ B signalling. EBERs induce IL-10, an autocrine growth factor for BL cells.

different functions for EBER1 and EBER2. Moreover, novel roles of the EBERs during EBV infection might be recognised and the individual roles for EBER1 or EBER2 in the prevention of apoptosis and tumorigenicity elucidated. The identification of EBER-regulated host cell genes in LCLs is a main focus of this thesis (section 1.3).

1.2.5.3 EBERs modulate innate immune signalling

As described above, RIG-I was reported to detect EBER1 and EBER2 in EBV-infected BL cells and to activate the transcription factors NF- κ B and IRF3, leading to the induction of IFN- α/β and inflammatory cytokines [284]. Signalling via IRF3 was found to initiate transcription of IL-10 and to enhance BL cell proliferation [287, 288] as illustrated in figure 1.7. Moreover, IL-10 can suppresses T-helper 1 and cytotoxic T cells.

The possible importance of RIG-I signalling for the induction of IGF1 in GC and NPC [104, 301] and IL-9 in T cells by EBER expression [302] has not been explored yet, but signalling via PKR is not involved in the enhanced expression of these autocrine growth

factors. However, the consequences of the simultaneous induction of IFN by RIG-I signalling could be prevented by the interaction of EBERs with PKR.

The prevention of PKR activation by EBERs might also contribute to oncogenesis. Injection of nude mice with murine NIH 3T3 cells containing mutated PKR with an inactive kinase domain causes large tumours rapidly within 1-2 weeks, whereas NIH 3T3 cells containing functional wt PKR do not cause tumours [305]. These results clearly demonstrate the strong tumorigenic potential of PKR inactivation. Inhibition of PKR by EBERs could therefore be directly responsible for the increased tumorigenicity observed for EBER-expressing cells in SCID mice [297, 300]. It seems plausible that the reduced responsiveness to apoptosis of cells expressing EBERs could contribute to tumourigenicity and that the effects of EBERs on apoptosis could be mediated by their interaction with PKR in addition to the discussed deregulation of BCL-2.

EBERs reduce apoptosis in response to cycloheximide, UV light, glucocorticoid, hypoxic stress and serum starvation in BL cells [297]. The involvement of PKR was not directly explored in this study. In any case, the diminished rate of cell death in reaction to poly(I:C) in NPC cells transfected with EBER expression plasmids could likely be the result of PKR deactivation [101]. Expression of EBERs in these cells resulted in a reduced phosphorylation and activation of p38 MAPK and Jun N-terminal kinase (JNK), both targets of PKR in response to poly(I:C) [306]. Furthermore, cleavage of apoptotic markers caspase-3 and poly(ADP)ribose polymerase (PARP) were lower in EBER-positive cells as determined by western blot [307].

Consistently, IFN- α -induced apoptosis is inhibited by the overexpression of EBERs or infection with wt EBV compared to infection with EBER deletion EBV or non-infected cells, both in BL and epithelial intestine 407 cells [274, 308]. EBER1 and EBER2 individually prevent autophosphorylation of PKR measured in *in vitro* kinase assays after immunoprecipitation of PKR from BL cells. At the same time, PKR phosphorylation is absolutely required to cause apoptosis as transfection of dominant-negative PKR results in resistance to apoptosis in response to IFN- α . Thus, averting the phosphorylation of PKR could be a direct way for EBERs to circumvent cell death caused by IFN- α .

A later study using phosphorylation-specific antibodies in western blots found no effect of EBERs on the phosphorylation status of PKR or its direct substrate eIF2 α in BL-cells, but still confirmed that EBERs mediate protection from IFN- α -induced apoptosis [309]. It is therefore possible that EBERs perform their anti-apoptotic function downstream of PKR or that EBERs alter phosphorylation of specific PKR targets by PKR instead of PKR autophosphorylation.

Moreover, EBERs confer resistance to FAS-mediated apoptosis in BJAB and epithelial cells, while treatment of normal cells with anti-FAS antibody leads to cell death [298, 308]. Expressing dominant-negative PKR in the same cells, it was shown that PKR phosphorylation is crucial to mediate apoptosis signalled by FAS and that EBERs prevent the cell from apoptosis by binding to PKR. FAS signalling is mediated through FAS associated death domain (FADD)-mediated activation of caspase 8 and caspase 9 [270]. The FAS receptor is a transmembrane protein member of the tumour necrosis factor (TNF) family, which PKR has been implicated to regulate during malignant transformation [310].

Preventing the activation of PKR activity would be a potent mechanism for EBV to inhibit antiviral and anti-apoptotic pathways in response to viral infection. Moreover, PKR is a key component of multiple stress signalling pathways activated by other stimuli that can result in the inhibition of translation and cell death. Expression of EBERs during latent infection could therefore prevent PKR-mediated responses of memory B cells to external stimuli and so secure the survival of the EBV host cell which provides a safe niche for EBV latency (figure 1.8).

EBER1 has recently been detected in the supernatant of EBV-infected BL Mutu cells, Akata cells and LCLs. A large fraction of extracellular EBER1 is in complex with La protein and might consequently be actively secreted together with the protein. Released EBER1 stimulates Toll-like receptor 3 (TLR3) signalling and induces an inflammatory IFN response via IRF3 and NF- κ B [259]. IFN- β production depends on the concentration of EBER1 and diminishes if TLR3 is silenced with siRNA or anti-TLR3 antibodies. TLR3

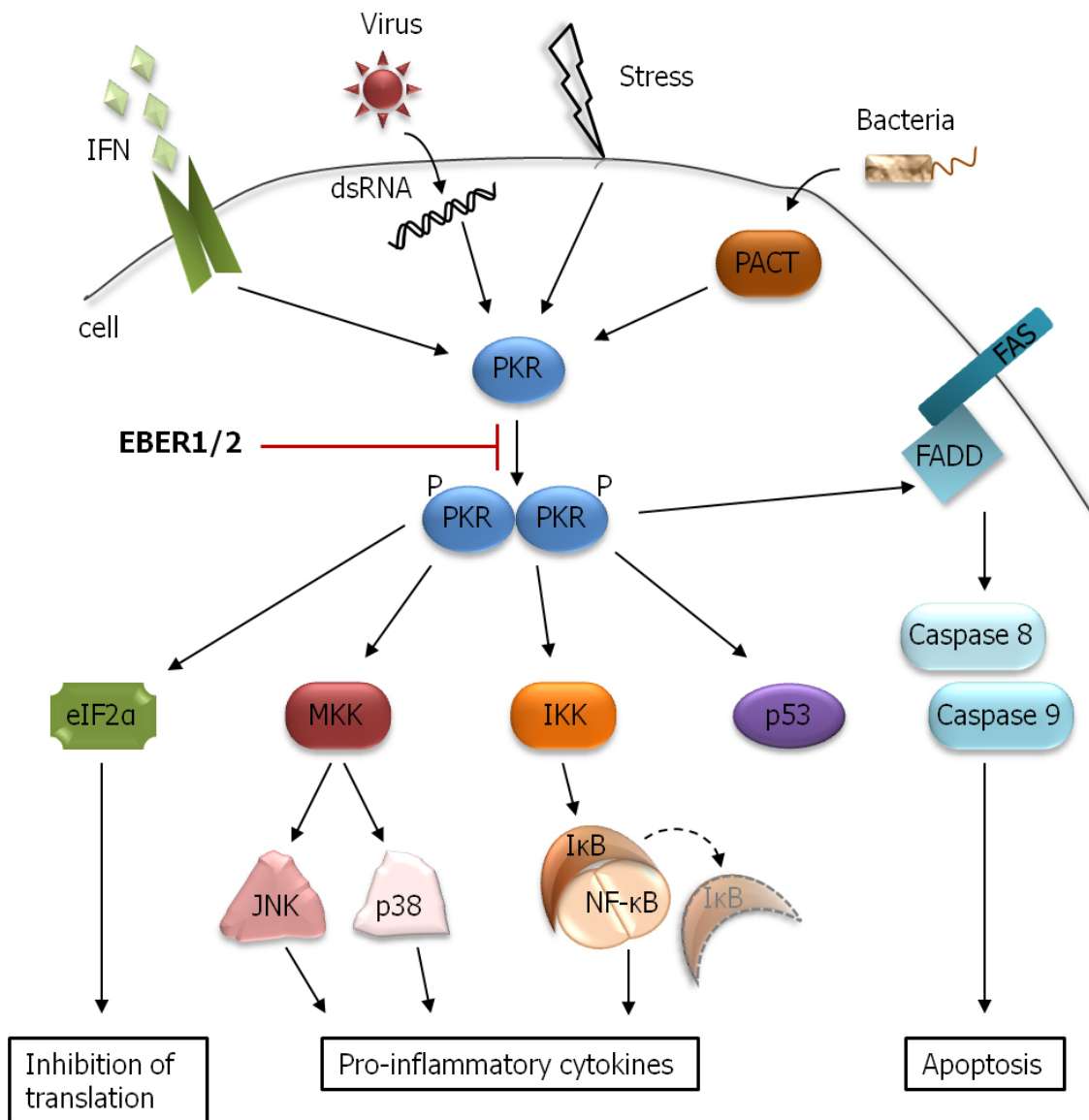


Figure 1.8: EBER interaction with PKR. PKR is a master mediator of cell stress responses. PKR dimerises and autophosphorylates in response to dsRNA binding, IFN, PACT, FAS signalling and other stress stimuli. Activated PKR phosphorylates eIF2 α and blocks the initiation step of translation. Additionally, PKR phosphorylates the IKK complex, which in turn phosphorylates I κ B. This releases transcription factor NF- κ B, which translocates to the nucleus to regulate the expression of antiviral genes. PKR further increases expression of pro-inflammatory cytokines by activating p38 MAPK and JNK signalling. PKR is moreover involved in the direct induction of apoptosis via the FAS receptor and FADD activated caspase cascade and potentially the p53 pathway. EBERs bind PKR to prevent activation of the kinase and subsequent antiviral cell responses.

signalling further increases expression of the maturation markers CD83 and CD86 on dendritic cells (DC). Activation of immune cells by extracellular EBER1 could potentially be responsible for pathological effects of active EBV infections.

During persistence, EBERs might be able not only to act within but also between cells. Generally, functional RNAs have many roles in the cells they originate from but can also have nonautonomous effects and spread from cell to cell ^[311]. In addition to secretion, RNA can be transferred actively and selectively between cells by cell to cell contact via membrane receptors such as SID-1 in *C.elegans* or membrane channels ^[312, 313]. Lately, BL cells were found to donate EBV viral miRNA to T cells in a contact dependent manner, potentially to modify T cell immune response ^[314]. Moreover, miRNA and mRNA can also be transported between cells in exosomes ^[315] or tumour shed microvesicles ^[316].

It is unlikely that EBERs released from latently infected memory B cells provoke activation of DC and other immune cells during persistent infection. So far, there are no reports on whether EBER1 is released from persistently infected cells *in vivo*. The possibility that EBERs regulate the environment of EBV-infected cells by modifying signalling and gene expression in bystander cells is however very intriguing and certainly merits further investigation (section 3.2.12).

1.3 Aims of the thesis

EBER RNAs are highly abundant during all forms of latent infection but their exact role in EBV infection has not yet been identified. This project focuses on functions of EBER RNAs during EBV infection within their host cells and the identification of possible mechanisms of action, specifically with respect to:

1. EBER-regulated host cell genes

- Which host cell genes are differentially regulated with EBER1 in LCLs?
- Which host cell genes are differentially regulated with EBER2 in LCLs?
- Is it possible to develop a system to identify novel EBER1 or EBER2 interaction partners?
- Are EBERs released from host cells?

2. Interaction of EBER1 with RPL22

- Does EBER1 delocalise RPL22 in EBV-infected cells?
- Does RPL22 delocalisation differ between cell lines?
- Does EBER1 alter host cell stress responses to chemotherapeutic agents?
- Does RPL22 alter host cell stress responses to chemotherapeutic agents?
- Is RPL22 depleted from ribosomes in EBV-infected cells?
- Is it possible to develop a system to study potential effects of EBER1 on host cell translation?

2 Materials & methods

2.1 Solutions and chemicals

All chemicals were supplied by BDH unless stated otherwise.

2.1.1 Solutions for cell culture protocols

- 0.8% (w/v) ammonium chloride: 8 g ammonium chloride (Sigma) was dissolved in tissue culture grade dH₂O (Sigma) in a final volume of 1 l.
- PBS: 137 mM NaCl, 2.7 mM KCl, 19 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.0.

2.1.2 Solutions for DNA protocols

- Agarose gel loading buffer: 10% (w/v) sucrose, 60 mM EDTA, 10 mM Tris-HCl pH 7.6, 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol FF, 60% (v/v) glycerol in dH₂O.
- Alkaline SDS: 1% (w/v) SDS, 0.2 M NaOH in dH₂O.
- Buffer EB: 10 mM Tris-HCl pH 8.5.
- Buffer N3 and P3: 3 M potassium acetate pH 5.5.
- Buffer P1: 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNaseA.
- Buffer P2: 200 mM NaOH, 1% (w/v) SDS.

- Buffer QBT: 750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100.
- Buffer QC: 1 M NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol.
- Buffer QF: 1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% (v/v) isopropanol.
- Buffer QN: 1.6 M NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol.
- 0.5 M EDTA pH 8.0: 186.1 g of EDTA was dissolved in 900 ml dH₂O, the pH adjusted with NaOH and dH₂O added to 1 l. The solution was autoclaved and stored at RT.
- KPB: 46 g KH₂PO₄ and 243 g K₂HPO₄ dissolved in 1 l dH₂O.
- Luria Bertani (LB) medium: 10 g bacto tryptone, 5 g bacto yeast extract and 10 g NaCl were dissolved in 1 l dH₂O. The pH was adjusted to 7.0 with NaOH and the solutions was autoclaved.
- Luria Bertani (LB) plates: 15 g of agar agar was dissolved in 1 l LB medium and autoclaved before pouring the plates.
- 5 M NaCl: 292.2 g of NaCl was dissolved in dH₂O in a final volume of 1 l and autoclaved.
- 3 M Sodium acetate pH 5.2: 408.1 g sodium acetate (3H₂O) was dissolved in 900 ml dH₂O and the pH was adjusted to 5.2 with acetic acid. The volume was adjusted to 1 l and autoclaved.
- STET: 8% (w/v) glucose, 5% (v/v) Triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 8.0 in dH₂O.
- Superbroth: 12 g bactotryptone, 24 g yeast extract and 4 ml glycerol were dissolved in 950 ml dH₂O and autoclaved. Subsequently, 50 ml sterile potassium phosphate buffer (KPB) was added.

- 10×TBE: 108 g Tris base, 55 g boric acid and 40 ml EDTA pH 8.0 were dissolved in 900 ml dH₂O, the volume adjusted to 1 l and the solution autoclaved.
- TE: 10 mM Tris-HCl pH 7.6 and 1 mM EDTA pH 8.0 in dH₂O.
- 1 M Tris-HCl, various pH values between 6.8 and 8.8: 121.1 g of Tris base was dissolved in 900 ml dH₂O, the pH adjusted with HCl and dH₂O added to 1 l. The solution was autoclaved and stored at RT.

2.1.3 Solutions for RNA protocols

- 50×Denhardt's: 1% (w/v) Ficoll (Sigma), 1% (w/v) Polyvinylpyrrolidone (Sigma) and 1% (w/v) bovine serum albumine (BSA, Fluka) were dissolved in Milli-Q dH₂O and filter sterilised.
- Diethylpyrocarbonate (DEPC) treated water: 0.1% (v/v) DEPC was added to Milli-Q dH₂O and incubated at RT o/n in a fume cabinet. The solution was autoclaved to inactivate the DEPC and stored at RT.
- Glyoxalation mix: 75.5% (v/v) DMSO, 15 mM sodium phosphate pH 7.0 and 21.5% (v/v) glyoxal. DMSO and glyoxal were de-ionised before use on Duolite MB6113 mixed resin and aliquots stored at -20°C.
- HGT agarose gel: 1.4 g RNase-free HGT agarose was melted in 100 ml of 10 mM sodium phosphate pH 7.0.
- RLN lysis buffer: 50 mM Tris-HCl pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40, 100 U/ml RNase inhibitor, 1 mM DTT.
- RNA running buffer: 10 mM sodium phosphate pH 7.0.
- RP labeling buffer: 200 μM each of dATP, dGTP and dTTP in 10 mM Tris-HCl pH 7.5, 10 mM β-mercaptoethanol and 10 mM MgCl₂.
- 20×SSC: 176.4 g C₆H₅Na₃O₇·2H₂O and 350.6 g NaCl were dissolved in 2 l dH₂O.

2.1.4 Solutions for protein protocols

- AB1: 200 mM KCl, 1 mM EDTA, 20 mM HEPES pH 7.9.
- AB2: 20 mM KCl, 1 mM EDTA, 20 mM HEPES pH 7.9.
- ABE: 20 mM KCl, 1 mM EDTA, 10 mM maltose, 20 mM HEPES pH 7.9, stored at 4°C.
- Coomassie Blue staining solution: 40% (v/v) methanol, 10% (v/v) acetic acid, 1 g/l R-250, stored at RT.
- Coomassie destaining solution: 40% (v/v) methanol, 10% (v/v) acetic acid, stored at RT.
- Dignam buffer: 20 mM HEPES pH 7.9, 100 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA pH 8, 20% (v/v) glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF, Sigma) in dH₂O.
- HB1: 1 mM EDTA, 20 mM HEPES pH 7.9.
- HB2: 1 M KCl, 1 mM EDTA, 20 mM HEPES pH 7.9.
- Isopropylthio- β -D-galactoside (IPTG): 0.238 g IPTG was dissolved in 10 ml dH₂O and sterilised by passing it through a 0.22 μ m Nalgene filter. IPTG was prepared fresh prior to use.
- Jenkins lysis buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 1 mM DTT, 1 mM NaF, 1 $\times$ Roche complete protease inhibitor cocktail (Roche) and 0.1 mM PMSF (Sigma).
- Laemmli buffer: 100 mM Tris-HCl pH 6.8, 0.02% (w/v) bromophenol blue, 200 mM DTT, 4% (w/v) SDS, 20% (v/v) glycerol.
- PBS-T: 0.1% (v/v) Tween-20 in PBS.

- Ribosome fractionation buffer: 140 mM KCl, 5 mM MgCl₂, 0.2 mM DTT, 20 mM Tris-HCl pH 8.0.
- Ribosome fractionation lysis buffer: 140 mM KCl, 1 mM MgCl₂, 0.2 mM DTT, 20 mM Tris-HCl pH 8.0, 1% (v/v) Triton X-100 and 40 U/ml RNasin (Promega).
- RIPA lysis buffer: 0.15 M NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris-HCl pH 8.0, 1 mM PMSF (Sigma) and 1×Roche complete protease inhibitor cocktail (Roche).
- 10% (w/v) SDS: 100 g SDS salt was dissolved in 1 l dH₂O by stirring at 50°C. The solution was then filtered and stored at RT.
- SDS-gels: Resolving gel: 10-20% (v/v) acrylamide/bis-acrylamide 29:1 (depending on the size of the protein of interest), 375 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS. Stacking gel: 5% (v/v) acrylamide/bis-acrylamide 29:1, 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS. 10 µl NNN'N'-tetramethylethylenediamine (TEMED) and 50 µl 10% (w/v) ammonium persulphate (APS) per 10 ml gel volume was used to polymerise the gels.
- 2×SDS sample buffer: 120 mM Tris-HCl pH 6.8, 0.01% (w/v) bromophenol blue, 2% (v/v) β-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol.
- 10-40% (w/v) sucrose gradients: 10, 15, 20, 25, 30, 35 and 40% filtered sucrose solutions in ribosome fractionation buffer were carefully layered and stored at -80°C. Frozen gradients were moved to 4°C for equilibration o/n before use.
- Western blot blocking solution for enhanced chemiluminescence protocol: 5% (v/v) skimmed milk powder (Marvel) or 5% (v/v) BSA (Sigma) in PBS-T, stored at 4°C.
- Western blot blocking solution for Odyssey Infrared Imaging System: 5% (v/v) skimmed milk powder (Marvel) in PBS, stored at 4°C.
- Western blot 10×SDS running buffer: 400 mM glycine, 50 mM Tris-HCl, 0.1% (w/v) SDS.

- Western blot stripping buffer: 2% (w/v) SDS, 62.5 mM Tris-HCl pH 6.8 in dH₂O, stored at RT. 100 mM β -mercaptoethanol was added immediately before use.
- Western blot transfer buffer: 200 mM glycine, 25 mM Tris base, 0.05% (w/v) SDS, 25% (v/v) ethanol, cooled to 4°C before use.

2.2 Cell culture protocols

2.2.1 Cell lines and cell maintenance

The cell lines used in this study and corresponding culture media are listed in table 2.1. All cells were grown in culture media (Gibco) + 2 mM L-Glutamate supplemented with 10% (v/v) heat inactivated (56°C, 20 min) foetal bovine serum (FBS) from Biosera in the presence of 200 U/ml penicillin and 200 U/ml streptomycin (pen/strep, Gibco) with the appropriate antibiotic selection and maintained at 37°C in a humidified incubator with 10% CO₂.

All non-adherent cells grown in suspension were fed 2-3 times per week by dilution in an equal volume of pre-warmed fresh medium.

Adherent cells were split 2-3 times per week by washing with warm PBS and trypsinisation (0.05% trypsin, Gibco) for 5 min at 37°C. Detached cells were washed in complete culture medium and a fraction of the cells was resuspended in fresh pre-warmed medium for further cultivation.

2.2.2 Viable cell counts

Cells were diluted 1:2 or 1:5 in Trypan Blue staining solution (Sigma) and the number of live cells was determined by counting unstained cells in a Neubauer counting chamber under a light microscope (Olympus CK2).

Table 2.1: Cell lines used in this study. pen/strep: 200 U/ml penicillin and 200 U/ml streptomycin (Gibco), glu: 2 mM L-Glutamate. G418: Gentamycin (Calbiochem), puro: puromycin (Sigma), hyg: hygromycin (Roche), RPMI: Roswell Park Memorial Institute 1640 (Gibco), DMEM: Dulbecco's Modified Eagle's Medium (Gibco).

Cells	Origin	Characteristics	Medium and selection	Reference
AGS	Gastric carcinoma	EBV-	Ham's F-12, pen/strep, glu	[317]
AGS/BX1	Gastric carcinoma	EBV+	Ham's F-12, pen/strep, glu, G418 (350 μ g/ml)	[18]
Akata 2003	Single cell clone of the Burkitt's lymphoma-derived Akata cell line	EBV+	RPMI, pen/strep, glu	[43, 88]
Akata 31	Single cell Akata clone, lost EBV genome	EBV-	RPMI, pen/strep, glu	[293, 318]
B95-8 LCL	B cells transformed with B95-8 strain of EBV	EBV+	RPMI, pen/strep, glu	[319]
BJAB	Burkitt's lymphoma	EBV-	RPMI, pen/strep, glu	[320]
HEK 293	Embryonic human kidney	EBV-	DMEM, pen/strep, glu	[321]
HEK 293 BAC	Embryonic human kidney	BAC EBV+, +/-EBER	DMEM, pen/strep, glu, hygromycin (100 μ g/ml)	This study, [66]
Hone-1	Nasopharyngeal carcinoma	EBV-	RPMI, pen/strep, glu	[322]
Hone-1 EBV+	Nasopharyngeal carcinoma	EBV+	RPMI, pen/strep, glu, G418 (500 μ g/ml)	[322]
IB4	EBV transformed umbilical cord B cells	EBV+	RPMI, pen/strep, glu	[323]
LCL	B cells transformed with EBV BAC	EBV+, +/-EBER	RPMI, pen/strep, glu	This study
MEF wt	Mouse embryonic fibroblasts	EBV-	DMEM, pen/strep, glu	[231, 324]
MEF Δ RPL22	Mouse embryonic fibroblasts	EBV-, RPL22-	DMEM, pen/strep, glu	[231, 324]
Raji	Burkitt's lymphoma	EBV+	RPMI, pen/strep, glu	[325]
U-937	Histiocytic lymphoma	EBV-	RPMI, pen/strep, glu	[326]

2.2.3 Cell freezing

Adherent cells were washed, trypsinised and resuspended in complete medium, then centrifuged at $390\times g$, 5 min, 4°C to collect the cells. Non-adherent cells were directly centrifuged using the same conditions. The pellet was resuspended in 1 ml 10% (v/v) DMSO in FBS for all B cell-derived cell lines or 10% (v/v) DMSO in 30% (v/v) FBS in DMEM for adherent cell lines. The cells were frozen slowly in cryovials in a Nalgene Cryo $1^{\circ}\text{C}/\text{min}$ freezing container o/n at -80°C . The cryovials were subsequently transferred into liquid nitrogen for long term storage.

2.2.4 Cell thawing

Cells were thawed rapidly at 37°C and diluted in complete medium without selective antibiotics. Cells were cultivated at 37°C and the required antibiotic selection was added after 24-48 h.

2.2.5 Cell harvesting

The desired number of cells in suspension was pelleted at $390\times g$ for 5 min at 4°C and washed twice in ice-cold PBS by centrifugation.

75-95% confluent adherent cells were washed in ice-cold PBS in the culture flask and detached into PBS with a cell scraper (Greiner) to be washed in PBS twice and pelleted ($390\times g$, 5 min, 4°C). Cell pellets were snap-frozen on dry-ice after aspiration of the supernatant and stored at -80°C .

2.2.6 Cell transfection

2.2.6.1 Lipofectamine 2000 transfection

The cells were transfected with sterile DNA (section 2.3.7) by lipofection with Lipofectamine 2000 (Invitrogen). The day before transfection, cells were split into 2 ml medium without antibiotics in 6-well plates to be 60-80% confluent at the time of

transfection (10^5 cells/500 μ l or 1 confluent 10 cm dish in 14 6-well plate wells). For one transfection, 10 μ l Lipofectamine 2000 was diluted in 250 μ l medium without serum and incubated for 5 min at RT. 4 μ g DNA was diluted in 250 μ l medium without serum before being added to the Lipofectamine solution. The mixture was incubated 20 min at RT and then added to the cells for 24 h. Cells were transfected in a 6-well plate format to establish stable cell lines and for transient expression in immunofluorescence microscopy experiments (section 2.6.1). 10 cm dishes were used for transient transfection for immunoprecipitation (section 2.5.7). The amounts of DNA and Lipofectamine used were multiplied by 6 to scale up from 6-well plates to 10 cm dishes.

2.2.6.2 Neon transfection of B cells

On the day before transfection, cells were diluted 1:2 in fresh tissue culture medium. For transfection, 2×10^6 cells per transfection were harvested ($390 \times g$, 5 min, $4^\circ C$), washed in Mg^{2+} Ca^{2+} -free DPBS (Invitrogen) and resuspended in 100 μ l Neon R solution (Invitrogen). The cell suspension was added to sterile plasmid DNA (generally 1, 4 or 10 μ g/transfection) in Eppendorf tubes. 100 μ l of plasmid-cell suspension was transferred to a Neon electroporation pipette. 3 ml Neon E2 solution was added to the electroporation tube in the electroporation unit and the Neon electroporation pipette inserted in the electroporation tube. LCLs were electroporated using condition 16 (pulse voltage: 1400 V, pulse width: 20 ms, pulse number: 2). For the expression of RPL22, various conditions were tested by performing the preset optimisation protocol. A pulse voltage of 1600 V with a pulse width of 10 ms and a pulse number of 3 was used for expression of MS2-EBER constructs. Cells were added to 3 ml pre-warmed supplemented medium without antibiotics immediately after transfection and resuspended by pipetting gently.

2.2.6.3 Amaxa nucleofection of U-937 cells

U-937 cells were diluted to 1×10^5 cells/ml 2 days prior to nucleofection. 8×10^6 cells/transfection were collected at $90 \times g$ and the supernatant removed completely before

resuspension of the cell pellet in 100 μ l room temperature Nucleofector solution C (Lonza). The cell suspension was combined with 4 μ g sterile DNA (section 2.3.7) and transferred to an Amaxa Nucleofector cuvette. U-937 cells were nucleofected using programme W-01. 500 μ l of pre-warmed medium was added immediately after transfection and the cells transferred to 12-well plates containing 1 ml supplemented medium/well for incubation at 37°C o/n.

2.2.7 Creation of stable cloned cell lines

2.2.7.1 Creation of stable cloned HEK 293 cell lines

60-70% confluent HEK 293 cells were transfected by lipofection (section 2.2.6) with the desired vector (table 2.5) in duplicate. Fresh medium without antibiotics was added to the cells 8 h after transfection and incubated o/n. The cells were split the next day into fresh non-selective medium at a ratio of 1:4, 1:8 and 1:16. Selective medium was added to the cells after further incubation o/n. The cells were monitored for 2-3 weeks to follow the selection process, changing the culture medium every 2-3 d. When necessary, confluent cells were split into fresh medium at a ratio of 1:4 and 1:8. Non-transfected cells under selection were used as negative control. After elimination of non-transfected cells, single cell colonies were picked from the culture dish with a Gilson P 200 into 96-well plates under a light microscope (Olympus CK2). BAC-transfected single cell clones were subsequently grown out to a larger scale and tested by PFGE (section 2.3.5), northern blot (section 2.4.5) and for virus production (section 2.2.9).

2.2.7.2 Creation of stable cloned B cell lines

LCLs and IB4s were transfected by Neon electroporation with the desired vector (table 2.5) in duplicate or triplicate as described in section 2.2.6. Selective medium was added to the cells after incubation o/n. The cells were monitored for 3-4 weeks to follow the selection process, slowly increasing the culture medium every 2-3 d if cell outgrowth was visible. Non-transfected cells under selection were used as negative control.

2.2.8 Immortalisation of mouse embryonic fibroblasts

Immortalised mouse embryonic fibroblasts (MEF) were selected in accordance with the 3T3 protocol. Every 3 days, cells were washed in warm PBS, trypsinised for 5 min at 37°C, resuspended in DMEM + 10% (v/v) FBS, counted in a Neubauer counting chamber and pelleted by centrifugation for 5 min at 390×g. 3.8×10^5 cells were resuspended in 10 ml fresh DMEM + 10% (v/v) FBS + 200 U/ml penicillin and 200 U/ml streptomycin (Gibco) in 25 cm² flasks (Greiner).

2.2.9 EBV virus production

For testing virus production, confluent HEK 293 cells containing EBV BACs were split into 2 ml fresh medium in 6-well plates and grown o/n at 37°C to ~70% confluence. The cells were transfected by lipofection with Lipofectamine 2000 (section 2.2.6) with 0.5 µg pBZLF1 (pCMV-EB1) and 0.5 µg pBALF-4 (table 2.5) in duplicate to induce the lytic cycle and virus production. The cells were incubated at 37°C for 4 days, after which the supernatant (containing virus) was passed through a 0.45 µm Nalgene syringe filter using a screw cap syringe. Filtered virus preparation was stored at 4°C.

2.2.10 Raji infection for virus quantification

Raji cells were split the day before infection at a ratio of 1:1 in fresh culture medium. A 10-fold serial dilution of each virus preparation (section 2.2.9) was prepared in 1 ml of supplemented RPMI medium in a 24-well plate. 10^5 Raji cells in 0.5 ml RPMI medium were added to each well containing virus. After incubation for 48 h, 0.5 ml RPMI containing 20 nM phorbol myristate acetate (PMA, Sigma) and 5 mM n-Butyrate (Sigma) was added to each well and incubated o/n. PMA activates protein kinase C and helps to induce EBV reactivation while n-Butyrate causes histone deacetylation and consequently leads to higher viral gene expression and GFP expression levels. Raji cells were resuspended as single cells by pipetting and were then incubated for 1 h at 37°C to settle down in the plate. The number of infected Raji cells was analysed by

quantification of GFP positive cells by fluorescence microscopy (Olympus IX 70).

2.2.11 B cell purification

B cells were purified from buffy coat residues (mixed-donors) by negative selection using the RosetteSep procedure (Stemcell technologies). 1250 μ l of RosetteSep Human B cell enrichment cocktail was added to 25 ml of blood and incubated at RT for 30 min. 25 ml of Mg^{2+} Ca^{2+} -free PBS (Invitrogen) supplemented with 2% (v/v) filtered FBS (0.22 μ m Nalgene filter) was added and the solution carefully layered on FicollPlaquePlus (Stemcell technologies) density medium at a ratio of 1:1 in 50 ml Falcon tubes. The solutions were centrifuged ($1200\times g$, 20 min, RT) with the brake off. Enriched B lymphocytes were recovered from the plasma interface and washed twice in 1 volume of Mg^{2+} Ca^{2+} -free PBS supplemented with 2% (v/v) filtered FBS ($311\times g$, 15 min, RT). Residual erythrocytes were lysed in 10 ml 0.8% (w/v) ammonium chloride for 10 min at RT. Then, 40 ml Mg^{2+} Ca^{2+} -free PBS supplemented with 2% (v/v) filtered FBS was added to restore the isotonic balance and the cells pelleted ($311\times g$, 10 min, RT). Lysis of erythrocytes was repeated once if necessary. Finally, the enriched cells were washed twice in Mg^{2+} Ca^{2+} -free PBS ($311\times g$, 10 min, RT) and resuspended in 5 ml RPMI supplemented with 20% (v/v) filtered FBS and pen/strep. B cells were plated at 1×10^6 cells/ml in 24-well plates at $37^{\circ}C$ o/n prior to infection.

2.2.12 B cell infection and establishment of LCLs

To establish LCLs, 1×10^6 primary B cells were infected with 5000, 2500 or 1250 GRU (section 2.2.10) of EBV BAC recombinant virus in 1 ml medium in 24-well plates. Infected cells were grown in RPMI supplemented with 20% (v/v) FBS and pen/strep. 200 μ l of medium was replaced every second day. The culture medium volume was increased slowly once LCLs started to grow out (cluster formation). Non-infected B cells were cultured in parallel as a negative control. After establishment of LCLs, FBS was reduced to 10% (v/v) and the cells put under hygromycin selection (150 μ g/ml) for

a period of 3 weeks.

2.2.13 Stress experiments

2.2.13.1 Mouse embryonic fibroblasts

MEFs were resuspended to 2.5×10^4 cells/ml. 2 ml cell suspension was added to each well of a 6-well plate for incubation at 37°C for 24 h. All medium was then replaced by 2 ml DMEM supplemented with 10% (v/v) FBS and pen/strep containing the drug of choice, cisplatin (David Bull laboratories), etoposide (Sigma), actinomycin D (Sigma), mitomycin C (Sigma) or no drug as control and the cells were incubated at 37°C. At this point, and different time points during the course of the experiment, cell samples were collected. For this, the cells were washed in warm PBS before trypsinisation with 200 μ l trypsin for 5 min at 37°C. In addition, a cell scraper was used to ensure detachment of all cells. The cells were then resuspended in 300 μ l DMEM supplemented with 10% (v/v) FBS and pen/strep by pipetting and were counted twice. Cells were collected (390 $\times$ g, 5 min, 4°C) and the pellet quick-frozen on dry ice. All samples were lysed and processed simultaneously at the end of the time course to avoid differences in protein extraction.

2.2.13.2 Lymphoblastoid cell lines

LCLs were split 1:2 in fresh culture medium the day before the start of the experiment. On the day of transfection, cells were resuspended at 2.5×10^5 cells/ml and the drug of choice was added: 20 μ g/ml cisplatin (David Bull laboratories), 20 μ g/ml etoposide (Sigma), 5 nM actinomycin D (Sigma) or no drug as control. 2 ml of cell suspension was incubated per well in 6-well plates at 37°C. Cells were collected at time points 0 h and 16 h (390 $\times$ g, 5 min, 4°C), washed in PBS, counted twice and washed again in PBS. Finally, the cell pellet was quick-frozen on dry ice.

2.3 DNA protocols, vectors and cloning

2.3.1 DNA quantification

Concentration and purity of DNA was determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer (Unicam He λ ios β). The concentration was calculated from the 260 nm reading assuming an absorbance of 1.0 at this wavelength corresponds to 50 μ g/ml of double-stranded DNA. The purity was calculated from the A260/A280 ratio. Pure DNA has a ratio of 1.8 in sterile dH₂O.

2.3.2 Plasmid DNA extraction from bacterial cultures

2.3.2.1 Small scale plasmid DNA extraction

The QIAprep Spin Miniprep Kit (Qiagen) was used for small scale DNA extraction. Single bacterial colonies were picked from selective LB agar plates and inoculated in 4 ml LB medium containing the appropriate antibiotic selection. The culture was incubated o/n at 37°C while shaking. 1.5 ml of the bacteria were pelleted (2500 $\times$ g, 10 min, 4°C) and resuspended in 250 μ l buffer P1. 250 μ l buffer P2 was added and mixed by inverting the tube. The cells were lysed at RT for 5 min. 350 μ l buffer N3 was added for neutralisation and precipitation. The sample was centrifuged (18,000 $\times$ g, 10 min, RT) and the supernatant was recovered and applied to a QIAprep column. The column was centrifuged for 1 min as before and washed in 750 μ l buffer PE. The DNA was eluted in 40 μ l dH₂O.

2.3.2.2 Large scale plasmid DNA extraction

The Endofree Plasmid Maxi Kit (Qiagen) was used for large scale DNA extraction. A 3 ml bacterial culture was grown in selective LB medium for 8-10 h and used to inoculate 250 ml o/n culture. The bacteria were harvested by centrifugation (2500 $\times$ g, 15 min, 4°C) and the pellet resuspended in 10 ml buffer P1. 10 ml buffer P2 was added and the cells lysed for 5 min at RT. 10 ml of ice-cold buffer P3 was then added and the lysate poured into

the barrel of a QIAfilter Cartridge for incubation at RT for 10 min to precipitate protein and genomic DNA. Subsequently, the lysate was filtered through the QIAfilter Cartridge into a 50 ml Falcon tube and 2.5 ml Qiagen ER buffer was added prior to incubation on ice for 30 min. The sample was applied to a QIAGEN-tip 500 column, which had been pre-equilibrated in 10 ml buffer QBT, and washed in 2×30 ml buffer QC. The DNA was eluted in 15 ml buffer QN. 10.5 ml isopropanol (VWR) was added to precipitate the DNA (3750 rpm, 60 min, 4°C). The DNA pellet was washed in 5 ml 70% (v/v) endotoxin-free ethanol and centrifuged again for 30 min. The pellet was air dried in a laminar flow chamber and dissolved in 200 µl sterile endotoxin-free TE buffer.

2.3.3 BAC DNA extraction

2.3.3.1 BAC DNA extraction from bacterial cultures

To extract large BACs from bacterial cultures, single bacterial colonies were picked from plates into 1.5 ml superbroth containing appropriate selection antibiotics and incubated for 8-10 h at 37°C. This preculture was used to inoculate a 10 ml o/n culture. Bacteria were harvested (2000×g, 10 min, RT) and the pellet resuspended in 70 µl STET. 200 µl alkaline SDS was added, followed immediately by 150 µl 7.5 M ammonium acetate. The supernatant was cleared after incubation on ice for 5 min (18,000×g, 20 min, 4°C). DNA was precipitated from the supernatant with 240 µl isopropanol (10,000×g, 10 min, RT). The DNA pellet was washed in 70% (v/v) ethanol, air-dried and resuspended in TE buffer.

2.3.3.2 BAC DNA extraction from eukaryotic cells

85% confluent HEK 293 cells (10 cm dish) were harvested and resuspended in 60 µl STET. Cells were lysed in 130 µl alkaline SDS and neutralised with 110 µl 7.5 M ammonium acetate. After incubation on ice for 5 min, the lysate was cleared by centrifugation (18,000×g, 30 min, 4°C). The supernatant was mixed with 300 µl phenol-chloroform-isoamylalcohol (25:24:1, Sigma) and centrifuged at 10,000×g for 5 min at RT in a microcentrifuge. The upper, aqueous phase containing the nucleic acids

was transferred to a fresh tube and extraction repeated twice with 300 μ l chloroform. DNA was subsequently precipitated with 670 μ l absolute ethanol, (10,000 $\times$ g, 5 min, RT) and washed with 70% (v/v) ethanol (10,000 $\times$ g, 5 min, RT). The pellet was air-dried and resuspended in 50 μ l TE buffer containing 5 μ g/ml RNase A.

2.3.4 Agarose gel electrophoresis

0.5-2% (w/v) agarose (Invitrogen), depending on the DNA size to be fractionated, was dissolved in 1 $\times$ TBE buffer by heating and poured into a gel casting tray after addition of 0.5 μ g/ μ l ethidium-bromide (Sigma). Gel loading buffer was added to the DNA samples before loading. A 1 kb or 100 bp ladder (New England Biolabs (NEB)) was used as a standard to determine the length of the DNA fragments. Electrophoresis was performed in TBE buffer at 60-120 V. DNA was stained with ethidium-bromide and visualised using a Syngene transilluminator ultraviolet (UV) light source and Gene-Snap software.

2.3.5 Pulsed field gel electrophoresis (PFGE)

PFGE was used to analyse large DNA fragments (5-100 kb). 5 μ l low molecular weight DNA (section 2.3.3.2) was digested with 0.5 μ l restriction enzyme in a total volume of 10 μ l. DNA sample buffer was added to the mixture and the total volume loaded on a pre-cooled (14°C) 1% (w/v) agarose gel in 0.5 $\times$ TBE. Samples were run in 0.5 $\times$ TBE for 14 h (CHEF-DR II, Bio-Rad, V/cm=6, Initial SW=1, Final SW=10) at 14°C. DNA was visualised on a Syngene UV transilluminator after incubation in 0.5 $\times$ TBE + 0.5 μ g/ μ l ethidium-bromide at RT for 30 min.

2.3.6 DNA extraction from agarose gels

The QIAquick gel extraction kit (Qiagen) was used to extract the desired DNA fragments from agarose gels. The slice of gel containing the DNA fragment of interest was excised using a scalpel under UV light and dissolved in three volumes of buffer QG (Qiagen) at 50°C for 10 min. The solution was loaded to a QIAquick spin column and centrifuged

at $18,000\times g$ for 1 min to bind the DNA. The column was washed with 750 μl buffer PE (Qiagen) and the DNA eluted in 40 μl buffer EB (Qiagen).

2.3.7 DNA sterilisation

DNA was precipitated with $0.1\times$ volume of 3 M sodium acetate pH 5.2 and $2.5\times$ volume of ethanol at -20°C for 1 h. DNA was pelleted by centrifugation ($18,000\times g$, 15 min, 4°C) and washed in 70% (v/v) ethanol. The washed DNA pellet was air-dried under sterile conditions in a laminar flow chamber and resuspended in sterile filtered TE buffer.

2.3.8 Digestion of DNA with restriction enzymes

Digestions were performed with NEB restriction enzymes (supplied at 10-20 U/ μl) and the corresponding buffers. BSA was added when required for the specific enzyme. Digestions were carried out at the recommended temperature for 1.5-2 h. Enzyme was usually added to give about a 10 fold over-digestion. Loading dye was added to the digestion mixture and the DNA fragments were separated by agarose gel electrophoresis (section 2.3.4) and extracted from the gel (section 2.3.6) if required.

2.3.9 Polymerase chain reaction (PCR)

DNA was amplified by PCR using GoTaq DNA polymerase (Promega) or KOD Hot Start DNA polymerase (Merck). The PCR reaction mixtures used are listed in tables 2.2 and 2.3. The corresponding primers are listed in table 2.4. PCR was performed on the Gene Amp System 9700 (Applied biosystems) as described below.

For colony PCR, single bacterial colonies were transferred directly into 50 μl GoTaq DNA polymerase PCR mixture (table 2.2). Subsequently, 20 μl of the PCR reaction was analysed by agarose gel electrophoresis (section 2.3.4).

GoTaq DNA polymerase:

Table 2.2: GoTaq DNA polymerase PCR reaction.

Component	Amount
Template	5 ng plasmid, 2 μ g genomic DNA, 2 μ l cDNA
5 $\times$ PCR GoTaq Flexi Buffer	10 μ l
MgCl ₂ (25 mM)	3 μ l (1.5 mM)
dNTPs mix (10 mM each)	1 μ l (0.2 mM)
Forward primer 10 μ M	1 μ l (0.2 μ M)
Reverse primer 10 μ M	1 μ l (0.2 μ M)
GoTaq DNA polymerase (5 U/ μ l)	0.25 μ l
Sterile water	to a final volume of 50 μ l

DNA was denatured at 95°C for 5 min before the PCR reaction was repeated in 24-35 cycles of denaturation at 95°C for 1 min, annealing for 30 s and extension at 72°C for 1 min/kb of product length. Final elongation was performed at 72°C for 5 min. The annealing temperature used was the melting temperature of the primers minus up to 5°C and was optimised by gradient PCR at several different temperatures.

KOD Hot Start DNA polymerase:

Table 2.3: KOD Hot Start DNA polymerase PCR reaction.

Component	Amount
Template	5 ng plasmid, 2 μ g genomic DNA
10 $\times$ KOD Hot Start PCR Buffer	5 μ l
MgSO ₄ (25 mM)	3 μ l (1.5 mM)
dNTPs mix (2 mM each)	5 μ l (0.2 mM)
Forward primer 10 μ M	1.5 μ l (0.3 μ M)
Reverse primer 10 μ M	1.5 μ l (0.3 μ M)
KOD Hot Start DNA polymerase (1 U/ μ l)	1 μ l
Sterile water	to a final volume of 50 μ l

DNA was denatured at 95°C for 2 min before the PCR reaction was repeated in 24-35 cycles of denaturation at 95°C for 20 s, annealing for 10 s and extension at 70°C for 10 s/kb of product length. Final elongation was performed at 70°C for 2 min. The annealing

temperature used was the melting temperature of the primers minus up to 5°C and was optimised by gradient PCR at several different temperatures.

2.3.10 Primers

All primers used in this study are listed in table 2.4.

Table 2.4: Primers used in this study.

Name	Sequence	Source	Use
CDC42BPA Fw	AATGGATTACAACCTTGCAC	Invitrogen	RT-PCR
CDC42BPA Rev	TTGAAGGATTTTCAGGAGAGA	Invitrogen	RT-PCR
CHL1 Fw	GATGACATCTCCACTCAAGG	Invitrogen	RT-PCR
CHL1 Rev	TTCTCCTTAGATCCAGCGTA	Invitrogen	RT-PCR
CXCR3 Fw	GGTCATGGCCTACTGCTAT	Invitrogen	RT-PCR
CXCR3 Rev	CTCTGGAGCCCTCTCTGGT	Invitrogen	RT-PCR
EBER1 Fw	TAATACGACTCACTATAGGGAG- GACCTACGCTGCCCTAGA	Invitrogen	RT-PCR
EBER1 Rev	AAAACATGCGGACCACCAGC	Invitrogen	RT-PCR
EBER2 Fw	TAATACGACTCACTATAGGGAG- GACAGCCGTTGCCCTAGT	Invitrogen	RT-PCR
EBER2 Rev	AAAATAGCGGACAAGCCGAAT- ACC	Invitrogen	RT-PCR
EBER1-MUT	GTACCAGCTGGTGGTCCACCGG-	Invitrogen	RT-PCR
AGEI 3' END Fw	TTTTGATCCAAACTTTTG		
EBER1-MUT	CAAAAGTTTGGATCAAAACCGG-	Invitrogen	RT-PCR
AGEI 3' END Rev	TGGACCACCAGCTGGTAC		
EBER2-MUT	GAAGGGTATTCGGCTTGTCCACC-	Invitrogen	RT-PCR
AGEI 3' END Fw	GGTTTTTTTGTGGCTAGTTTTGC		

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Name	Sequence	Source	Use
EBER2-MUT	GCAAAACTAGCCACAAAAAACC-	Invitrogen	RT-PCR
AGEI 3' END Rev	GGTGGACAAGCCGAATACCCTTC		
EPDR1 Fw	TTTCAGATTGACCAAGCCACC	Invitrogen	RT-PCR
EPDR1 Rev	TCGCTCATCTTCTCCAGTTGG	Invitrogen	RT-PCR
GAPDH Fw	TGAAGGTCGGAGTCAACGGATTG	Invitrogen	RT-PCR
GAPDH Rev	GCCATGGAATTTGCCATGCCATG- GGTGG	Invitrogen	RT-PCR
IFNA5 Fw	GGACAGACATGACTTTGGAT	Invitrogen	RT-PCR
IFNA5 Rev	CATGATTTCTGCTCTGACAA	Invitrogen	RT-PCR
IGF1 Fw	CCTCGCATCTCTTCTACCT	Invitrogen	RT-PCR
IGF1 Rev	TACATCTCCAGCCTCCTTAG	Invitrogen	RT-PCR
IGSF4 Fw	CAGGAAAGTTACACCACCAT	Invitrogen	RT-PCR
IGSF4 Rev	AATGTGCACTTGAGGCTTAT	Invitrogen	RT-PCR
IL12RB2 Fw	TGGAAATGTTCTACCTCTGC	Invitrogen	RT-PCR
IL12RB2 Rev	GATTACAGGCATGAGCTACC	Invitrogen	RT-PCR
IL6-R Fw	CAGAAGTTCTCCTGCCAGTT	Invitrogen	RT-PCR
IL6-R Rev	GTGGATGACACAGTGATGCT	Invitrogen	RT-PCR
IRF5 Fw	AGACAGACCCCTCTGCCATGAA	Invitrogen	RT-PCR
IRF5 Rev	GCCTTCGGTGTATTTCCCTGTC	Invitrogen	RT-PCR
KovKanFw	GAGTTGAAGGATCAGATCACG	Operon	PCR
L22FW-BamHI- FLAG	CCGGATCCGCCATGGATTACAA- GGATGACGACGATAAGGCTCCT- GTGAAAAAGCTTGT	Invitrogen	Cloning
L22FW-EcoRI- FLAG	CGGAATTCGCCATGGATTACAA- GGATGACGACGATAAGGCTCCT- GTGAAAAAGCTTGT	Invitrogen	Cloning

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Name	Sequence	Source	Use
L22FW-KpnI-FLAG	GCGGTACCGCCATGGATTACAA- GGATGACGACGATAAGGCTCCT- GTGAAAAAGCTTGT	Invitrogen	Cloning
L22FW-XbaI-FLAG	GCTCTAGAGCCATGGATTACAA- GGATGACGACGATAAGGCTCCT- GTGAAAAAGCTTGT	Invitrogen	Cloning
L22Rev-EcoRI	CCGAATTCTTAATCCTCGTCTT- CCTCCTC	Invitrogen	Cloning
L22Rev-NotI-long	ATAAGAATGCGGCCGCTTTAAT- CCTCGTCTTCCTCCTC	Invitrogen	Cloning
L22RT Fw	CTGTGAAAAAGCTTGTGGTG	Invitrogen	RT-PCR
L22RT Rev	CCTCTTCTTCGTCCTGGTTA	Invitrogen	RT-PCR
MUM1L1 Fw	CGAAAAGAGAGGAAAGCAAG	Operon	RT-PCR
MUM1L1 Rev	TTCATTATGCAGCTTTGGAA	Operon	RT-PCR
Primer1	GCCGGATCCTCCTCACCCTCG- AAACT	Operon	Cloning
Primer3	GCCTCTAGACCAAACCTTTTGTT- TTAGGA	Operon	Cloning
Primer8	GCCGTCGACGAGTGGGAGTGCT- ATCCT	Operon	Cloning
Primer10	GCCGGATCCTTTTCTGAGGTTA- TCCCC	Operon	Cloning
Primer 5201	TCCTGACTTGCAAATGCTCT	Operon	Cloning
P0364	AAAACATGCGGACCACCAGC	Invitrogen	Northern blot

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Name	Sequence	Source	Use
P0365	AGGACCTACGCTGCCCTAGA	Invitrogen	Northern blot
P4112	TGCCCTAGTGGTTTCGGACA	Invitrogen	Northern blot
P4114	CAGAGGGATTAGAGAATCCT	Invitrogen	Northern blot
TCEAL3 Fw	AGGGAAAGAGAGAGGATGAG	Operon	RT-PCR
TCEAL3 Rev	CAAAGGCATTAAAGGTATGG	Operon	RT-PCR
tRNA-MUT AGEI	CGAAACCATCCTCTGCTAACCG-	Invitrogen	RT-PCR
3' End Fw	GTCCTTTTTTTTTTCTCCCC		
tRNA-MUT AGEI	GGGGAGAAAAAAAAAAGGACCG-	Invitrogen	RT-PCR
3' End Rev	GTTAGCAGAGGATGGTTTCG		
WWC3 Fw	GTGGATAGCGGCTGTAGCAACT	Operon	RT-PCR
WWC3 Rev	CTGCAAACATACTGGCTTCGTG	Operon	RT-PCR
3-MS2-LONG	GCAGAAGACCGGTCCTAAGTCC-	Invitrogen	RT-PCR
EBERS Fw	AACTACCAAAC		
3-MS2-LONG	ATGCATCACCGGTTAGAATAGG-	Invitrogen	RT-PCR
EBERS Rev	GCCCTCTAGAC		
5-MS2-LONG	GCAGAAGAGATCTCCTAAGTCC-	Invitrogen	RT-PCR
EBERS Fw	AACTACCAAAC		
5-MS2-LONG	ATGCATCAGATCTTAGAATAGG-	Invitrogen	RT-PCR
EBERS Rev	GCCCTCTAGAC		

2.3.11 Vectors

All vectors used in this study are listed in table 2.5. Cloning strategies for RPL22 expression vectors, EBV bacterial artificial chromosomes (BAC) and MS2-EBER expression vectors are described below in sections 2.3.12 to 2.3.14.

2.3.11.1 EBER expression vectors

pHEBo-SUPER plasmid

pHEBo-SUPER vectors had perviously been cloned in our laboratory as follows: EBER1 and EBER2 were PCR amplified from Bluescript plasmids pBSJJI and pBSJJ2^[327] using primers CCAGATCTCCAGGACCTACGCTGCCCT and CCAAGCTTGGATGCATAAATCCTAA or primers CCAGATCTCCAGGACAGCCGTTGCCCT and CCAAGCTTGGGTGCAAACTAGCCA respectively, which include a BglII site at the 5' end and a HindIII site at the 3' end. The PCR products were cloned into pSUPERIOR (OligoEngine) using BglII and HindIII sites. The pSUPER BamHI-to-XhoI fragments containing the H1 promoter and EBER1 or EBER2 were subsequently cloned between the BamHI and SalI sites of pHEBo^[328], which harbours oriP and hygromycin resistance, to create pHEBo-SUPER vectors.

Puro/oriP-pSUPER plasmid

Puro/oriP-pSUPER vectors are pHEBo-SUPER vectors in which the hygromycin resistance cassette has been replaced by a puromycin resistance gene by Prof Paul J Farrell.

Table 2.5: Vectors used in this study. amp: ampicillin (50 $\mu\text{g/ml}$), cam: chloramphenicol (50 $\mu\text{g/ml}$), G418 (200 $\mu\text{g/ml}$), hygro: hygromycin (150 $\mu\text{g/ml}$), kan: kanamycin (50 $\mu\text{g/ml}$), puro: puromycin (1 $\mu\text{g/ml}$), tet: tetracyclin (10 $\mu\text{g/ml}$).

Vector	Selection	Use
EBV BAC	cam, hygro	Recombinant EBV (2.3.13)
FLAG-RPL22-MEP4	amp, hygro	FLAG-RPL22 expression (2.3.12.4)
FLAG-RPL22-pBK2-CMV	kan, G418	FLAG-RPL22 expression (2.3.12.1)
FLAG-RPL22-p294-CMV/W	amp, hygro	FLAG-RPL22 expression (2.3.12.2)
FLAG-RPL22-p3.1W	amp, G418	FLAG-RPL22 expression (2.3.12.3)
Puro/oriP-pSUPER-EBER1	amp, puro	EBER1 expression (2.3.11.1)
Puro/oriP-pSUPER-EBER2	amp, puro	EBER2 expression (2.3.11.1)
Puro/oriP-pSUPER-HI	amp, puro	Empty vector control (2.3.11.1)
pBALF-4		Virus production (2.2.9)
pcDNA3.1 FLAG-Schlafen	amp, G418	FLAG-Schlafen expression
pCMV-EB1 (BZLF1)	kan, G418	Virus production (2.2.9)
pCR2.1-TOPO	kan	TA cloning (2.3.12.1)
pDF2.5-tet-RecA	tet	Cloning EBER2-rev BAC (2.3.13)
pEN101 pcDNA3.1	amp	MS2 PCR template (2.3.14)
pHEBo-3'MS2-E1	amp, hygro	MS2-EBER1 expression (2.3.14)
pHEBo-3'MS2-E2	amp, hygro	MS2-EBER2 expression (2.3.14)
pHEBo-5'MS2-E1	amp, hygro	MS2-EBER1 expression (2.3.14)
pHEBo-5'MS2-E2	amp, hygro	MS2-EBER2 expression (2.3.14)
pHEBo-5'MS2-H1	amp, hygro	MS2-H1 expression (2.3.14)
pHEBo-3'MS2-tRNA	amp, hygro	MS2-tRNA expression (2.3.14)
pHEBo-5'MS2-tRNA	amp, hygro	MS2-tRNA expression (2.3.14)
pIFN- β pIFDlucifer ^[329]		Luciferase assay (2.5.9)
pKov-KanF-SucS-EBER1	kan	Cloning EBER1-rev BAC (2.3.13)
pKov-KanF-SucS-EBER2	kan	Cloning EBER2-rev BAC (2.3.13)
pMalc-MBP-MS2	amp	MS2-MPB purification (2.5.10)

2.3.12 Cloning strategies for FLAG-RPL22 expression vectors

2.3.12.1 FLAG-RPL22-pBK2-CMV expression vector

pBK2-CMV plasmid

pBK2-CMV originates from pBK-CMV plasmid (Stratagene). pBK-CMV was modified to pBK2-CMV by Prof Paul J Farrell to remove an additional methionine codon in the lac promoter located between the CMV promoter and the multiple cloning site (MCS) in pBK-CMV. The methionine could potentially result in preinitiation. Digestion of pBK-CMV with NheI and SacI removed the T3 promoter so an adaptor was cloned in to reintroduce the T3 promoter site. The forward and reverse oligonucleotides for the adaptor are CTAGCGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGA-GCT and CCAGCTTTTGTTCCTTTAGTGAGGGTTAATTCGAGCG.

pBK2-CMV harbours a kanamycin and a neomycin resistance gene, the CMV promoter, the SV40 polyadenylation signal and an MCS containing BamHI and EcoRI.

FLAG-RPL22 was cloned between the BamHI and EcoRI restriction sites of pBK2-CMV as follows (figure 2.1), resulting in FLAG-RPL22-pBK2-CMV.

Cloning of FLAG-RPL22-pBK2-CMV expression vector

1. TA-cloning

RPL22 was amplified from total HEK 293 cDNA by PCR with GoTaq polymerase (figure 2.1 a, section 2.3.9) using forward primer L22FW-BamHI-FLAG and reverse primer L22Rev-EcoRI (table 2.4) to introduce an N-terminal FLAG sequence as well as BamHI and EcoRI restriction sites to the PCR product. The PCR amplicon was ligated into pCR2.1-TOPO vector (Invitrogen) which contains single 3'-thymidine overhangs. GoTaq polymerase adds single deoxyadenosine residues to 3' ends of PCR products in a template independent manner, allowing the PCR inserts to ligate efficiently into the pCR2.1-TOPO vector.

The amount of PCR product needed for ligation with 50 ng pCR2.1-TOPO was calculated using the following formula:

$$\text{PCR product (ng)} = \text{size of PCR product (bp)} \times 50 / 3900 \text{ bp (size pCR2.1-TOPO)}$$

Ligations were performed in a final volume of 10 μl , using 1 μl of T4 DNA ligase (NEB, 400 U/ μl) and incubation at 16°C o/n. A ligation reaction without insert was used as negative control. 2 μl of the ligation reaction was used to transform 100 μl of competent *E.coli* XL1 Blue cells and positive transformants were grown on selective LB agar plates (50 $\mu\text{g/ml}$ kanamycin). Single bacterial colonies were used to extract DNA (section 2.3.6) and correct insertion of FLAG-RPL22 in the target vector was confirmed by analytical digest with BamHI and EcoRI as well as HindI and EcoRI (figure 2.1 b, section 2.3.8). Clones 2, 4, 6 and 11 in figure 2.1 b displayed the correct digestion fragments. Established FLAG-RPL22-pCR2.1-TOPO plasmids were sequenced to ensure that no mutations had occurred in the FLAG-RPL22 sequence.

2. Subcloning of FLAG-RPL22 into pBK2-CMV expression vector

FLAG-RPL22-pCR2.1-TOPO and pBK2-CMV were digested with EcoRI and BamHI (section 2.3.8), the digestion products separated on an agarose gel (section 2.3.6) and the desired FLAG-RPL22 and pBK2-CMV backbone extracted (section 2.3.6) for ligation with T4 DNA ligase. Ligations were performed with a molar vector:insert ratio of 1:3. Reactions were performed in a final volume of 10 μl , using 1 μl of T4 DNA ligase (400 U/ μl , NEB) and incubation at 16°C o/n. A ligation reaction without insert was used as negative control.

2 μl of the ligation reaction was used to transform 100 μl of competent *E.coli* XL1 Blue (section 2.3.16) and positive transformants were grown on selective LB agar plates (50 $\mu\text{g/ml}$ kanamycin). Single bacterial colonies were used to extract DNA (section 2.3.6) and the obtained pBK2-CMV-FLAG-RPL22 plasmids were tested by restriction digestion (figure 2.1 c, section 2.3.8). Established plasmids were sequenced to ensure that no mutations had occurred in the FLAG-RPL22 sequence.

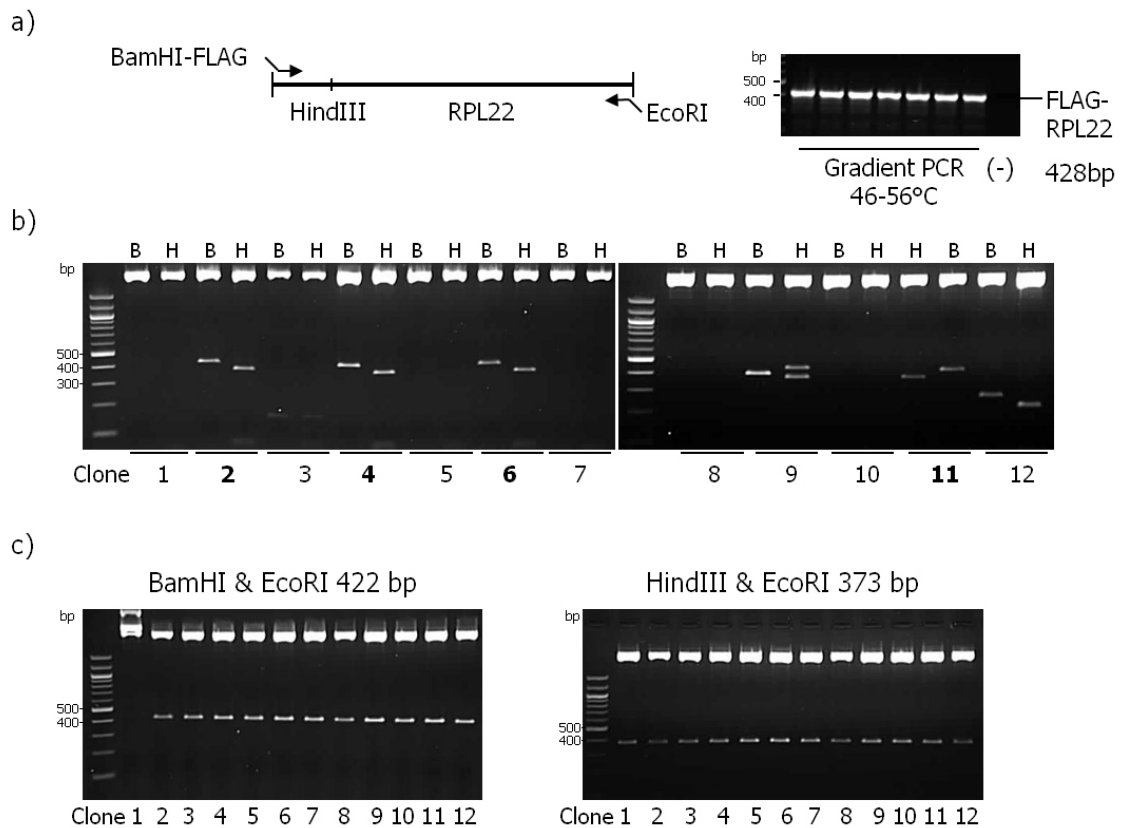


Figure 2.1: Cloning of FLAG-RPL22-pBK2-CMV expression vector.

a) RPL22 was amplified by PCR with GoTaq polymerase from HEK 293 cDNA. The primers used attached the FLAG sequence, BamHI and EcoRI restriction sites to the PCR amplicon. Gradient PCR using annealing temperatures of 46-54°C is shown on the right. Only the specific PCR product was amplified for all annealing temperatures. b) Correct ligation of FLAG-RPL22 into pCR2.1-TOPO was tested by analytical digestion with BamHI and EcoRI (B) and HindIII and EcoRI (H). Clones 2, 4, 6 and 11 (bold) contained FLAG-RPL22. c) FLAG-RPL22 was subcloned from pCR2.1-TOPO vector into pBK2-CMV vector and correct insertion was tested by analytical digestion with BamHI and EcoRI and HindIII and EcoRI. All plasmids tested showed the expected restriction fragments.

2.3.12.2 FLAG-RPL22-p294-CMV/W expression vector

pSuperInt1 plasmid

pSuperInt1 had previously been cloned in our laboratory by Dr Laila Cancian. The plasmid includes one EBV BamHI W repeat between BglII and EcoRI restriction sites followed by the EBNA2 coding sequence between EcoRI and NotI (figure 2.2 a). The W-EBNA2 sequence was inserted into the BglII and NotI sites of the commercially available pSuper vector (OligoEngine).

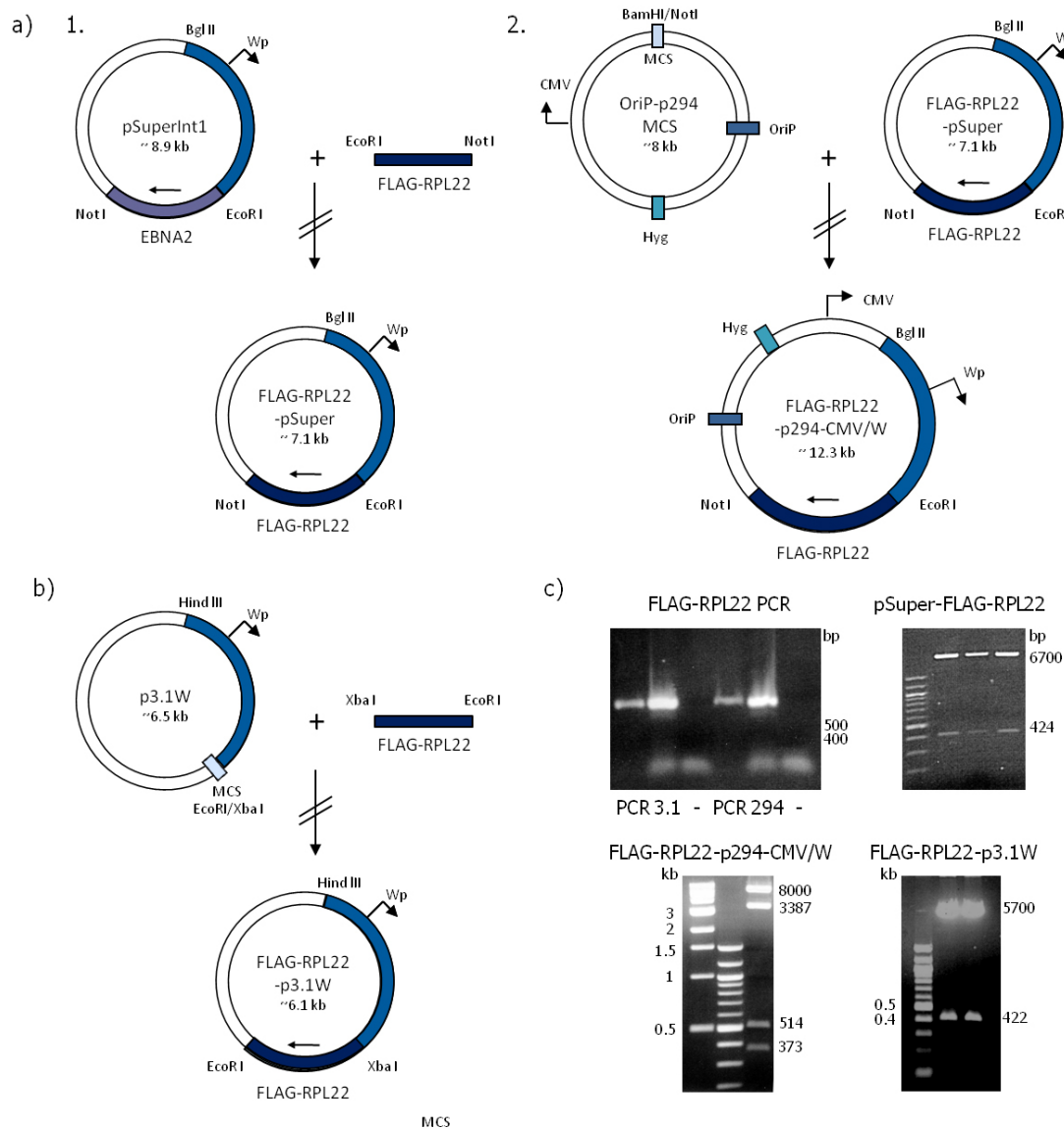


Figure 2.2: Cloning of FLAG-RPL22 expression vectors.

a) Cloning strategy for FLAG-RPL22-p294-CMV/W2 expression vector. 1. FLAG-RPL22 with flanking EcoRI and NotI restriction sites was amplified from total HEK 293 cDNA by PCR with KOD Hot Start DNA polymerase and ligated into pSuperInt1. 2. The W-FLAG-RPL22 fragment was excised from the constructed FLAG-RPL22-pSuper (BglIII/NotI) and subcloned into oriP-p294 (BamHI/NotI). Note: BamHI and BglIII have compatible ends. b) Cloning strategy for FLAG-RPL22-p3.1W expression vector. FLAG-RPL22 with flanking XbaI and EcoRI restriction sites was amplified from total HEK 293 cDNA by PCR with KOD Hot Start DNA polymerase and ligated into p3.1W. c) FLAG-RPL22 PCR products (top left) for cloning of FLAG-RPL22-p294-CMV/W (PCR 294) and FLAG-RPL22-p3.1W (PCR 3.1). Analytical digests are shown of pSuper-FLAG-RPL22 (EcoRI/NotI, top right), FLAG-RPL22-p294-CMV/W (HindIII/NotI, bottom left) and FLAG-RPL22-p3.1W (XbaI/EcoRI, bottom right). All plasmids tested showed the expected restriction digest pattern.

oriP-p294 plasmid

The oriP-p294 MCS plasmid used in this study was available in our laboratory and was created by inserting an MCS as a HindIII-BamHI fragment into the CMVpEBNA-1 plasmid described in Sugden *et al* [330]. The vector contains oriP, which allows the vector to replicate as a plasmid in EBNA1-positive cells. Further, the plasmid harbours a hygromycin resistance gene, an ampicillin (amp) resistance gene and the CMV promoter. The MCS includes HindIII, BamHI and NotI sites (figure 2.2 a).

Cloning of FLAG-RPL22-p294-CMV/W expression vector**1. Cloning of FLAG-RPL22-pSuper plasmid**

The cloning strategy for FLAG-RPL22-pSuper is outlined in figure 2.2 a 1. RPL22 was amplified from total HEK 293 cDNA by PCR with KOD Hot Start DNA polymerase (figure 2.2 c, top left) using primers L22FW-EcoRI-FLAG and L22Rev-NotI-long (table 2.4) which include the FLAG sequence and EcoRI and NotI restriction sites. The PCR product and pSuperInt1 were digested with EcoRI and NotI (section 2.3.8). The digestion products were separated on an agarose gel and the desired FLAG-RPL22 and pSuperInt1 backbone extracted (section 2.3.6) for ligation with LigaFast Rapid DNA Ligation System (Promega), thus replacing the EBNA2 sequence with FLAG-RPL22 in the pSuper vector. Ligations were performed with a molar vector:insert ratio of 1:3 in a final volume of 10 μ l, using 1 μ l of T4 DNA ligase (400 U/ μ l) and incubation at RT for 5 min, followed by heat inactivation of the ligase at 70°C for 10 min. A ligation reaction without insert was included as negative control.

5 μ l of the ligation reaction was used to transform 50 μ l One Shot TOP10 *E.coli* (Invitrogen, section 2.3.16) and positive transformants were grown on selective LB agar plates (50 μ g/ml amp). Single bacterial colonies were used to extract DNA (section 2.3.6). The correct fragments of 6700 kbp and 424 kbp were observed in analytical digests of FLAG-RPL22-pSuper with EcoRI and NotI (figure 2.2 c, top right).

2. Subcloning of FLAG-RPL22 into oriP-p294 expression vector

The cloning strategy for FLAG-RPL22-p294-CMV/W is outlined in figure 2.2 a 2. FLAG-

RPL22-pSuper was digested with BglII and NotI and oriP-p294 was cut with BamHI and NotI (section 2.3.8). The digestion products were separated on an agarose gel and the desired W-FLAG-RPL22 and oriP-p294 backbone extracted (section 2.3.6) for ligation with LigaFast Rapid DNA Ligation System (Promega). BamHI and BglII have compatible ends. Ligations were performed with a molar vector:insert ratio of 1:3. Reactions were performed in a final volume of 10 μ l, using 1 μ l of T4 DNA ligase (400 U/ μ l) and incubation at RT for 5 min, followed by heat inactivation of the ligase at 70°C for 10 min. A ligation reaction without insert was included as negative control.

5 μ l of the ligation reaction was used to transform 50 μ l One Shot TOP10 *E.coli* (Invitrogen, section 2.3.16) and positive transformants were grown on selective LB agar plates (50 μ g/ml amp). Single bacterial colonies were used to extract DNA (section 2.3.6) and the obtained FLAG-RPL22-p294-CMV/W plasmids were tested by restriction digestion with HindIII and NotI. The desired restriction fragments of 8 kbp, 3387 kbp, 514 kbp and 373 kbp were obtained in the analytical digest (figure 2.2 c, bottom left). Established plasmids were sequenced to ensure that no mutations had occurred in the FLAG-RPL22 sequence.

2.3.12.3 FLAG-RPL22-p3.1W expression vector

p3.1W plasmid

Plasmid p3.1W originates from pUC19 ^[331] and was available in our laboratory. Wt EBV B95-8 sequence 42017 to 45089 containing the W promoter was inserted between HindIII sites to obtain p3.1W. The expression vector includes ampicillin resistance, the SV40 polyadenylation signal and an MCS containing XbaI and EcoRI (figure 2.2 b).

Cloning of FLAG-RPL22-p3.1W expression vector

The cloning strategy for FLAG-RPL22-p31W is outlined in figure 2.2 b. RPL22 was amplified from total HEK 293 cDNA by PCR with KOD Hot Start DNA polymerase (figure 2.2 c, top left) using primers L22FW-XbaI-FLAG and L22Rev-EcoRI (table 2.4) which include the FLAG sequence and XbaI or EcoRI restriction sites.

The PCR product and p3.1W were digested with EcoRI and XbaI (section 2.3.8). The digestion products were separated on an agarose gel and the desired FLAG-RPL22 and p3.1W backbone extracted (section 2.3.6) for ligation with T4 DNA ligase and the LigaFast Rapid DNA Ligation System (Promega). Ligations were performed with a molar vector:insert ratio of 1:3. Reactions were performed in a final volume of 10 μ l, using 1 μ l of T4 DNA ligase (400 U/ μ l) and incubation at RT for 5 min, followed by heat inactivation of the ligase at 70°C for 10 min. A ligation reaction without insert was included as negative control.

5 μ l of the ligation reaction was used to transform 50 μ l One Shot TOP10 *E.coli* (Invitrogen, section 2.3.16) and positive transformants were grown on selective LB agar plates (50 μ g/ml amp). Single bacterial colonies were used to extract DNA (section 2.3.6) and the obtained FLAG-RPL22-p3.1W plasmids were tested by restriction digestion (figure 2.2 c). Established plasmids were sequenced to ensure that no mutations had occurred in the FLAG-RPL22 sequence.

2.3.12.4 FLAG-RPL22-MEP4 expression vector

pMEP4 plasmid

Plasmid pMEP4 (Invitrogen) comprises an MCS under the control of the metallothionein promoter. This allows the controlled induction of protein expression with cadmium chloride. The vector contains hygromycin and ampicillin resistance cassettes for selection in eukaryotic and prokaryotic cells and the SV40 polyadenylation signal. Additionally, pMEP4 contains oriP, which allows the vector to replicate as a plasmid in EBNA1-positive cells (figure 2.3).

Cloning of FLAG-RPL22-MEP4 expression vector

RPL22 was amplified from total HEK 293 cDNA by PCR with GoTaq polymerase (section 2.3.9). An N-terminal FLAG-tag, KpnI and NotI restriction sites were added to PCR product by using primers L22FW-KpnI-FLAG and L22Rev-NotI-long (table 2.4). The PCR amplicon was ligated into pCR2.1-TOPO vector (Invitrogen) as described in

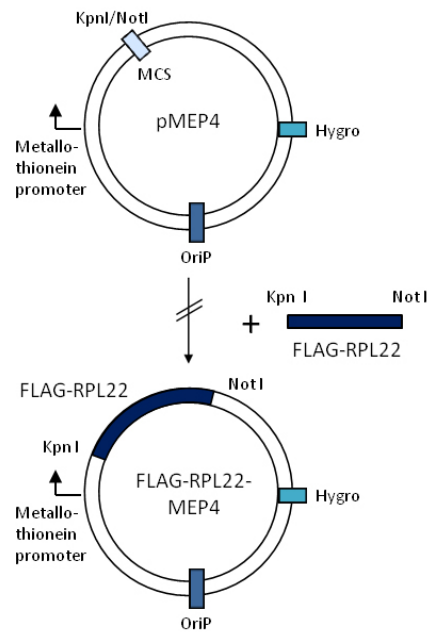


Figure 2.3: Cloning of FLAG-RPL22-MEP4 expression vector.

Cloning strategy for FLAG-RPL22-MEP4 expression vector. FLAG-RPL22 with flanking KpnI and NotI restriction sites was amplified from total HEK 293 cDNA by PCR with GoTaq polymerase and inserted into pMEP4 plasmid via pCR2.1-TOPO cloning.

section 2.3.12.1. The sequenced FLAG-RPL22 fragment was further subcloned into pMEP4 using KpnI and NotI restriction sites. The final FLAG-RPL22-MEP4 plasmids were analysed by restriction digestion and sequencing.

2.3.13 Cloning strategies for EBV bacterial artificial chromosomes

EBER deletion EBV BACs were constructed by Prof Paul J Farrell. EBER revertant BACs were constructed by Prof Paul J Farrell and myself.

2.3.13.1 EBER deletion bacterial artificial chromosomes

EBER1 or EBER2 were deleted individually from the B95-8 EBV BAC ^[332] (kindly provided by Prof Wolfgang Hammerschmidt) by RecA-mediated homologous recombination. Targeting constructs were cloned between the BamHI and SalI sites of pKov-Kan-Cm (kanamycin resistant, sucrose sensitive) as described previously ^[333]. EBER1 deletion replaced EBV bases 6599 to 6798 with TCTAGA XbaI restriction site

sequence. The EBER1 targeting plasmid contained EBV bases 6066 to 7322 as flanking sequence (BamHI was added at the 6066 end). The EBER2 deletion replaced EBV bases 6950 to 7130 with TCTAGA XbaI restriction site sequence. The EBER2 targeting plasmid contained EBV bases 6669 to 7413 as flanking sequence (BamHI was added at the 6669 end). EBER sequences were replaced by XbaI restriction sites to monitor the deletion.

Cointegration

EBER targeting plasmids and pDF2.5-tet-RecA were transformed into bacteria containing wt EBV BAC (section 2.3.16). pDF2.5-tet-RecA expresses RecA, tetracycline resistance and contains a temperature sensitive origin of replication that only allows replication at 30°C. Transformed bacteria were selected on LB plates containing chloramphenicol (cam, 50 µg/ml), kanamycin (kan, 25 µg/ml) and tetracyclin (tet, 10 µg/ml) o/n at 30°C. Colonies obtained were cured of RecA by growth on cam/kan (50 µg/ml, 25 µg/ml) at 42°C o/n. Correct cointegrants were confirmed by PCR (section 2.3.9) and tested by restriction digest and PFGE (section 2.3.5).

Production of chemically competent cointegrants

Bacteria were grown in 10 ml LB cam/kan (50 µg/ml, 25 µg/ml) o/n at 43°C. 100 µl bacteria were transferred into 10 ml of fresh selective medium for 3 h. Bacteria were subsequently incubated on ice for 30 min, pelleted (2000×g, 15 min, 4°C) and resuspended in 2 ml 100 mM CaCl₂. Bacteria were incubated on ice for 30 min, centrifuged again and the pellet resuspended in 200 µl 100 mM CaCl₂, 15% (v/v) glycerol. The obtained competent bacteria were snap-frozen in aliquot and stored at -80°C.

Resolving cointegrants

pDF2.5-tet-RecA was reintroduced (section 2.3.17) into characterised competent cointegrants to resolve the cointegrants. Transformed bacteria were selected on cam/tet (50 µg/ml, 10 µg/ml) plates o/n at 30°C to allow RecA activity. Single colonies were

grown in 1 ml LB cam/tet (50 μ g/ml, 10 μ g/ml) plus 5% (w/v) sucrose for 4 h at 30°C to cure the bacteria of the pKov-Kan-Cm targeting vector sequence, followed by plating on sucrose and cam and growth at 42°C o/n to remove the RecA plasmid. Single colonies were plated in replica on cam/kan and cam only plates (50 μ g/ml, 25 μ g/ml) and incubated o/n. Bacteria with a mutated SacB gene are able to grow under sucrose selection but correctly resolved BACs are identified by being sensitive to kan. The constructed Δ EBER BACs were tested by colony PCR (section 2.3.9) and analysed by restriction digestion and PFGE (section 2.3.5).

2.3.13.2 EBER-revertant bacterial artificial chromosomes

EBER-revertant BACs were constructed from EBER deletion BACs to revert effects of EBER deletion on host cell gene expression. EBERs were integrated into the EBER deletion BACs from pKov-KanF-SucS-EBER1 containing wt EBV bases 6066 to 7322 or pKov-KanF-SucS-EBER2 containing wt EBV bases 6669 to 7413 (available in our laboratory).

Cointegration

Competent *E.coli* cells containing EBER deletion BAC were transformed with pDF2.5-tet-RecA and pKov-KanF-SucS-EBER plasmid as pKov-Kan-Cm described in section 2.3.16. Transformed bacteria were grown on cam/kan/tet (50 μ g/ml, 25 μ g/ml, 10 μ g/ml) plates o/n at 30°C to allow cointegration of pKov-KanF-SucS-EBER into EBER deletion BACs by RecA activity. Selected single colonies were plated on cam/kan (50 μ g/ml, 25 μ g/ml) plates and incubated at 43°C to allow loss of pDF2.5-tet-RecA. Integration of pKov-KanF-SucS-EBER into EBER deletion BAC was confirmed by colony PCR (section 2.3.9). BAC-pKov-KanF-SucS-EBER cointegrants were extracted (section 2.3.6) and tested by restriction digest and PFGE (section 2.3.5).

Production of chemically competent cointegrants

Bacteria were grown in 10 ml LB cam/kan (50 μ g/ml, 25 μ g/ml) o/n at 42°C. 100 μ l

bacteria were transferred into 10 ml of fresh selective medium for 3 h. Bacteria were subsequently incubated on ice for 30 min, pelleted ($2000\times g$, 15 min, 4°C) and resuspended in 2 ml 100 mM CaCl_2 . Bacteria were incubated on ice for 30 min, centrifuged again and the pellet resuspended in $200\ \mu\text{l}$ 100 mM CaCl_2 , 15% (v/v) glycerol. The obtained competent bacteria were snap-frozen in aliquot and stored at -80°C .

Resolving cointegrants

pDF2.5-tet-RecA was reintroduced (section 2.3.17) into competent *E.coli* containing BAC-pKov-KanF-SucS-EBER to resolve the cointegrants. Transformed bacteria were selected on cam/tet (50 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$) plates o/n at 30°C to allow RecA activity. Single colonies were grown in 1 ml LB cam/tet (50 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$) plus 5% (w/v) sucrose for 4 h at 30°C . To select against bacteria containing integrated pKov-KanF-SucS-EBER plasmids, 100 μl of this culture was subsequently transferred into 1 ml fresh selective medium for o/n incubation at 30°C . 5 μl of the o/n culture was streaked out on a 14 cm LB 5% (w/v) sucrose plus cam (50 $\mu\text{g/ml}$) plate and incubated o/n at 43°C to lose pDF2.5-tet-RecA. Single colonies were plated in replica on cam/kan and cam only plates (50 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$) and incubated o/n. Bacteria with a mutated SacB gene are able to grow under selection but correctly resolved BACs are identified by being sensitive to kan. The constructed EBER-revertant BACs were tested by colony PCR (section 2.3.9) and analysed by restriction digestion and PFGE (section 2.3.5).

2.3.14 MS2-EBER expression vectors

MS2 sequence was cloned 3' and 5' of EBER1, EBER2 and tRNA(Met) or alone under control of the H1 promoter in pHEBo-SUPER vectors (section 2.3.11.1) as outlined in figure 2.4.

6 MS2 repeats were amplified by PCR (section 2.3.9) using KOD Hot Start DNA polymerase (Merck) from pEN101 pcDNA3.1 (kind gift of Dr Ian Goodfellow) using primers 5-MS2-LONG EBERS forward and reverse containing BglIII restriction sites for

5' cloning or 3-MS2-LONG EBERS forward and reverse containing AgeI restriction sites for 3' cloning (table 2.4). The MS2 PCR product was gel purified (section 2.3.6), digested with BglII or AgeI (section 2.3.8) and gel purified again before insertion into the pHEBo-SUPER vector backbone.

For 5' cloning, pHEBo-SUPER plasmids were used directly as the vectors already contain a BglII restriction site 5' of the EBERs. For 3' cloning, an AgeI restriction site was introduced into the pHEBo-SUPER vectors by site directed mutagenesis (section 2.3.15) using EBER1-MUT AGEI 3' END forward and reverse primers for EBER1, EBER2-MUT AGEI 3' END forward and reverse primers for EBER2 and tRNA-MUT AGEI 3' END forward and reverse primers for tRNA (table 2.4).

500 ng of the pHEBo-SUPER vectors was digested with BglII or AgeI (section 2.3.8), gel purified (section 2.3.6) and eluted in 40 μ l dH₂O. 35 μ l vector was dephosphorylated by addition of 1 μ l (5 U) antarctic phosphatase (NEB) plus 4 μ l antarctic phosphatase buffer 10 $\times$ (NEB) for 30 min at 37°C. The reaction was stopped at 65°C for 5 min. The ligations were performed with a molar vector:insert ratio of 1:2 in a final volume of 10 μ l, using 1 μ l of T4 DNA ligase (400 U/ μ l, NEB) and incubation at 16°C o/n. Alternatively, LigaFast Rapid DNA Ligation System (Promega) with 1 μ l of T4 DNA Ligase (Promega) in a final volume of 10 μ l and incubation at RT for 10 min followed by heat inactivation of the ligase at 70°C for 10 min was used. A ligation reaction without insert was performed as negative control.

4 μ l of each ligation reaction was used to transform 50 μ l of competent One Shot TOP10 chemically competent *E.coli* (Invitrogen, section 2.3.16) and positive transformants were grown on selective LB agar plates (50 μ g/ml amp). Single colonies were used to extract DNA (section 2.3.6) and the resulting plasmids pHEBo-5'MS2-H1, pHEBo-5'MS2-E1, pHEBo-5'MS2-E2, pHEBo-3'MS2-E1, pHEBo-3'MS2-E2, pHEBo-5'MS2-tRNA and pHEBo-3'MS2-tRNA were tested by restriction digestion (section 2.3.8) and sequencing.

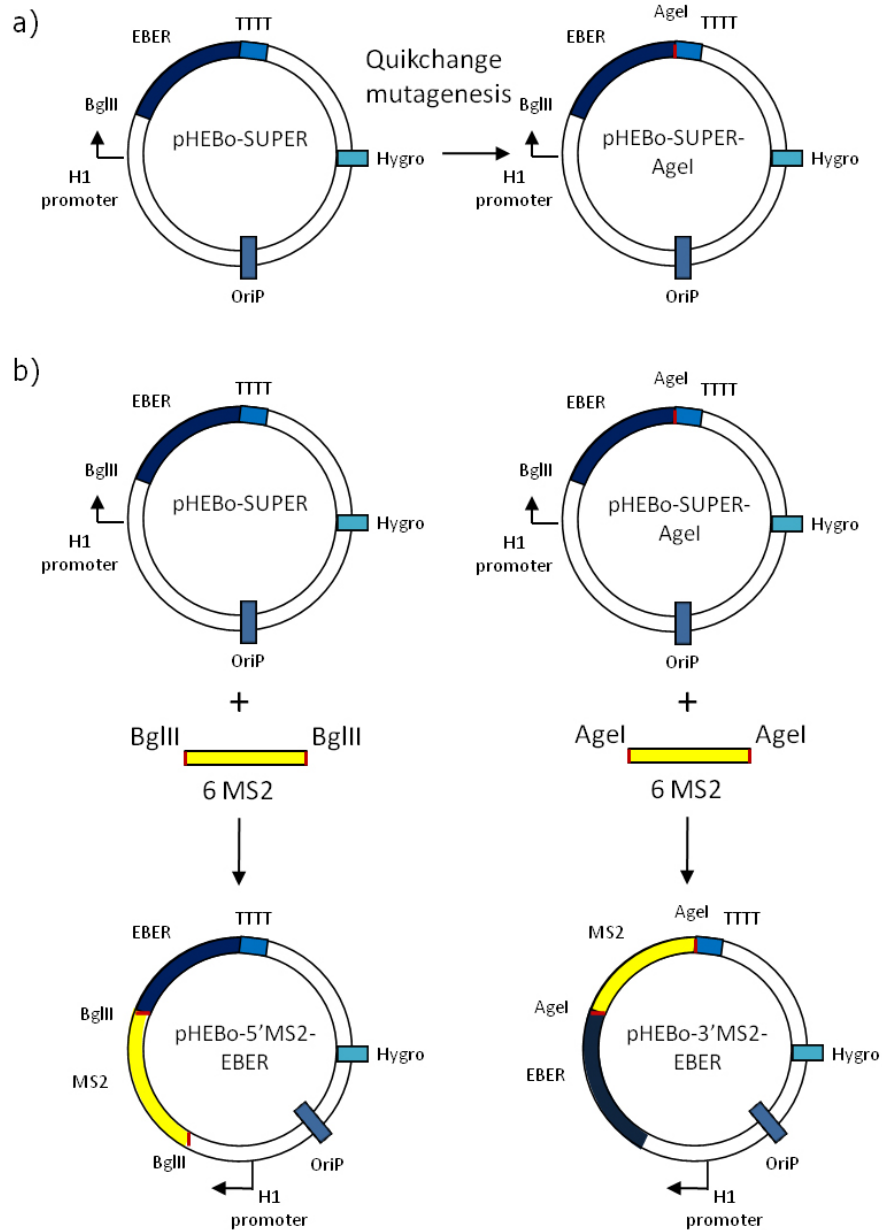


Figure 2.4: Cloning of MS2-EBER expression vectors.

a) An AgeI restriction site was introduced into pHEBo-SUPER vectors by site directed mutagenesis 3' of the EBER sequence but before the poly-thymidine RNA polymerase III termination signal (TTTT). b) 6 MS2 repeats were amplified by PCR with primers containing BglIII or AgeI restriction sites. The BglIII-MS2 PCR product was cloned into a BglIII restriction site 5' of the EBERs in pHEBo-SUPER vectors (left). The AgeI-MS2 PCR product was cloned into the AgeI restriction site introduced in a) 3' of the EBERs in pHEBo-SUPER vectors (right).

2.3.15 Site directed mutagenesis

DNA mutations were introduced using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Sets of complementary primers containing the mutation of choice were designed. Primers were 40-45 bases long with a melting temperature of $\leq 74.9^{\circ}\text{C}$. The melting temperature was estimated using the following formula:

$$\text{Melting temperature} = 81.5 + 0.41(\%GC) - 675/n - \% \text{ mismatch}$$

n = length of the primer in bases

The reaction was set up as follows in table 2.6. The PCR was performed on the Gene Amp System 9700 (Applied biosystems) according to the thermal cycling conditions outlined in table 2.7.

Table 2.6: QuikChange site directed mutagenesis reaction.

Component	Amount
10×reaction buffer	5 μl
Template DNA	10 ng
Forward primer	125 ng
Reverse primer	125 ng
Nucleotide mix	1 μl
QuikSolution	3 μl
PfuUltra HF DNA polymerase (2.5 U/ μl)	1 μl
Nuclease-free water	to a final volume of 50 μl

Table 2.7: QuikChange site directed mutagenesis thermal cycling conditions.

Segment	Cycles	Temperature	Time
Initial denaturation	1	95°C	2 min
Denaturation	18	95°C	50 s
Annealing	18	68°C	50 s
Extension	18	68°C	1 min/kb
Final extension	1	68°C	7 min

Methylated parental DNA was digested using 1 μ l DpnI (10 U/ μ l, Stratagene) and incubation at 37°C for 1 h. 2 μ l of XL10-Gold β -mercaptoethanol mix (Stratagene) was added to 45 μ l XL10-Gold ultracompetent cells (Stratagene) and incubated on ice for 10 min, swirling gently every 2 min. 2 μ l of the Dpn I-treated DNA was added to the bacteria and incubated on ice for 30 min before heat shock at 42°C for 30 s followed by recovery on ice for 2 min. 0.5 ml of SOC broth was added to the transformation mix and incubated at 37°C for 1 h while shaking at 225–250 rpm. 50 μ l of bacteria was then plated on selective agar plates and incubated o/n at 37°C. Colonies were analysed by restriction enzyme digestion of plasmid DNA (section 2.3.2) and sequencing.

2.3.16 Transformation of chemically competent bacteria

XL1 Blue or One Shot TOP10 (Invitrogen) chemically competent *E.coli* were thawed on ice. 2 μ l of ligation reaction or plasmid DNA was added to 100 μ l of bacteria and incubated on ice for 30 min. The bacteria were heat-shocked for 30 s at 42°C before incubation on ice for 2 min. 250 μ l of LB or SOC medium (Invitrogen) respectively, was added and the bacteria were incubated on a shaker at 225 rpm at 37°C for 1 h. 10% and 90% of bacteria were then plated on selective LB agar plates and incubated o/n at 37°C.

2.3.17 Transformation of electrocompetent bacteria

E-shot DH10B-T1 electrocompetent *E.coli* (Invitrogen) in electroporation cuvettes were thawed on ice and mixed with 2 μ l of low molecular weight DNA (section 2.3.3.2). Bacteria were electroporated with a Bio-Rad Genepulser 200 (25 μ F, 2 kV) and 500 μ l SOC medium (Invitrogen) was added immediately. The transformed bacteria were incubated at 37°C for 1 h while shaking, pelleted (2000 $\times$ g, 3 min, RT) and plated on selective agar plates (25 μ g/ml, cam).

2.4 RNA protocols

2.4.1 RNA quantification

Absorbance at 260 nm in dH₂O and 280 nm in TE buffer was measured using a spectrophotometer (Unicam He λ ios β) to quantify RNA concentration and determine RNA purity. The concentration was calculated from the 260 nm reading assuming an absorbance of 1.0 at this wavelength corresponds to 40 μ g/ml of RNA. The purity was calculated from the absorbance A₂₆₀/A₂₈₀ ratio. Pure RNA has a ratio of 2 in 10 mM Tris-HCl pH 7.5.

2.4.2 RNA extraction

2.4.2.1 Total RNA extraction

Total RNA was extracted using Trizol reagent (Invitrogen). Approximately 10⁷ cells were pelleted (390 $\times$ g, 5 min, 4°C) and resuspended in 1 ml Trizol reagent. The homogenised samples were incubated for 5 min at RT to allow dissociation of nucleoprotein complexes. 0.2 ml chloroform was added and the sample shaken for 15 s prior to incubation for 3 min at RT. Samples were centrifuged (12,000 $\times$ g, 15 min, 4°C) and thereby separated into a phenol-chloroform phase, an interphase and an upper aqueous phase containing RNA. The aqueous phase was recovered and RNA precipitated with 0.5 ml isopropanol and incubation for 10 min at RT. RNA was pelleted (12,000 $\times$ g, 10 min, 4°C) and washed in 1 ml 75% (v/v) ethanol (10,000 $\times$ g, 5 min, 4°C). The RNA pellet was air-dried and dissolved in RNase-free dH₂O.

2.4.2.2 Cytoplasmic RNA extraction

Cytoplasmic RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen). Cells were harvested and washed in PBS and the cell pellet was resuspended in 175 μ l RLN lysis buffer (Qiagen) for 5 min on ice. The supernatant was cleared by centrifugation (300 $\times$ g, 2 min, 4°C) and mixed with 600 μ l RLT buffer (Qiagen) and 430 μ l ethanol (96-100%).

The solution was transferred to an RNeasy spin column and RNA bound by centrifugation ($12,000\times g$, 30 min, RT). The column was washed with 350 μ l RW1 buffer (Qiagen) and the RNA treated with DNase I (10 μ l DNase I stock solution plus 70 μ l RDD buffer, Qiagen) for 15 min at RT. The column was subsequently washed with 350 μ l RW1 buffer (Qiagen) and twice with 500 μ l RPE buffer (Qiagen) before elution of RNA into RNase-free dH₂O.

2.4.2.3 Extracellular RNA extraction

RNA released from LCLs was extracted from cell supernatant using Trizol LS reagent (Invitrogen). 2×10^5 cells/ml were grown for 4 days and pelleted ($390\times g$, 5 min, 4°C). The top 80% of supernatant was collected and 0.75 ml Trizol LS added per 0.25 ml of supernatant volume. Samples were homogenised by pipetting before incubation at RT for 5 min. The supernatant was filtered through a 0.22 μ m Nalgene syringe filter using a screw cap syringe before addition of Trizol LS if so indicated. 0.2 ml chloroform was added and the sample shaken for 15 s prior to incubation for 15 min at RT. Samples were centrifuged ($12,000\times g$, 15 min, 4°C) and thereby separated into a phenol-chloroform phase, an interphase and an upper aqueous phase containing RNA. The aqueous phase was recovered and 0.5 μ l GlycoBlue (Invitrogen) added as co-precipitant prior to RNA precipitation with 0.5 ml isopropanol and incubation for 10 min at RT. RNA was pelleted ($12,000\times g$, 10 min, 4°C) and washed in 1 ml 75% (v/v) ethanol ($10,000\times g$, 5 min, 4°C). The RNA pellet was air-dried and dissolved in RNase-free dH₂O at 55°C for 10 min.

2.4.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

Cytoplasmic RNA (section 2.4.2) was transcribed into cDNA using the first strand cDNA synthesis kit (Protoscript). 1 μ g RNA in 10 μ l dH₂O was mixed with 2 μ l primer dT₂₃VN (50 μ M) and 4 μ l dNTP mix (2.5 mM each) and incubated for 5 min at 70°C. 2 μ l 10 $\times$ RT buffer (Protoscript), 1 μ l RNase inhibitor (10 U/ μ l) and 1 μ l M-MULV reverse transcriptase (25 U/ μ l) was added to the RNA mixture. The sample was incubated for 1

h at 42°C to allow reverse transcription and then for 5 min at 95°C to inactivate enzyme activity. The cDNA was diluted to 50 μ l in dH₂O and 2 μ l was used for PCR reactions as described in section 2.3.9.

2.4.4 DNase treatment of RNA

RNA was treated with RQ1 RNase-free DNase (Promega) to avoid DNA contamination before RT-PCR, if necessary. 1 U DNase per μ g RNA was incubated for 30 min at 37°C in a total volume of 10 μ l. To purify nucleic acids from the reaction, the solution was diluted to 50 μ l with DEPC dH₂O. 50 μ l of phenol-chloroform-isoamylalcohol (25:24:1, Sigma) was added and mixed thoroughly by vortexing for 1 min. After centrifugation (15,000 $\times$ g, 5 min, RT), the upper, aqueous phase was transferred into a fresh Eppendorf tube. The RNA was precipitated by addition of 1:10 volumes of 3 M sodium acetate pH 5.2 and 2.5 volumes of 100% ethanol, incubation at -20°C for 45 min and centrifugation at 15,000 $\times$ g for 15 min at 4°C. The RNA was then washed with 70% (v/v) ethanol (15,000 $\times$ g, 5 min, 4°C), air-dried and resuspended in 10 μ l DEPC treated Milli-Q water (Ambion).

2.4.5 Northern blotting

2 μ g RNA in 4 μ l dH₂O was denatured with 7.2 μ l glyoxalation mix for 10 min at 50°C in a total volume of 11.2 μ l. 2 μ l 6 $\times$ loading dye (NEB) plus Orange G (Sigma) was added and the total RNA sample separated on a thin HGT agarose gel in 10 mM sodium phosphate pH 7.0 running buffer. A capillary blotting system was used to transfer the RNA to the membrane. The gel was placed on top of three 3 MM Whatman filter papers previously saturated in SSC. The gel was covered with a Pall biodyne 0.2 μ m nylon membrane cut to the exact size of the gel and three Whatman filter papers saturated in dH₂O. Paper towels were added on top and covered with weights for transfer of the RNA o/n at RT. The next day, RNA was UV crosslinked to the membrane (1200 J/cm², spectrolinker XL-1500, Spectros corporation) and deglyoxylated by addition of boiling 20 mM Tris-HCl pH 8.0 and cooling down to RT on a shaker for 30 min. The membrane

Table 2.8: Northern blot hybridisation mix.

Component	Amount
20×SSC	15 ml
50×Denhardt's	10 ml
10% (w/v) SDS	2.5 ml
0.5 M EDTA pH 8.0	1 ml
Salmon sperm DNA (Sigma)	0.5 ml
Sterile Milli-Q water	21 ml

was pre-hybridised for 2-3 h in hybridisation mix (table 2.8) at 65°C in rotating hybridisation bottles.

The Amersham MegaPrime DNA Labeling System (GE healthcare) was used to prepare radioactively labeled probes. The EBER template was created by PCR using primers P0364 and P0365 for EBER1 or primers P4112 and P4114 for EBER2 (table 2.4) [334]. 2 µl DNA template PCR product for EBER1 or EBER2 was added to 29 µl dH₂O and 5 µl random primer. The mixture was heated to 100°C for 5 min before 10 µl RP labeling buffer and 2 µl Klenow polymerase (1 U/µl) were added on ice. Subsequently, 2 µl 3000 Ci/mmol 10 mCi/ml [α^{32} -P]dCTP (Perkin Elmer) was added and incubated for 20 min at 37°C. 5 µl 0.2 M EDTA pH 8.0 was added to stop the reaction. A micro bio-spin chromatography column (Bio-Rad) was prepared by centrifugation (1000×g, 4 min, RT) and used to clear unincorporated dCTP from the probe (1000×g, 4 min, RT). The probe was boiled for 5 min, added to the membrane in fresh hybridisation mix (table 2.8) and incubated o/n at 65°C on a rotator. The membrane was then washed 2×10 min at RT in 250 ml 2×SSC, 0.2% (w/v) SDS and 2×30 min at 65°C in 250 ml 0.1×SSC, 0.2% (w/v) SDS. The membrane was covered in saran wrap and exposed to medical x-ray film (Fujifilm) for 3 h or o/n at -80°C using an intensifying screen.

2.4.6 Microarray analysis

Microarray analysis was performed using Agilent G4112F ID 014850 whole human genome oligonucleotide microarrays. Four to six independent EBV wt, EBER deletion or EBER-revertant cell lines were used for the analysis of cytoplasmic RNA and two to three independent cell lines per virus type for the analysis of ribosome-bound RNA. Synthesis of Cy3-labeled cRNA, cRNA fragmentation, hybridisation and washing were performed using one color Quick Amp labeling kit according to the manufacturer's recommendations (Agilent Technologies). Slides were then scanned on an Agilent MicroArray Scanner (G2565CA) at photomultiplier tube settings 100% and 5% to increase the dynamic range of the measurements (extended-dynamic-range mode) and the data extracted and processed using the recommended default extraction protocol file of Agilent's feature extraction software v9.5.3.1. The data was normalised using an inter-array approach (global linear scaling to one arbitrary reference array, according to the 75th percentiles of intensity distribution). 47 was used as a surrogate value for all measurements below this lower-intensity threshold. Microarray data analysis was performed using Genomics Suite software (Partek) and Genedata Expressionist software (Genedata AG). Principal component analysis was performed with Partek Genomics Suite software using the covariance method. Differences in gene expression levels correlating with EBER expression were identified using analysis of variance (ANOVA) in Partek and Genedata software. To identify differentially regulated genes, expression levels in EBER deletion mutant cell lines were compared to wt and revertant LCLs. A fold change of 2 with a p-value of 0.001 was used as minimal requirement and a false discovery rate of 1% was applied to the p-value.

Microarray hybridisations were performed by Dr Oliver Dittrich-Breiholz (Medizinische Hochschule Hannover) as part of the INCA EU grant consortium. Bioinformatic analysis of the data was performed by Dr Rainer Russ (Genedata, Basel) and our own laboratory.

2.5 Protein protocols

2.5.1 Protein extraction

Cells were harvested as described in section 2.2.5. The cell pellet was resuspended completely in 2-3×cell volume cold RIPA lysis buffer and lysed on ice for 20 min. Cell debris and nuclei were removed by centrifugation (18,000×g, 15 min, 4°C) and the cleared lysate recovered for further investigation. Alternatively, cell pellets were resuspended directly in 2×SDS sample buffer and sonicated on ice for 2×15 s at a sonication strength of 5 (XL sonicator, Heat systems).

2.5.2 Protein quantification

Protein concentrations were determined using the BioRad DC protein assay (Bio-Rad). 5 µl of protein sample was diluted in 15 µl lysis buffer. Bovine serum albumin dilutions (0, 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 µg/µl) were prepared to produce a protein concentration standard curve. 100 µl of reagent A (Alkaline copper tartrate solution) and 2 µl of reagent S were added to each sample, followed by 800 µl of reagent B (Folin reagent). Samples were mixed by inversion and incubated at RT for 15 min. The protein concentrations were measured by reading the absorbance at 750 nm using a spectrophotometer (Unicam Heliosβ) and comparing it with the standard curve samples. Protein extracts were quick-frozen on dry ice and stored at -80°C.

2.5.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein separation was performed by SDS-PAGE using the Bio-Rad Mini Protean II system. 10-20% (v/v) resolving polyacrylamide gels combined with a 5% (v/v) stacking gel were used for protein separation depending on the size of the protein of interest. 10 µl TEMED and 50 µl 10% (w/v) APS per 10 ml gel volume were used to polymerise the gels. Protein samples were mixed with 2×SDS sample buffer or Laemmli buffer and

boiled for 5 min at 95°C. 10-30 μ g total protein was loaded on the gel per well. 5 μ l full Range Rainbow Molecular Weight Marker (Amersham) or NEB protein ladder (NEB) or Novex Sharp prestained protein standard (Invitrogen) were used as standards to determine protein sizes. Gels were run at 120-160 V in SDS running buffer.

2.5.4 Coomassie staining

Proteins on SDS-gels were stained in Coomassie Blue for 1-4 h and destained in 40% (v/v) methanol, 10% (v/v) acetic acid o/n.

2.5.5 Western blotting

2.5.5.1 Western blotting using enhanced chemiluminescence

Proteins were fractionated by SDS-PAGE and electrotransferred to a nitrocellulose membrane (Schleicher & Schuell) or PVDF membrane (Immobilon) for detection of specific proteins. SDS-PAGE gels were equilibrated in transfer buffer for 30 min before the proteins were blotted at 100 V for 1 h in a cooled Bio-Rad blotting chamber in transfer buffer. PVDF membranes were pre-wetted in methanol for 1 min, washed in dH₂O and equilibrated in transfer buffer for 30 min before use. Membranes were blocked in 5% (v/v) skimmed milk powder (Marvel) or 5% (v/v) BSA (Sigma) in PBS-T for 1 h at RT on a slow shaker. The membranes were incubated with primary antibody (table 2.9) in blocking solution for 1 h at RT or at 4°C o/n. Subsequently, the membranes were washed 3×10 min in PBS-T on a fast shaker at RT. The second antibody (table 2.9) conjugated to horse-radish peroxidase (HRP) was diluted 1:2000 in blocking solution and applied to the membrane for 1 h at RT, before the membranes were washed 3×10 min in PBS-T. Enhanced chemiluminescence (ECL) reagent (Amersham) was added to the membrane for 1 min and the bound proteins visualised by exposure on autoradiography film (Fujifilm).

2.5.5.2 Western blotting using Odyssey Infrared Imaging System

Proteins were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane (Schleicher & Schuell) for detection of specific proteins. SDS-PAGE gels were equilibrated in transfer buffer for 30 min before the proteins were blotted at 100 V for 1 h in a cooled Bio-Rad blotting chamber in transfer buffer. The membrane was blocked in 5% (v/v) skimmed milk powder (Marvel) in PBS-T for 1 h at RT on a slow shaker. Membranes were incubated with primary antibody (table 2.9) in blocking solution at 4°C o/n. Subsequently, membranes were washed 3×5 min in PBS-T on a fast shaker at RT. The IRDye infrared dye-conjugated secondary antibody (table 2.9) was diluted 1:20,000 in blocking solution and applied to the membrane for 1 h at RT in the dark before the membrane was washed 3×5 min in PBS-T and 5 min in PBS only to remove residual Tween-20. The membranes were then patted dry on filter paper, wrapped in aluminium foil and stored at 4°C. Bound proteins were visualised by scanning the appropriate channels in the infrared wavelength range using an Odyssey Infrared Imaging System (LI-COR).

2.5.5.3 Stripping of western blot membranes

To reprobe a western blot membrane for a second protein of interest, the first primary and secondary antibodies were removed by incubating the membrane in 50 ml western blot stripping solution at 55°C for 45 min in a tightly closed plastic box. The membrane was washed in PBS-T for 3×5 min in a fume cupboard followed by 3×10 min on a fast rocker to remove all traces of β -mercaptoethanol. The membrane was then blocked and incubated with different antibodies as described above (section 2.5.5).

2.5.6 Antibodies

Antibodies used in this study are listed in table 2.9 below.

Table 2.9: Antibodies used in this study. SCB: Santa Cruz Biotechnology, XA: ExAlpha Biologicals, IF: immunofluorescence microscopy, WB: western blot.

Antigen	Antibody	Source	Dilution
EBNA1	Human serum		1:1000 WB
EBNA3A	Sheep polyclonal	XA	1:1000 WB
EBNA3B	Sheep polyclonal	XA	1:350 WB
EBNA3C A10	Mouse monoclonal	Hybridoma cell line	1:10 WB
EBNA-LP JF186	Mouse monoclonal	[335]	1:500 WB
FLAG M2	Mouse monoclonal	Sigma	1:2000 WB, IF
Goat IgG	Rabbit-HRP	Sigma	1:2000 IF
Human ABCB1	Rabbit polyclonal	Abcam	1:200 WB
Human ARHGEF4	Goat polyclonal	Abcam	1:250 WB
Human β -actin	Mouse monoclonal	Sigma	1:2000 WB
Human Fibrillarin	Rabbit polyclonal	Abcam	1:100 IF
Human IL1R2	Mouse polyclonal	Abcam	1:500 WB
Human P53 DO1	Mouse monoclonal	SCB	1:1000 WB
Human P53 PAb240	Mouse monoclonal	Abcam	1:2000 WB
Human P73	Mouse monoclonal	Abcam	1:5000 WB
Human Δ N P73	Rabbit polyclonal	Abcam	1:1000 WB
Human RPL22	Rabbit polyclonal	Prof Steitz	1:40 IF 1:15000 WB
Human N-term RPL22	Goat polyclonal	SCB	1:2000 WB
Human RPL26	Rabbit polyclonal	Abcam	1:500 IF
LMP1 CS 1-4	Mouse monoclonal	DAKO	1:500 WB
Mouse IgG	Goat-DyLight 680LT	LI-COR	1:20000 WB

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Antigen	Antibody	Source	Dilution
Mouse IgG	Goat-TRITC	Sigma	1:10000 IF
Mouse IgG	Sheep-HRP	GE healthcare	1:2000 WB
Mouse P53	Goat polyclonal	SCB	1:1000 WB
Rabbit IgG	Goat-DyLight 800	LI-COR	1:20000 WB
Rabbit IgG	Goat-HRP	DAKO	1:2000 WB
Rabbit IgG	Goat-TRITC	Sigma	1:1000 IF

2.5.7 Immunoprecipitation

One 10 cm dish of 80-95% confluent HEK 293 cells or 4×10^6 LCLs were harvested (section 2.2.5) and subsequently lysed (section 2.5.1) in 3-5 $\times$ pellet volume of Jenkins lysis buffer. Protein G Sepharose beads (Sigma) were equilibrated by washing 3 $\times$ with 1 ml cold lysis buffer (1000 $\times$ g, 2 min, 4°C). 10 μ g selective antibody (Sigma) was bound to the beads by incubation at 4°C for 1 h before washing 3 $\times$ with 1 ml cold lysis buffer (1000 $\times$ g, 2 min, 4°C). 1 mg protein extract was pre-cleared on equilibrated beads at 4°C for 1 h and the supernatant pulled down on antibody coupled beads at 4°C for 2 h. Pulled down protein was washed in 3 $\times$ 1 ml cold lysis buffer (1000 $\times$ g, 2 min, 4°C) and attained in 100 μ l sample buffer for 15 min at 4°C before heating to 95°C for 5 min. Beads were spun down (2000 $\times$ g, 2 min 4°C) and free protein used for further investigation.

2.5.8 Ribosomal fractionation

LCLs were diluted to 6×10^5 cells/ml and incubated for 48 h at 37°C. 7×10^7 cells were treated with 0.1 mg/ml cycloheximide (Sigma) for 5 min at RT and harvested as described (section 2.2.5). The cell pellet was lysed in 250 μ l ribosome fractionation lysis buffer containing 1% (v/v) Triton X-100 and 40 U/ml RNasin (Promega) for 20 min on ice and the lysate cleared by centrifugation (12,000 $\times$ g, 10 min, 4°C). The lysate was carefully loaded on top of a 10-40% (w/v) sucrose gradient and centrifuged at 50,000 rpm for

33 min in an SW55Ti rotor in a precooled Sorwall Discovery SE ultracentrifuge. The gradient was fractionated (Akta prime) using an external peristaltic pump (P3 pharmica fine chemicals) at a marked flow rate of 2.1 at 4°C while measuring and recording (Rec 112, Amersham Bioscience) the absorbance at 280 nm. Total RNA in all fractions was analysed by Trizol extraction and gel electrophoresis as described in section 2.4.2. RNA for microarrays was extracted from pooled polysomal fractions using the RNaeasy Kit (Qiagen). RPL22 distribution was analysed by western blotting (section 2.5.5).

2.5.9 Luciferase assay

Cells were harvested (section 2.2.5), the cell pellet washed in cold PBS and lysed in 100 μ l reporter lysis buffer (Promega) for 5 min at RT. Cell suspensions were frozen at -20°C o/n to aid cell lysis. Lysates were thawed on ice and cell debris was removed by centrifugation (18,000 $\times$ g, 4°C, 3 min) in a microcentrifuge. 20 μ l of sample supernatant (5 μ l for 293 transfections) was assayed for 10 s on a Luminometer Omega Fluostar with 50 μ l of luciferase assay substrate (Promega).

2.5.10 MBP-MS2 purification

2.5.10.1 MBP-MS2 purification on amylose

5 ml LB supplemented with 100 μ g/ml amp and 2% (w/v) glucose was inoculated with a single bacterial colony of XL1 Blue containing pMalc-MBP-MS2 (kind gift of Prof Josep Vilardell) and incubated o/n at 37°C while shaking. Glucose is essential for the purification process as it suppresses the expression of bacterial amylase which can degrade amylose on the affinity beads. The o/n culture was used to inoculate 500 ml LB supplemented with 100 μ g/ml amp and 2% (w/v) glucose and the bacteria were grown at 37°C while shaking to an OD₆₀₀ of 0.5 before induction with 1.5 ml of fresh 0.1 M IPTG (0.3 mM final concentration). The bacteria were grown for an additional 2 h and harvested by centrifugation (7000 $\times$ g, 10 min, 4°C). The pellet was resuspended in 10 ml cold AB1 plus PMSF (Sigma) at a final concentration of 1 mM/g bacteria. The cell

suspension was frozen at -20°C o/n and thawed in an ice bath the next day. Cells were broken open by sonication on ice using an ultrasonic processor XL sonicator (Heat systems). 15 s pulses with 5 s breaks at a sonication strength of 5 was used for a total sonication time of 2 min. Release of protein was monitored by Bradford assay (Bio-Rad). All following steps were performed at 4°C in a cold room or on ice. Insoluble components were removed by centrifugation (14,000×g, 20 min, 4°C) and the supernatant was loaded on a 5 ml amylose resin (NEB) column (50 ml syringe plugged with glass wool) equilibrated with AB1 by washing twice in 50 ml AB1. The column was washed with 40 ml of AB1, followed by 10 ml of AB2 to lower the salt concentration in preparation for heparin chromatography. The protein was eluted with 20 ml ABE collected in 1 ml fractions. Peak fractions were identified by Bradford assay and pooled.

2.5.10.2 MBP-MS2 purification on heparin

A 15 ml Heparin Sepharose 6 Fast Flow (GE Healthcare) column (50 ml syringe plugged with glass wool) was equilibrated by washing twice with 50 ml AB2. The protein obtained in the amylose purification (section 2.5.10.1) was added to the column and the flow through was collected and loaded again to enhance binding of the protein to the column. This reloading procedure was repeated. The column was washed in 50 ml AB2 and the protein eluted in a step gradient of 5 ml of 40, 50, 55, 60, 65, 100, 200 and 400 mM KCl, made by mixing HB1 and HB2. 1 ml fractions were collected. The MBP-MS2 protein eluted around 60 mM KCl. Peak fractions were identified by Bradford assay (Bio-Rad), pooled and the protein concentration determined.

2.6 Immunofluorescence microscopy protocols

2.6.1 Immunofluorescence microscopy of cell cultures

Sterile cover slips (baked at 150°C o/n, VWR) were treated with gelatine or Poly-L-Lysine (Sigma) 1:5 in PBS for 5 min at RT and washed twice in PBS for 2 min in 6-well plates. 5×10^5 cells/well were added and incubated o/n. Cells were washed twice in PBS, fixed with 4% (v/v) paraformaldehyde (PFA, Electron Microscopy Sciences) in PBS for 45 min at RT and washed $3 \times$ in PBS before quenching with 50 mM ammonium chloride (Sigma) for 10 min at RT. After three PBS washes, the cells were permeabilised with 0.2% (v/v) Triton X-100 for 7 min, washed $4 \times$ in PBS and blocked for 30 min in 1% or 5% (w/v) BSA (Sigma) in PBS. Cells were stained with primary antibody (table 2.9) for 45 min and washed in PBS 3×5 min before incubation with secondary antibody (table 2.9). The cells were washed 3×5 min in PBS and once in dH₂O before being counterstained in 2 µg/ml DAPI and mounted in Moviol (Calbiochem) to a carrier slide. Slides were stored at 4°C in the dark and analysed with a confocal microscope (Zeiss LSM Pascal or Zeiss LSM 510 Meta).

To quantify different staining patterns by confocal fluorescence microscopy, a total of 100 cells/slide were evaluated in 2-4 stochastically chosen fields.

2.6.2 Immunofluorescence microscopy of NPC sections

Biopsies were embedded in Jung tissue freezing medium OCT (Leica instruments) and sections of 6 µm cut with a Leica CM 3050-Cryostat. Specimens on slides were stored at -80°C. Before staining, the slide was dried for 2 h at 40°C. Compartments were drawn on the slide using a Mini PapPen (Zymed) and drying for 10 min at 40°C. Specimens were washed $3 \times$ in PBS, fixed in 4% (v/v) PFA (Electron Microscopy Sciences) in PBS for 10 min and washed $3 \times$ in PBS before permeabilisation in 0.2% (v/v) Triton X-100 for 10 min. The slides were washed 3×5 min in PBS and blocked for 1 h in 5% (w/v) BSA (Sigma) in PBS. Cells were stained with primary antibody (table 2.9) o/n at RT

and washed with PBS 3×5 min before incubation with secondary antibody (table 2.9). Unbound antibody was washed off 3×5 min in PBS and the sections counterstained in 2 µg/ml DAPI with additional washing in PBS 3×5 min. The specimens were mounted in Dako mounting medium (Dako), covered with coverslips and sealed with nail polish. Slides were stored at 4°C in the dark and analysed by confocal microscopy (Zeiss LSM 510 Meta).

3 EBER-regulated host cell genes

The aim of this chapter is to identify reliable and significant changes in host cell gene expression correlating with EBER1 or EBER2 expression in lymphoblastoid cell lines.

3.1 Introduction

The ability of EBERs to increase cell proliferation, enhance tumourigenicity and at the same time circumvent immune reactions and induction of apoptosis in response to the viral infection and cell transformation suggest an important role for EBERs in growth transformation and establishment of EBV-associated cancers. Some of the reported functions of EBERs are certainly performed through binding to previously described interaction partners such as RPL22, PKR, RIG-I and La protein [192, 223, 260, 284]. However, the mechanism of action of EBERs has not been elucidated in detail so far. EBERs potentially interact with multiple additional cell proteins and might control a significant number of host cell genes crucial for oncogenesis or immune evasion.

This study aims to define changes in the host cell as a consequence of EBER1 or EBER2 during EBV infection. Therefore, gene expression profiles dependent on EBER1 or EBER2 expression in a wt EBV background in LCLs were examined by microarray analysis for the first time. Microarray analysis is a powerful tool for highly efficient analysis of genome-wide transcriptional changes in response to specific stimuli. Thousands of genes and complex regulatory networks can be evaluated in an individual experiment. Analysis of changes in host cell gene expression, during infection or

disease, delivers valuable insights into immune response, pathogenicity or can define diagnostic markers [336].

The EBER target genes revealed by this approach may help to elucidate EBER functions during EBV infection and might propose a mechanism for previously suggested roles of the EBERs. Furthermore, clarification of the mechanism of EBER action might assist the understanding of the mechanism of action of other functional RNAs.

EBERs are abundantly expressed in EBV-associated malignancies. Understanding EBER function and mechanism of action might therefore also help to define new therapeutic targets and lead to the development of novel therapeutic approaches against EBV-associated cancers.

3.2 Results

3.2.1 Experimental design for the analysis of EBER effects on host cell gene expression using recombinant EBV BACs

Changes in host cell gene expression correlating with EBER expression were determined, using the experimental approach outlined in figure 3.1. In order to identify individual effects of EBER1 or EBER2, novel recombinant EBV bacterial artificial chromosomes (EBV BAC) with deletions of EBER1 (Δ EBER1) or EBER2 (Δ EBER2) were constructed (section 2.3.13.1). This allowed the investigation of specific roles of EBER1 or EBER2 in host cell gene regulation in a normal wt EBV B95-8 background for the very first time. For Δ EBER1, both the EBER1 sequence and the EBER1 promoter region were deleted to avoid interference of the EBER1 promoter sequence with the EBER2 promoter in the recombinant virus BAC. Only the EBER2 sequence was deleted in Δ EBER2 recombinant virus BAC, while the promoter region was left intact.

In addition, EBER-revertant BACs were constructed (section 2.3.13.2) to complement the EBER deletion phenotype and assign a reliable set of truly EBER-regulated host cell genes. Lymphoblastoid cell lines (LCLs), EBV immortalised primary B lymphocytes,

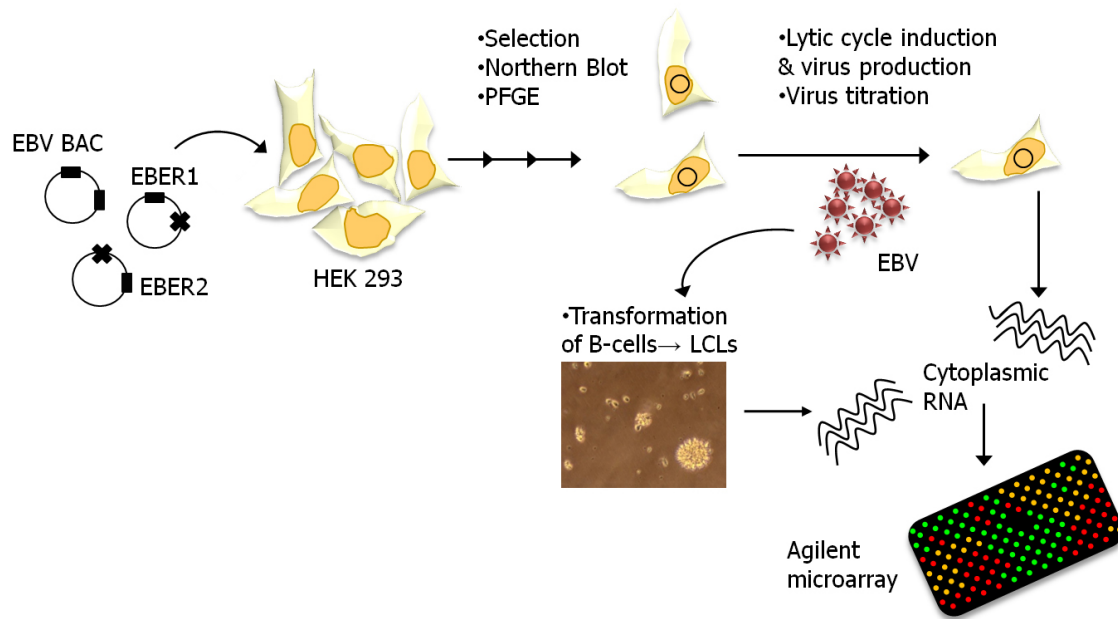


Figure 3.1: Schematic illustration of the creation of cell lines for microarray analysis.

HEK 293 cells were transfected with wt EBV, EBER deletion or EBER-revertant BACs and single clone cell lines grown under antibiotic selection. The obtained cell lines were analysed by northern blot, PFGE and virus production. Virus titers were determined by Raji cell infection and infectious virus used to transform primary B cells. Cytoplasmic RNA of selected HEK 293 and LCL cell lines was profiled by microarray analysis.

were chosen as model system ^[84]. LCLs are an ideal model system, as they represent natural EBV host cells, are continuously proliferating and easily cultivated once established. LCLs express a latency III gene expression pattern ^[5, 47] and therefore resemble the early stages of B cell infection *in vivo* (figure 1.3).

In a first step towards obtaining the desired LCLs, the constructed BACs were transfected into HEK 293 cells and single clone cell lines grown out under selection. To ensure the quality of the transfected cell lines, EBER expression was tested by northern blotting and the composition of the BACs in the cell lines was controlled by PFGE. In a next step, lytic cycle and release of viral particles from the HEK 293 cell lines was induced by transfecting the cells with BZLF1 and BALF4 expression vectors (section 2.2.9) ^[337, 338]. Subsequently, BL-derived Raji cells were infected with the obtained viral particles to determine the amount of functional, infectious virus produced. The titered virus was used to immortalise primary B cells into LCLs. Finally, cytoplasmic RNA of selected cell lines was analysed on Agilent whole human genome microarray chips (figure 3.1).

This labour-intensive part of the project was performed as a team in cooperation with Dr Goran Gregorovic. The contribution of the different members of the team will be accredited here for clarity. Prof Paul J Farrell and I constructed EBER BACs. HEK 293 single clone cell lines were produced and tested by PFGE and for virus production by Dr Goran Gregorovic and me. I performed northern blot analysis of all cell lines, whereas Dr Goran Gregorovic established the LCLs used in the microarrays. All figures in this thesis are the direct result of my own work, unless indicated otherwise.

The subsequent sections in this chapter will follow the experimental design for microarray analysis outlined in figure 3.1.

3.2.2 Construction of EBER-revertant BACs

EBER-revertant BACs were constructed from EBER deletion BACs to revert effects of EBER deletion on host cell gene expression. EBER1 or EBER2 was reintroduced into the corresponding EBER deletion BAC from pKov-KanF-SucS-EBER1 containing wild-type EBV bases 6066 to 7322 or pKov-KanF-SucS-EBER2 containing wild-type EBV bases 6669 to 7413. An overview of the cloning process is illustrated in figure 3.2.

E.coli cells containing EBER deletion BACs were co-transformed with pDF2.5-tet-RecA and pKov-KanF-SucS-EBER plasmid to mediate cointegration of pKov-KanF-SucS-EBER into EBER deletion BACs by RecA activity. Selected single colonies were subsequently cured of pDF2.5-tet-RecA on selective plates at 43°C. Integration of pKov-KanF-SucS-EBER into EBER deletion BAC was confirmed by colony PCR (section 2.3.9) using primers KovKanFw and 5201, described in table 2.4. The PCR was analysed by agarose gel electrophoresis and the expected product of 411 bp detected in the majority of plasmids tested as shown for EBER2 cointegrant in figure 3.3. 26 colonies were analysed and 20 found to have the EBER2 shuttle plasmid integrated.

Chemically competent bacteria were established from colonies containing correct cointegrants to allow reintroduction of RecA (pDF2.5-tet-RecA) to resolve the cointegrants. Transformed bacteria were selected on chloramphenicol/tetracyclin plates

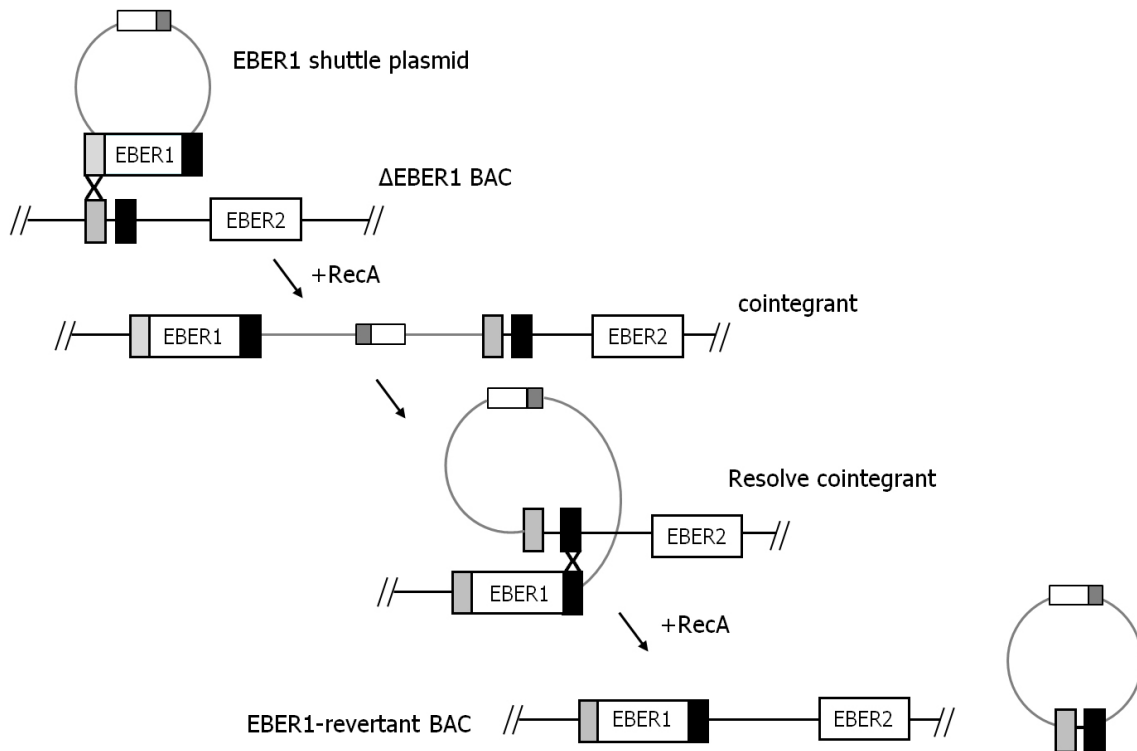


Figure 3.2: Schematic illustration of EBER-revertant construction.

pKov-KanF-SucS-EBER1 shuttle plasmid is integrated into Δ EBER1 BAC by RecA activity. Subsequently, RecA mediates a second recombination, whereby correctly resolved BACs maintain EBER1 reintegrated in the BAC backbone, restoring wt sequence.

at 30°C to allow RecA activity. Colonies were then selected against integrated pKov-KanF-SucS-EBER2 plasmids on sucrose plus chloramphenicol plates and incubated o/n at 43°C to lose pDF2.5-tet-RecA. Single colonies were plated in replica on chloramphenicol/kanamycin and chloramphenicol only plates. Bacteria with a mutated SacB gene are able to grow under sucrose selection but correctly resolved BACs were identified by being sensitive to kanamycin.

Correct integration of EBERs into the EBER deletion BACs was confirmed by PCR over

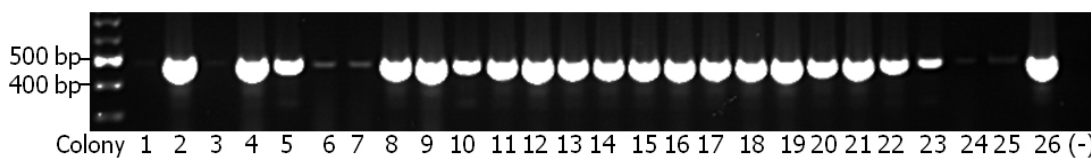


Figure 3.3: Colony PCR of EBER2 cointegrants.

The integration of pKov-KanF-SucS-EBER2 into EBER2 deletion BAC was confirmed by colony PCR using primers KovKanFw and 5201 in the first step of EBER2-revertant BAC cloning. (-): no DNA control.

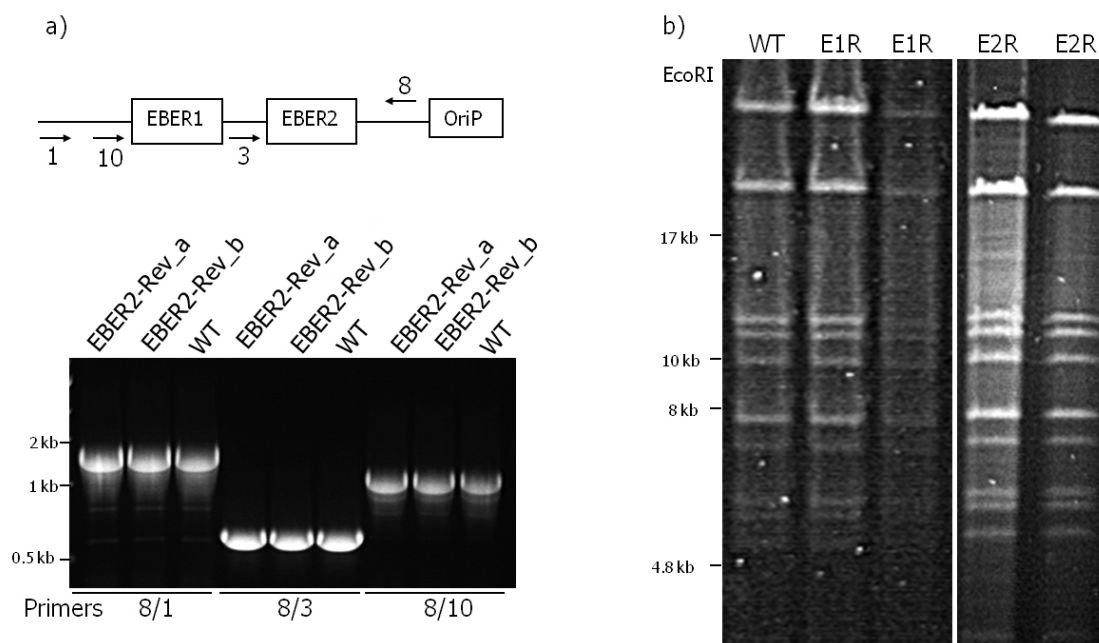


Figure 3.4: Analysis of EBER-revertant BACs.

a) PCR analysis of EBER2-revertant BACs. Schematic demonstration of the restored EBER region in EBER-revertant BACs, showing primers used to PCR the EBER region of the revertant constructs and the wt EBV BAC. PCR analysis is shown below. Products of the expected sizes were obtained and no difference between the revertant constructs and wt was found. b) PFGE analysis of EBER-revertant BACs. BAC DNA extracted from *E.coli* was digested with EcoRI and separated by PFGE. Two constructs for EBER1-revertant (E1R) and EBER2-revertant (E2R) BACs are shown. Restriction digest patterns were identical in wt, EBER1-revertant and EBER2-revertant BAC constructs.

the deletion site, as can be seen for the example of EBER2 in figure 3.4 a. Three different primer sets were used to control for the correct insertion of EBER2. Reverse primer 8 was combined with forward primers 1, 3 or 10 (table 2.4). Target sequences for primers 1 and 10 are located upstream of EBER1 while the target sequence of primer 3 is located between EBER1 and EBER2 sequences, as indicated in the top part of figure 3.4 a. PCR was performed on EBER-revertant BACs and wt EBV BACs to compare the size of the products obtained. PCR products amplified from the revertant (figure 3.4 a, first two lanes for all primer combinations) were the same size as PCR products amplified from wt EBV BACs (figure 3.4 a, third lane for all primer combinations). Products of the expected size were obtained for all primer pairs, 1347 bp for primers 8 and 1, 614 bp for primers 8 and 3 and 1082 bp for primers 8 and 10, respectively. The PCR confirmed the correct integration of EBER2 and restoration of wt EBV sequence in the Δ EBER2 BAC.

Additionally, selected EBER-revertant constructs were compared to wt EBV BAC by restriction digest using BamHI, EcoRI or AgeI. PFGE analysis of two examples of correct EBER1-revertants and EBER2-revertants digested with EcoRI is shown in figure 3.4 b. Restriction digest patterns of all four revertant constructs and the wt sequence were identical, as expected, which confirmed that no secondary deletions or rearrangements of the BAC backbone occurred during the cloning process. These revertant constructs were subsequently used to establish HEK 293 cell lines for virus production.

3.2.3 Establishment of EBV BAC-transfected stable single clone cell lines in HEK 293 cells

HEK 293 cells were used as virus producer cell lines to obtain infectious recombinant virus for B cell transformation. To this end, HEK 293 cells were transfected by lipofection (section 2.2.6) with wt, Δ EBER1, Δ EBER2, EBER1-revertant or EBER2-revertant BACs. The BACs confer hygromycin and chloramphenicol drug resistance, allowing for selection in both eukaryotic and prokaryotic cells. The cells were grown under selection and mock transfected cells were used as negative control. After elimination of non-transfected cells, single cell colonies were picked and cultured in 96-well plates, followed by outgrowth to a larger scale. Several BAC constructs per virus type were used for transfection of HEK 293 cells. More than 20 independent cell lines were established for each wt, Δ EBER1, Δ EBER2, EBER1-revertant or EBER2-revertant BAC and at least ten cell lines per BAC type were analysed as described below.

Firstly, BAC DNA from successfully established single clone cell lines was thoroughly analysed by rescuing BACs into *E.coli*, analytical digest and PFGE (section 2.3.5). To this end, low molecular weight DNA was extracted from single clone HEK 293 cell lines and used to transform competent *E.coli*. The enriched BACs were inspected by restriction digest and PFGE and digestion patterns of revertant BACs were compared to wt BACs.

During the cloning process, an XbaI site was introduced at the point of EBER deletion to

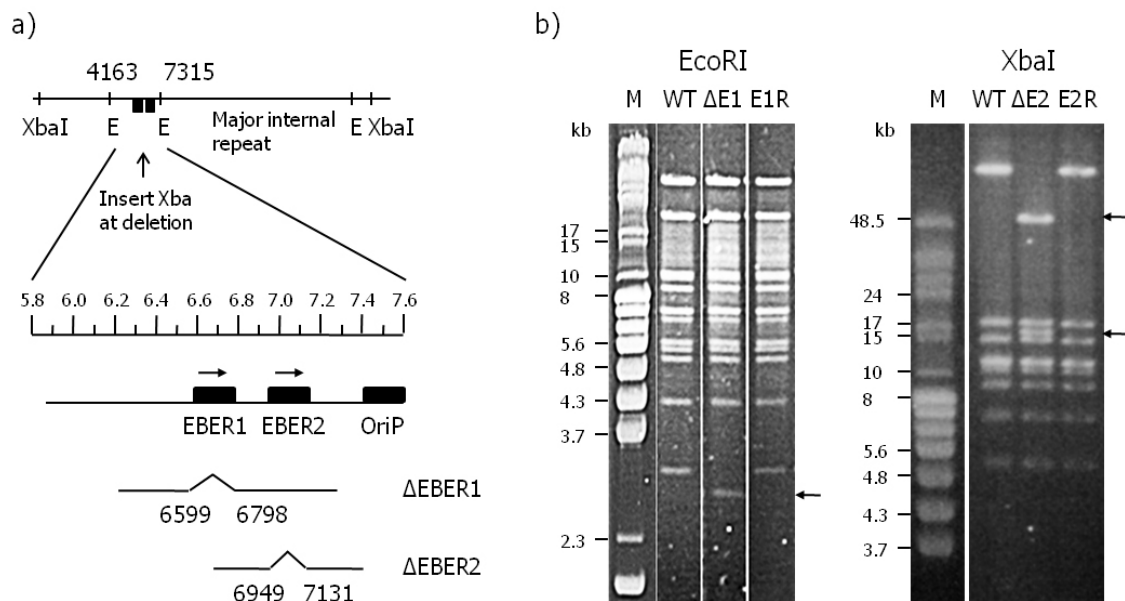


Figure 3.5: Pulsed field gel electrophoresis of EBV BACs.

a) Schematic illustration of EBER deletions introduced into wt EBV BAC. EBERs (black boxes), EcoRI (E) and XbaI restriction sites in the EBV genome are indicated. Below, EBV genome sequence (bases 5800 to 7600) is magnified and deletion of EBERs illustrated. Deletion of EBER1 includes the EBER1 sequence plus the EBER1 promoter (bases 6599 to 6798). Only the EBER2 sequence is affected in the deletion of EBER2 (bases 6949 to 7131). An additional XbaI site was introduced at the point of deletion for both EBER1 and EBER2. b) Analytical restriction digest of BAC DNA analysed by PFGE. Left panel: EcoRI digest of wt, Δ EBER1 and EBER1-revertant (E1R) BACs recovered from transfected 293 cells. The EcoRI digest fragment from bases 4163 to 7315, containing the EBER genes, was reduced in the Δ EBER1 mutant (arrow) but restored to wt length in the revertant. Right panel: XbaI digest of wt, Δ EBER2 and EBER2-revertant (E2R) BACs recovered from transfected 293 cells. An XbaI site was introduced at the point of EBER deletion. As a result, the largest XbaI fragment was split into two fragments (arrows) in the Δ EBER2 construct. The XbaI digest was performed by Dr Goran Gregorovic.

permit easy recognition of correct recombinant BACs by restriction digest and PFGE. A schematic illustration of the EBER deletion is shown in figure 3.5 a.

In the left panel of figure 3.5 b, an example of an EcoRI analytical digest of wt, Δ EBER1 and EBER1-revertant BAC recovered from single clone HEK 293 is shown. The EcoRI digest fragment from EBV bases 4163 to 7315 was reduced in size in the Δ EBER1 BAC, as indicated by the arrow, due to the absence of the EBER1 and EBER1 promoter sequence. The fragment size was restored to wt length in the EBER1-revertant BAC.

An example of an XbaI digest of a Δ EBER2 and EBER2-revertant BAC recovered from

single clone HEK 293 is displayed in the right panel of figure 3.5 b. The additional XbaI site, introduced at the point of EBER deletion, resulted in an additional band in the analytical XbaI digest and a reduction of the size of the largest XbaI fragment as marked by the arrows. The analytical digest clearly showed the changes in the restriction digest pattern expected as a result of EBER deletion, while EBER-revertants demonstrated a reversion to wt BAC sequence. Moreover, the largest EcoRI and XbaI restriction fragments spanning the major internal repeat of EBV was not changed between wt and revertant BACs, indicating that no loss of internal repeat sequences had occurred in the recombinations. The analysis demonstrates that the cloning of EBER deletion and EBER-revertant BACs was successful.

A large number of single clone HEK 293 cell lines were established and tested by PFGE. Examples of wt, Δ EBER1 and EBER1-revertant cell lines tested by analytical restriction digestion with EcoRI are shown in the top panel of figure 3.6. Correct constructs identified in the digest (white arrows) were retested by AgeI digestion (figure 3.6, lower gel) and confirmed to have maintained the correct EBV BAC sequence.

In a parallel analytical step, correct EBER expression in the single clone HEK 293 cell lines was ensured by northern blotting (section 2.4.5). Cells are able to integrate fragments of the EBV BAC, instead of containing it as an episome, to gain hygromycin resistance without EBER expression. Of the examples shown, the desired EBER expression was confirmed for all wt, Δ EBER1, Δ EBER2, EBER2-revertant and nine out of ten EBER1-revertant BAC-transfected cell lines (figure 3.7). The corresponding ethidium-bromide stain of rRNA is shown for a subset of the samples to confirm equal RNA loading on the gel. Probe specific activities and hybridisation efficiencies gave minimally weaker signals for EBER1 than EBER2 on these blots. Variations of the strength of the EBER signals observed were largely due to differences in the amount of RNA loaded (figure 3.7).

This experiment not only confirmed the absence of EBER expression in the deletion mutants, but also showed wt expression of the remaining EBER in the deletions and

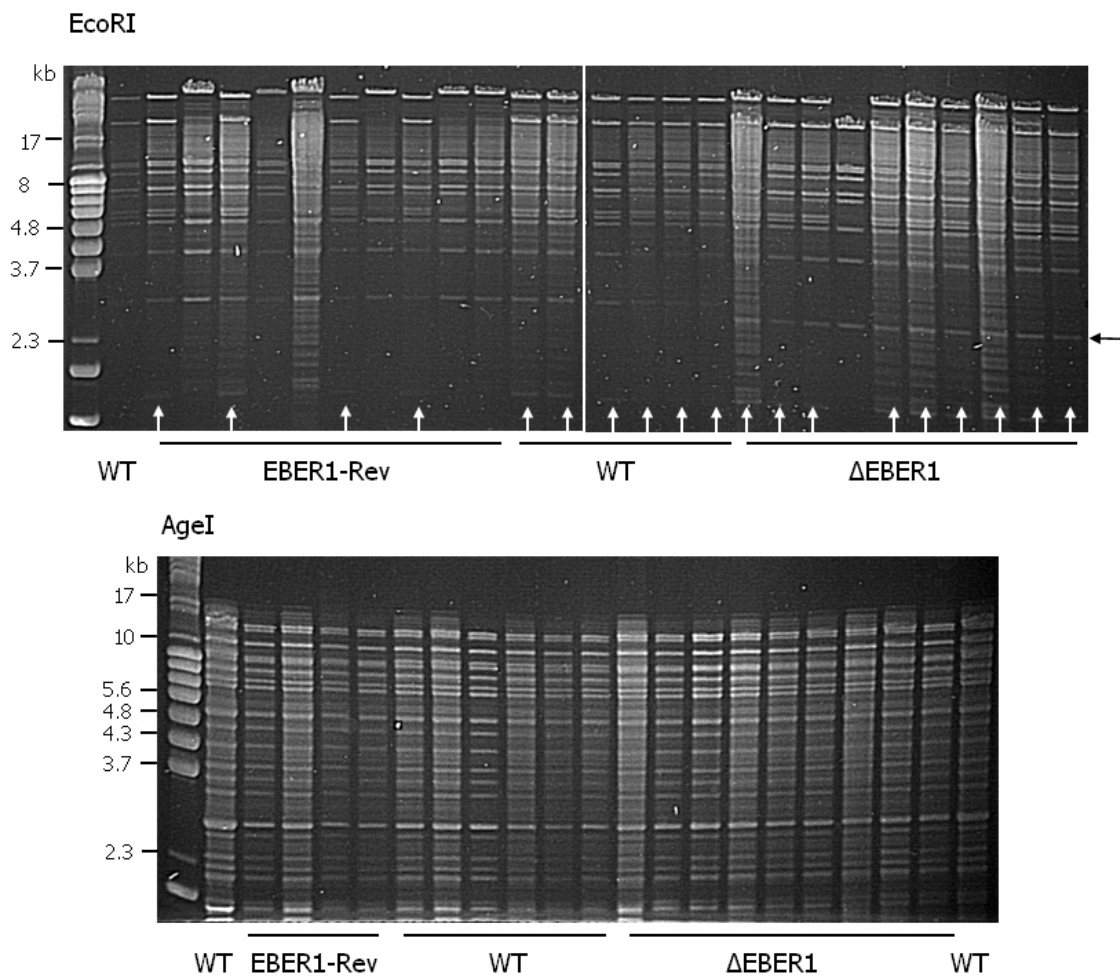


Figure 3.6: Pulsed field gel electrophoresis of EBV BACs from transfected HEK 293 cell lines. Wt, Δ EBER1 and EBER1-revertant cell lines were tested by diagnostic restriction digest with EcoRI (top gel). The EcoRI digest fragment from EBV base 4163 to 7315 was reduced in the Δ EBER1 BAC, as indicated by the black arrow, as a consequence of the absence of the EBER1 promoter and EBER1 sequence. The fragment size was restored to wt length in the EBER1-revertant BAC. Correct BACs (white arrows) were reanalysed by AgeI digest (bottom gel). All BACs tested in the second digest were confirmed to have maintained the desired BAC sequence.

restoration of EBER expression to wt levels in the revertant BAC-transfected cells.

HEK 293 cells with the correct EBER expression and BAC restriction digest pattern were subsequently used to produce infectious virus as described below (section 2.2.9).

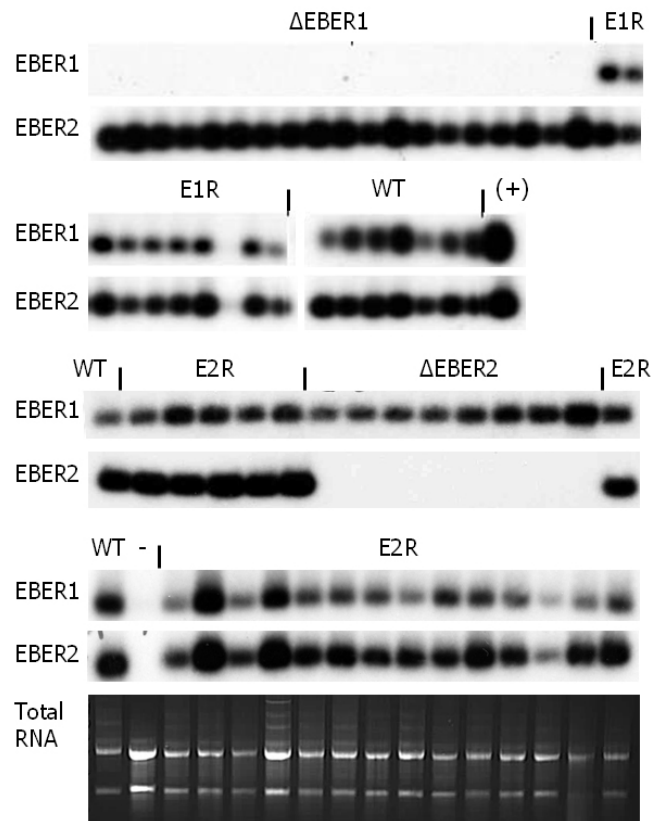


Figure 3.7: Northern blot analysis of EBER expression in HEK 293 cells.

a) EBER1 and EBER2 expression in HEK 293 single clone cell lines transfected with wt EBV, Δ EBER1, EBER1-revertant (E1R), Δ EBER2 or EBER2-revertant BACs (E2R). Stable transfection and EBER expression was confirmed in the majority of the cell lines. Variations in EBER signals were due to differences in the amounts of RNA used in the experiment.

3.2.4 Generation and titration of EBV BAC virus

The ability of single clone HEK 293 EBV BAC cell lines to produce infectious virus was tested by expressing BZLF1 and BALF4 to induce the lytic cycle ^[337, 338]. Virus was harvested from the supernatant, filtered and Raji cells were infected to quantify infectivity of the virus produced in Green Raji Units (GRU). GRUs correspond to the number of Raji cells containing GFP-expressing EBV BACs per μ l virus 4 days post-infection and are determined by fluorescence microscopy, as exemplified in figure 3.8.

GRU quantified in three independent titrations are summarised in table 3.1. Due to the complex nature of the procedure, slight variations are normally observed between independent experiments, depending on the Raji cells infected. GRUs determined for the same virus preparation of wt 1a varies from 1190 to 1640 in the independent titrations

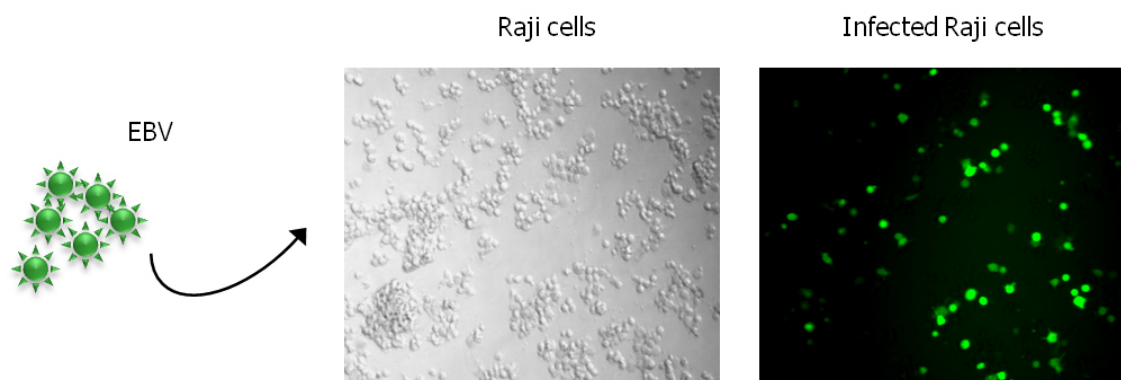


Figure 3.8: Raji cell infection for virus titration.

a) Virus harvested from HEK 293 cell supernatant after lytic cycle induction was used to infect Raji cells in dilution series. The amount of infected Raji cells with GFP-expressing EBV was quantified by fluorescence microscopy 4 days post-infection to determine Green Raji Units (GRU). The microscopy images are courtesy of Dr Goran Gregorovic.

(table 3.1). The ability of cell lines to produce virus varied strongly, ranging from 1640 GRUs for wt 1a to 0.1 GRUs for EBER2 revertant 5 in the examples shown here. However, the goal of this experiment was to define cell lines able to produce infectious virus in any quantity to transform primary B lymphocytes into LCLs. Cell lines unable to produce virus might have EBV BACs integrated into the genome.

Table 3.1: Green Raji units (GRU) produced by HEK 293 cell lines upon lytic cycle induction determined in three independent Raji infections, titration 1-3. The amount of virus produced varied between different cell lines. (-) no PMA and n-Butyrate control.

Titration 3		Titration 2		Titration 1	
Cell line	GRU	Cell line	GRU	Cell line	GRU
wt 1a	1190	wt 1a	1640	wt 1a	1550
wt 2	130	wt 1b	970	wt 1a (-)	1070
wt 3	66	wt 4	0.4	wt 1b	500
Δ EBER1 1	56	EBER2-Rev 1	0.5	EBER2-Rev 1	0.9
Δ EBER1 2	9	EBER2-Rev 2	0	EBER2-Rev 5	0.1
Δ EBER1 3	533	EBER2-Rev 3	0	EBER2-Rev 6	0
EBER1-Rev 1	8	EBER2-Rev 4	0	EBER2-Rev 7	0

3.2.5 Microarray analysis and identification of EBER-regulated host cell genes in LCLs

LCLs were established by infecting primary B cells with infectious virus derived from HEK 293 cells ^[66]. Four to six independent cytoplasmic RNA samples for each EBV BAC background were analysed on Agilent microarray whole human genome gene chips. This allowed the investigation of expression profiles of more than 41,000 unique genes and transcripts. The LCLs used for this experiment were established using B cells from buffy coat from the same pool of donors to avoid differences in gene expression profiles as a result of variations in the B cell background.

Microarray hybridisations were performed by Dr Oliver Dittrich-Breiholz (Medizinische Hochschule Hannover) as part of the INCA EU grant consortium. Bioinformatic analysis of the data was performed by Dr Rainer Russ (Genedata, Basel) and our own laboratory, using both Genedata Expressionist and Partek Genomics Suite software.

Unsupervised clustering of the data by principal component analysis (PCA), using the covariance method, showed very clear differences in LCL gene expression patterns correlating with EBER expression ^[66]. PCA is a powerful tool to highlight similarities and differences and identify patterns in large amounts of data by compressing the given information to three dimensions. PCA of LCL gene expression can be seen in figure 3.9 a. Every dot in the PCA represents an individual cytoplasmic RNA sample. Expression patterns of Δ EBER1 and Δ EBER2 EBV transformed LCLs separated away from wt LCLs and a strong aggregation of the Δ EBER2 samples was observed. The clustering of Δ EBER1 LCL samples was less pronounced, but the samples were still evidently isolated from wt or revertant samples. This demonstrates a systematic difference in gene expression patterns in dependence of EBER1 or EBER2.

Analysis of variance (ANOVA) was used to identify genes whose expression levels changed with EBER expression, comparing wt or revertant cytoplasmic RNA levels with the corresponding EBER deletion RNAs. High statistical significance was achieved by applying a fold-change threshold of 2, a p-value of <0.001 and a false discovery rate of

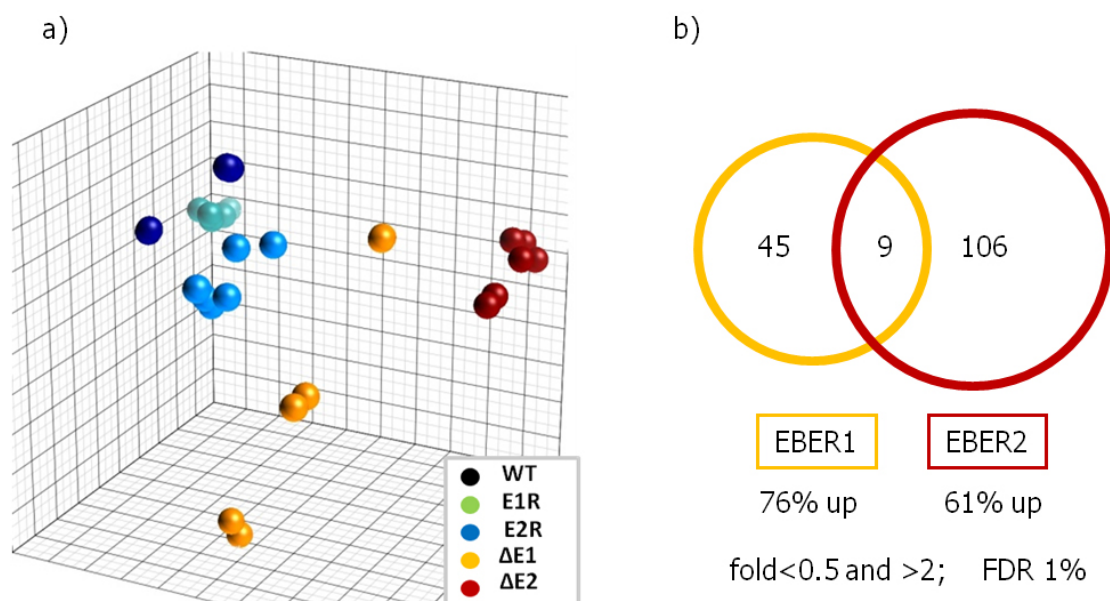


Figure 3.9: Microarray analysis.

Four to six independent cytoplasmic RNA samples of wt, Δ EBER1 (Δ E1), Δ EBER2 (Δ E2), EBER1-revertant (E1R) and EBER2-revertant (E2R) transformed LCLs were profiled by Agilent microarray analysis. a) Principal component analysis. Unsupervised clustering of gene expression patterns disclosed differences in host cell gene expression correlating with EBER expression. Δ EBER1 and Δ EBER2 deletion samples separate away from wt and revertant samples. b) Venn diagram of the number of host cell genes regulated with EBER1 or EBER2 expression. FDR: false discovery rate.

1% to the p-value. Expression levels of 54 genes were identified to change significantly with EBER1 expression by comparing Δ EBER1 with wt or revertant LCLs (appendix table 6.1). Noticeably more host cell genes were consistently regulated by EBER2, which modified expression of 115 genes, if our strict statistical criteria were applied (appendix table 6.2). Interestingly, only nine genes were regulated by both EBER1 and EBER2. This highlights the independence of EBER1 and EBER2 and the importance of investigations to identify separate, individual functions of the two RNAs. These results are summarised in a Venn diagram in figure 3.9 b.

76% of genes identified to be regulated by EBER1 were upregulated and only 24% were downregulated during EBV infection. For EBER2, 61% of genes were upregulated and 39% downregulated during EBV infection. The fact that EBERs enhance as well as decrease expression of host cell genes confirms a specific regulation of EBER target genes, in contrast to a general reduction in gene expression as a result of antiviral cell

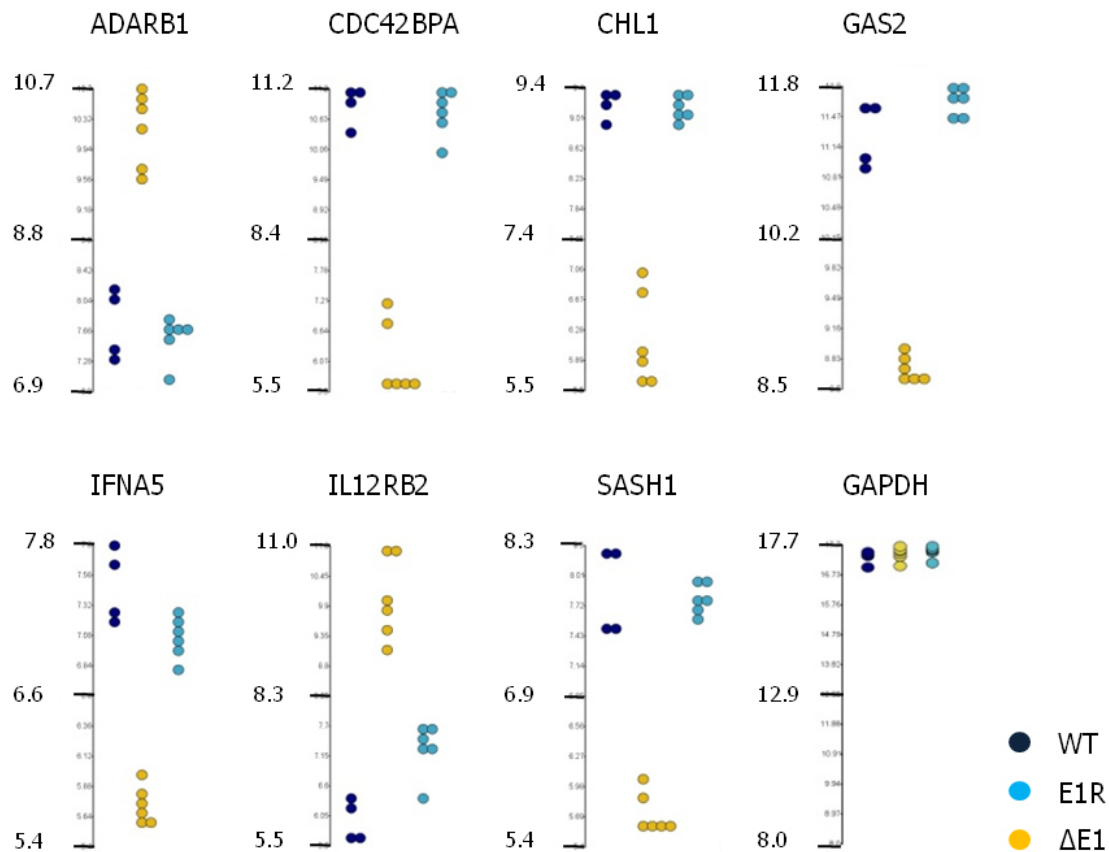


Figure 3.10: Dot plots of selected EBER1 target genes.

Each dot represents the expression level of the given gene in an individual LCL. Wt LCL expression levels (dark blue), differed significantly from Δ EBER1 expression levels (yellow). Gene expression was restored to wt level in revertant LCLs (bright blue). Given is the relative fluorescence intensity of the microarray analysis on a log 2 scale. GAPDH is shown as negative control.

responses. The complete lists of EBER-regulated host cell genes can be found in tables 6.1 and 6.2 in the appendix.

The relative expression levels of EBER targets were further studied in dot plots for both EBER1 (figure 3.10) and EBER2 (figure 3.11). Every dot represents the expression level of a given gene in an independent LCL sample. Clear differences between wt and EBER deletion LCL gene expression were observed. Moreover, the expression of individual genes was restored to wt expression levels in the revertant LCLs, proving a specific effect of EBERS on host cell gene expression. These results identified genes reliably and significantly regulated with EBER expression in an otherwise wt EBV background in a relevant host cell line for the very first time.

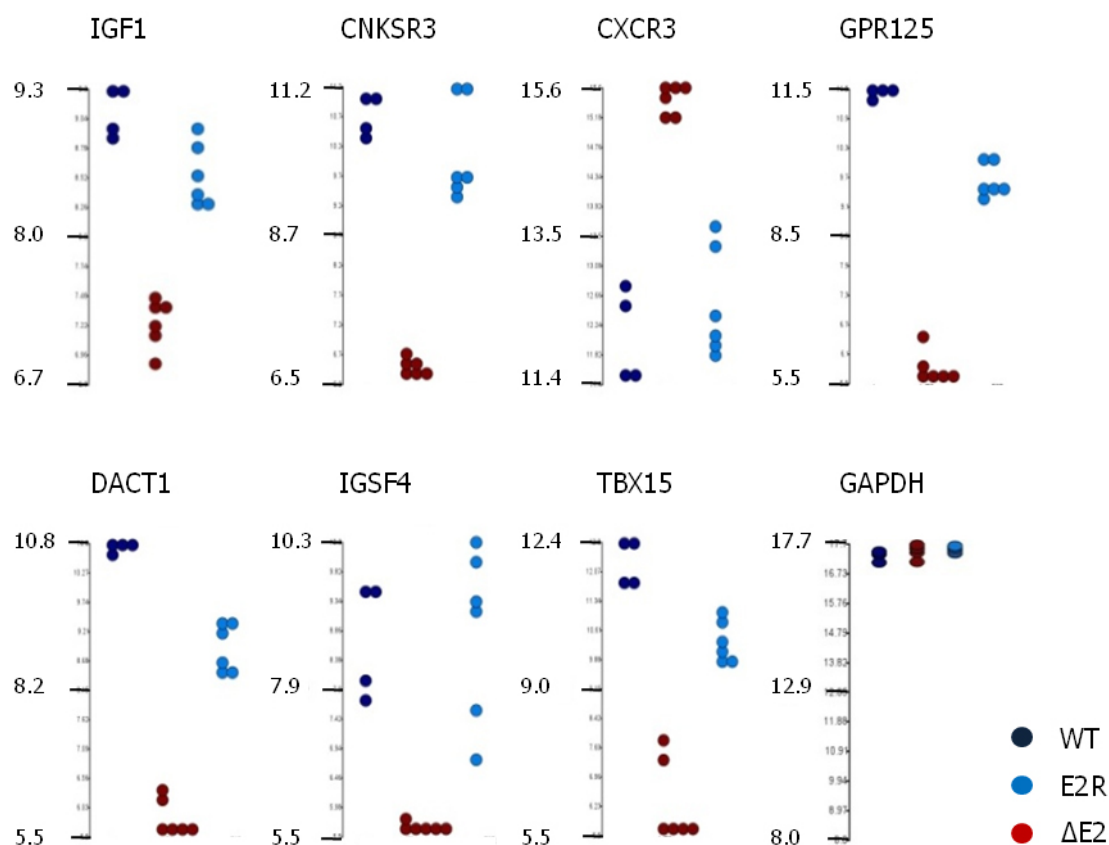


Figure 3.11: Dot plots of selected EBER2 target genes.

Each dot represents the expression level of the given gene in an individual LCL. Wt LCL expression levels (dark blue) differed significantly from ΔEBER2 expression levels (red). Gene expression was restored to wt level in revertant LCLs (bright blue). Given is the relative fluorescence intensity of the microarray analysis on a log₂ scale. GAPDH is shown as negative control.

3.2.6 Functional annotation of EBER-regulated host cell genes in LCLs

The genes regulated by EBER1 and EBER2 presented in tables 6.1 and 6.2 in the appendix were further classified into functional groups using the online Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7). Genes regulated with EBER1 expression were found to be frequently involved in antiviral responses. For example, interferon alpha 4, interferon alpha 5, interferon alpha 6 (IFNA, all upregulated with EBER1 expression) and interleukin 12 receptor beta 2 (IL12RB2, downregulated with EBER1 expression) are deregulated by EBER1. Additionally, genes regulated by

EBER1 were identified to be important in the regulation of metabolic processes, for instance RNA-specific adenosine deaminase B1 (ADARB1) was downregulated with EBER1 expression. Gene expression was altered up to 29.4-fold with EBER1 (CDC42BPA, appendix table 6.1).

EBER2 alters the expression of genes which regulate apoptosis, shown by the examples of insulin-like growth factor 1 (IGF1, upregulated with EBER2 expression), tumor necrosis factor superfamily member 10 (TNFSF10, upregulated with EBER2 expression) and tumor necrosis factor receptor superfamily member 19 (TNFRSF19, downregulated with EBER2 expression). Moreover, a significant amount of genes regulated by EBER2 are membrane signalling molecules. Examples are G protein-coupled receptor 125 (GPR125, upregulated with EBER2 expression), C-X-C motif chemokine receptor 3 (CXCR3, downregulated with EBER2 expression) and interleukin 12 receptor beta 2 (IL12RB2, downregulated with EBER2 expression). Host cell gene expression was altered up to 65.4-fold with EBER2 (TBX15, appendix table 6.2).

The nine genes regulated with both EBER1 and EBER2 are involved in a variety of biological functions and are listed in table 3.2. The gene expression levels were modified to different extents, reaching up to 43.7-fold change depending on EBER presence. The fold-change differences in gene expression with EBER1 and EBER2 were similar for seven of the nine genes. IL12RB2 and SAM and SH3 domain containing 1 (SASH1) were 1.8- and 16.8-fold stronger regulated with EBER2 than with EBER1 (table 3.2). However, EBER1 and EBER2 might have accumulative effects on these genes *in vivo*.

3.2.7 Confirmation of EBER-regulated host cell genes in LCLs

To validate the microarray results, a selection of EBER-regulated target genes was confirmed by RT-PCR. CDC42BPA, close homologue of L1 (CHL1), IFNA5 and IL12RB2 RNA levels are shown in figure 3.12 a for EBER1. The microarray analysis predicted a fold-change upregulation of 29.4 for CDC42BPA, 8.4 for CHL1 and 3.3 for IFNA5 by comparing wt expression levels with Δ EBER1 expression levels. Consistently,

Table 3.2: Genes regulated with both EBER1 and EBER2. The fold-change (Change) with EBER1 (E1) or EBER2 (E2) is listed.

Description	Gene symbol	Change with E1	Change with E2
Argininosuccinate synthetase	ASS	5.7	6.8
Carbohydrate sulfotransferase 6	CHST6	-2.1	-3.2
Coiled-coil domain containing 74B	CCDC74B	-7.8	-7.0
Homeobox hematopoietically expressed	HHEX	-4.8	-6.5
Interleukin 12 receptor beta 2	IL12RB2	-17.0	-30.5
Neurotrophic tyrosine kinase receptor type 2	NTRK2	3.9	4.2
Ring finger protein 43	RNF43	4.8	3.1
SAM and SH3 domain containing 1	SASH1	2.6	43.7
Solute carrier family 7, member 11	SLC7A11	3.0	2.3

a clear reduction of the RT-PCR products was seen in Δ EBER1 transformed LCLs (Δ E1) in comparison with wt LCL (wt) and EBER1-revertant LCLs (E1R) for all three genes. IL12RB2 was downregulated with EBER1 expression (17.0-fold) and a clear increase in the gene expression was confirmed by RT-PCR (figure 3.12 a, fourth panel) in Δ EBER1 LCLs (Δ E1) compared to wt and revertant LCLs. Expression levels of EBER2-regulated genes immunoglobulin superfamily member 4 (IGSF4), IGF1, CXCR3 and IL12RB2 were analysed in figure 3.12 b. Calculated fold upregulation with EBER2 expression was 8.1-fold for IGSF4 and 3.6-fold for IGF1. The RT-PCR results clearly confirmed the predicted effect of EBER2 on the expression of those two genes. Additionally, the downregulation of CXCR3 (10.4-fold) and IL12RB2 (30.5-fold) with EBER2 expression was also supported by the RT-PCR, which produced an increased product band in Δ EBER2 LCLs (Δ E2) compared to wt and revertant LCLs (figure 3.12 b).

The PCR was performed using freshly purified cytoplasmic RNA of individual cell lines, while the predicted fold-change in the microarray analysis is the average of at least four independent LCLs. This accounts for differences between changes in RT-PCR band intensity and calculated microarray fold-change. These results confirm the significant changes in host cell gene expression with EBER expression identified by microarray analysis.

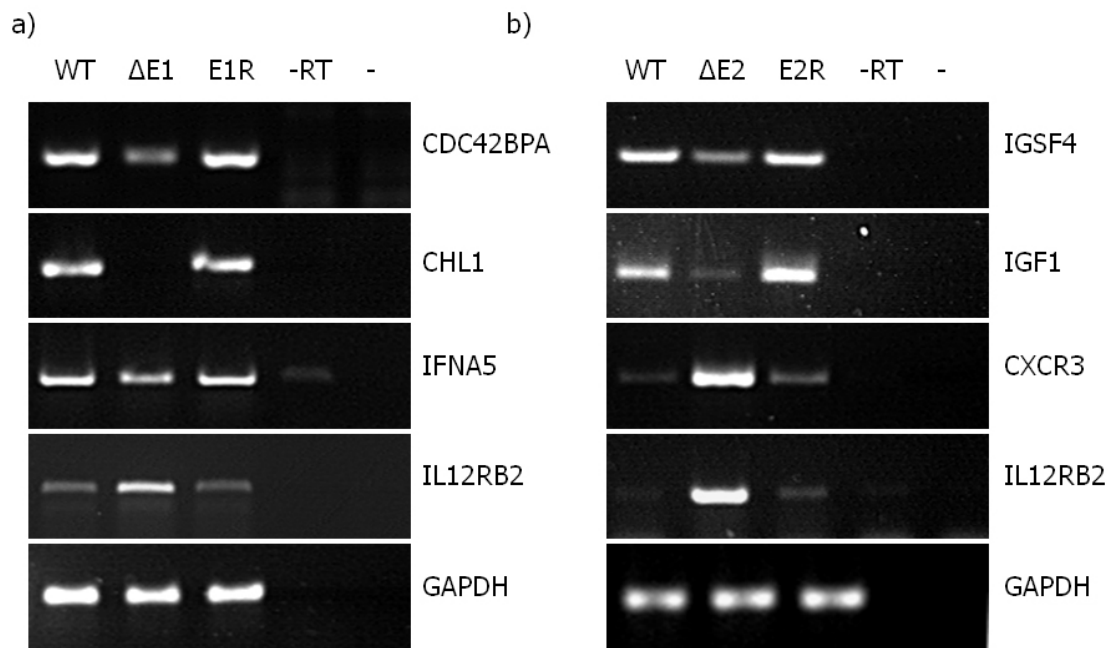


Figure 3.12: RT-PCR of EBER target genes.

a) RT-PCR of EBER1 target genes. CDC42BPA, CHL1, IFNA5 and IL12RB2 expression levels in wt, Δ EBER1 (Δ E1) and EBER1-revertant LCLs (E1R) are shown. Upregulation of CDC42BPA, CHL1, IFNA5 and downregulation of IL12RB2 with EBER1 expression was confirmed. b) RT-PCR of EBER2 target genes. IGSF4, IGF1, CXCR3 and IL12RB2 expression levels in wt, Δ EBER2 (Δ E2) and EBER2-revertant LCLs (E2R) are shown. Upregulation of IGSF4 as well as IGF1 and downregulation of CXCR3 and IL12RB2 with EBER2 expression was confirmed. GAPDH levels were included as control. -RT: no reverse transcriptase control, -: no cDNA control.

3.2.8 Microarray analysis and identification of EBER-regulated host cell genes in HEK 293 cells

Gene expression profiles of 293 cells established for virus production were also analysed on Agilent microarray chips as described above for the LCLs. Four wt, Δ EBER1, Δ EBER2, EBER1-revertant and EBER2-revertant BAC-transfected single clone cell lines were employed in the experiment. Interestingly, no significant differences in gene expression levels with EBER expression could be identified after exclusion of other variables, using the stringent criteria of a fold-change threshold of 2, a p-value of <0.001 and a false discovery rate of 1% to the p-value. This suggests a weaker effect of EBERs on gene regulation in these cells compared to LCLs, which was masked by the high statistical demands. Alternatively, EBERs might require the presence of cell type

specific molecules or cell responses to EBV infection to perform their function. This result strongly underlines the importance of the use of natural EBV host cell lines in microarray studies.

3.2.9 Attempt to complement EBER deletion effects on host cell gene expression by stable EBER expression from Puro/oriP-pSUPER plasmid in HEK 293 cells

Expression profiling of a small, independent set of HEK 293 cells transfected with EBER deletion BACs had been performed previously in our laboratory in 2007 and had identified changes in HEK 293 gene expression. This analysis did not contain EBER-revertant BACs. The next logical step in the analysis was to complement identified changes in host cell gene expression due to the absence of EBERs by reintroducing EBER1 or EBER2 expression vectors into the cells. To this end, HEK 293 cells infected with EBER deletion BACs were transfected with Puro/oriP-pSUPER plasmids carrying EBER1 or EBER2 and stable cell lines were established. EBER expression was tested in the outgrown cell lines by northern blotting and correct EBER expression patterns were confirmed in all cell lines analysed (figure 3.13). It should be noted that EBER expression levels from stably transfected Puro/oriP-pSUPER plasmids were lower than EBER expression levels from EBV BACs.

Selected host cell target genes were subsequently tested for restoration to wt EBV-infected host cell gene expression levels by RT-PCR. EBER1 target ependymin related protein 1 (EPDR1) and EBER2 targets interleukin 6 receptor (ILR6), interferon regulatory factor 5 (IRF5) and transcription elongation factor A protein like 3 (TCEAL3) were downregulated with EBERs in the microarray analysis. However, gene expressions were not changed by transfected EBER expression (figure 3.14).

WWC family member 3 (WWC3) and mutated melanoma-associated antigen 1 like protein (MUM1L1) gene expressions were also not changed by transfected EBER expression and not restored to wt levels (figure 3.15). However, downregulation of

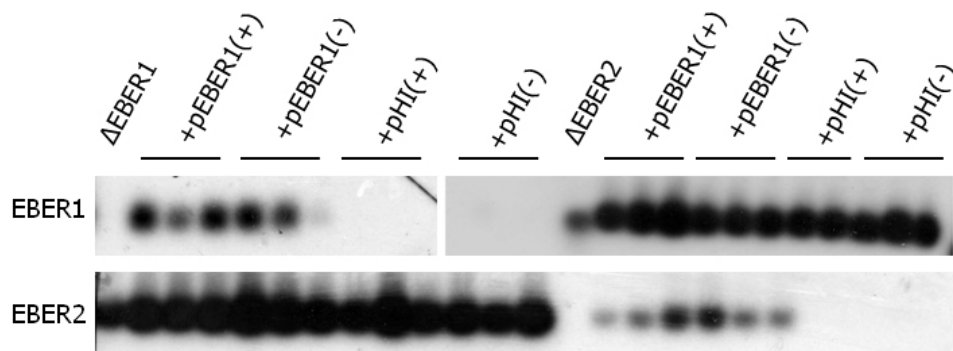


Figure 3.13: Northern blot analysis of EBER expression in HEK 293 cells.

a) EBER1 and EBER2 expression in Δ EBER1 and Δ EBER2 cells stably transfected with Puro/oriP-pSUPER-EBER1 (pEBER1), Puro/oriP-pSUPER-EBER2 (pEBER2) or Puro/oriP-pSUPER-HI promoter plasmid (pHI) as control. The inserts are carried in both possible orientations (+) and (-) and three independent cell lines were tested per plasmid type. EBER expression was confirmed after transfection with EBER carrying plasmids.

WWC3 and upregulation of MUM1L1 gene expression in wt EBV BAC-infected HEK 293 in contrast to Δ EBER2-infected cells was verified (figure 3.15) by comparing wt EBV BAC-transfected cells in lane 4 with EBER2 deletion EBV BAC-transfected cells in lane 6 (Δ E2). GAPDH levels were used to control for equal cDNA input into the PCR reaction.

The lack of complementation in this experiment indicates that EBER dependent host cell gene regulation might require EBER expression at a specific stage of viral infection or in a concentration dependent manner, as transfection with Puro/oriP-pSUPER-EBER resulted in EBER expression levels lower than EBER expression levels resulting from EBV BAC (figure 3.13). Moreover, identified differences in gene expression in this analysis might have been due to other unknown factors instead of EBER presence. Repetition of expression profiling in 2010 (section 3.2.8), including revertant BACs, did not identify significant and reliable changes with EBER expression in HEK 293. This emphasises the significance of EBER-revertant BACs to complement EBER deletion effects in the more relevant natural EBV host LCLs and the importance of the large numbers of samples employed in the subsequent microarrays (3.2.5).

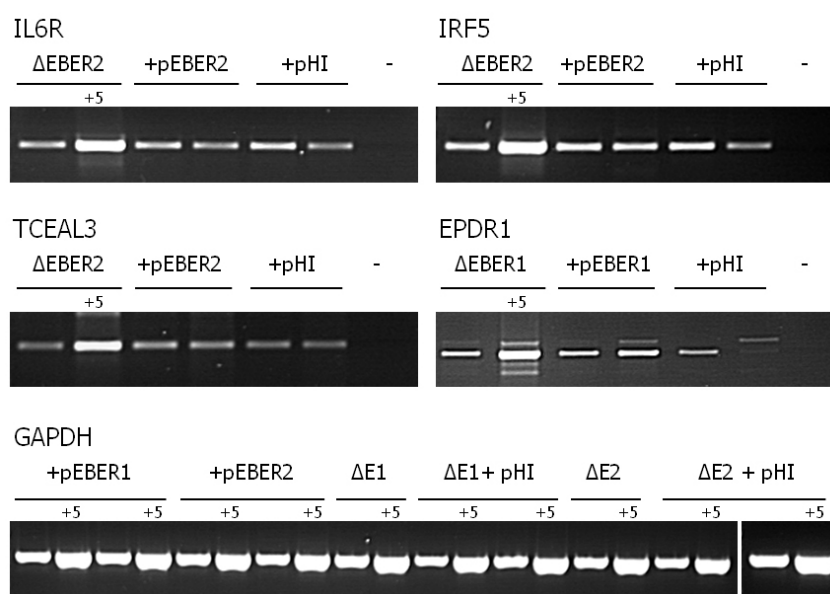


Figure 3.14: RT-PCR analysis of EBER dependent host cell gene expression in HEK 293 cells. IL6R, IRF5, TCEAL3 and EPDR1 expression in ΔEBER2 (ΔE2) or ΔEBER1 (ΔE1) cells after restoration of EBER expression by transfection with Puro/oriP-pSUPER-EBER2 (pEBER2) or Puro/oriP-pSUPER-EBER1 (pEBER1). Puro/oriP-pSUPER-HI promoter plasmid (pHI) was used as control. No difference in host cell gene expression was found after restoration of EBER expression.

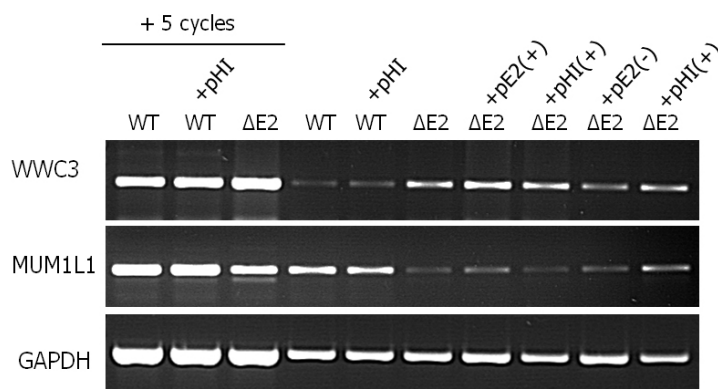


Figure 3.15: RT-PCR analysis of EBER dependent expression of WWC3 and MUM1L1 in HEK 293 cells.

WWC3 and MUM1L1 expression in ΔEBER2 HEK 293 cells (ΔE2) after restoration of EBER2 expression by transfection with Puro/oriP-pSUPER-EBER2 (pE2) in comparison with wt EBV BAC HEK 293 cells. Puro/oriP-pSUPER-HI promoter plasmid (pHI) transfection was used as control. The particular inserts are carried in both possible orientations (+) and (-). Changes in host cell gene expression after EBER2 deletion compared to wt EBV-infected cells was confirmed but EBER2 expression from the plasmid did not restore target expression levels back to wt levels.

3.2.10 Experimental design for the identification of novel EBER interaction partners in LCLs

EBER1 and EBER2 have strong effects on host cell transcription in LCLs and regulate the expression of specific sets of genes (section 3.2.5). The mechanism by which EBERs modify the expression of the identified genes is not clear yet, but EBERs could mediate their effects by interacting with cellular or viral transcription factors. The genes regulated with EBER1 and EBER2 differ significantly and consequently, the interaction with cellular proteins could be expected to differ between the two RNAs. So far, five interaction partners have been described for the EBERs (section 1.2.4). Identifying the complete sets of EBER interaction partners might elucidate the mechanism by which EBERs modulate gene expression, give new insights into additional functions of EBERs during infection and help understand the individual roles of EBER1 and EBER2.

To identify novel EBER interaction partners *in vivo*, a maltose-binding protein (MBP)-MS2 affinity purification system was established. The system is based on the strong interaction of *E.coli* bacteriophage MS2 protein with its target MS2-RNA-hairpin structure [339]. MS2 protein is a small 13.7 kDa bifunctional protein that acts as a structural component of the phage coat and is involved in translational repression of the bacteriophage replicase mRNA.

Coupling any RNA of interest to the MS2-RNA sequence provides a powerful tool for the purification of RNA-protein complexes or RNA-RNA complexes thanks to the reliable binding of MS2 to the constructed RNA [340, 341]. To enable this study, MS2-EBER expression vectors were constructed to allow expression of MS2-EBER fusion RNA in LCLs (section 2.3.14). The MS2-EBER RNA and any interaction partners bound *in vivo* can then be isolated from the LCL lysate by MS2-affinity purification (figure 3.16). To enable EBERs to fold properly into their complex secondary structures, the MS2 sequence was added either 3' or 5' of the EBERs. Further, MS2-H1 promoter and MS2-tRNA(Met) constructs were designed as negative controls to identify unspecific or MS2-RNA interaction partners.

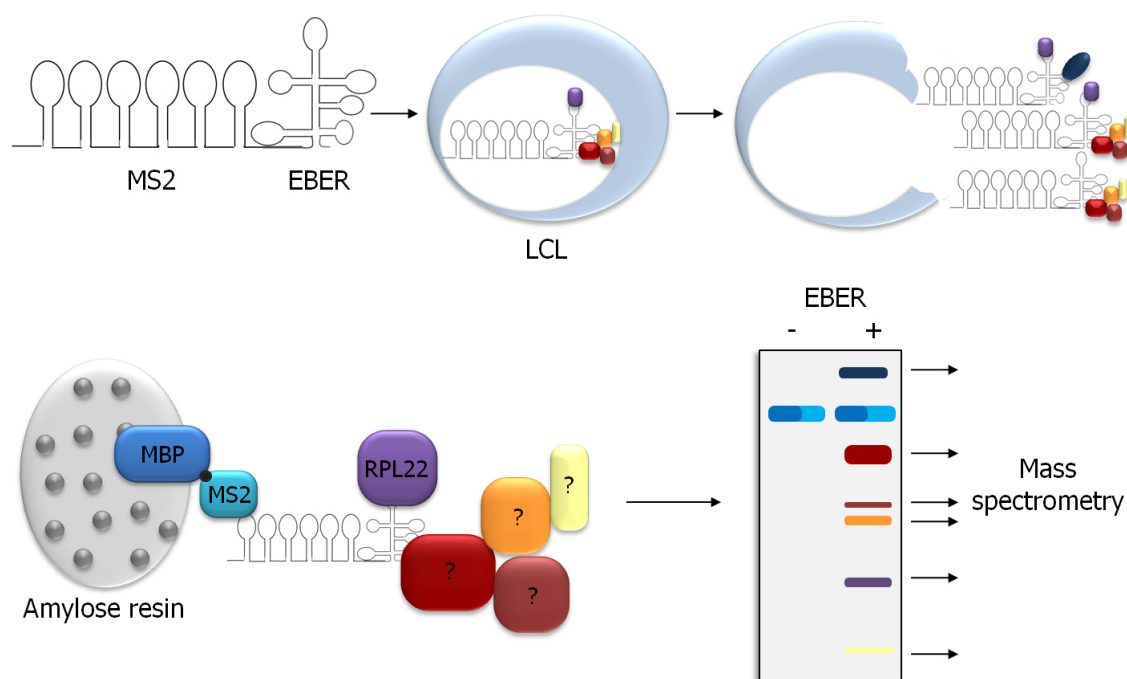


Figure 3.16: Experimental design for the identification of novel EBER interaction partners in LCLs.

MS2-EBER expression vectors were constructed and stably transfected LCLs are being outgrown currently. MS2-EBER RNAs complexed with cellular interaction partners can be purified from LCL extracts on amylose affinity resin using recombinant MBP-MS2 fusion protein. EBER interaction partners can be identified by mass spectrometry.

To purify MS2-EBER RNA-protein complexes efficiently, recombinant affinity tag adapter protein MBP-MS2 was expressed from pMalc-MBP-MS2 (kind gift of Prof Josep Vilardell) which contains MBP N-terminal of MS2, separated by a Xa protease factor recognition site. pMalc-MBP-MS2 harbours MBP encoded by the *E.coli* male gene under control of the strong, isopropylthio-b-D-galactoside (IPTG)-inducible tac promoter. The signal sequence of male which guides MBP through the cytoplasmic membrane into the periplasm is deleted in pMalc-MBP-MS2, leading to cytoplasmic expression of the fusion protein.

The combination of MS2 with MBP enables an efficient purification of the MS2 bound MS2-RNA complexes on amylose affinity resin via MBP binding, while the rest of the cell extract is washed through the amylose column. Advantageously, MBP can be released from the amylose column with free maltose under very mild conditions which are not disruptive to the other RNA-protein or protein-protein interactions. Proteins purified by

the MBP-MS2 affinity purification can subsequently be identified by mass spectrometry (figure 3.16).

Cloning of the expression vectors is described in detail in section 2.3.14. The MS2-constructs were transfected into LCLs for 24 h and 48 h using the Neon transfection system and 10 or 20 μ g vector DNA. Successful expression of the constructs was validated by RT-PCR for MS2 (figure 3.17). All constructs expressed MS2-EBER RNAs or MS2-tRNA efficiently. For pHEBo-5'MS2-E1, pHEBo-3'MS2-E1, pHEBo-5'MS2-E2 and pHEBo-5'MS2-tRNA, the best expression was achieved by transfecting 20 μ g DNA for 24 h (figure 3.17, condition 3). Generally, expression levels were stronger after 24 h than 48 h. pHEBo-3'MS2-tRNA was best expressed after 48 h using 10 μ g DNA and was stronger expressed than all the other constructs. 3'MS2-tRNA RNA might be more stable than the other MS2 RNAs. However, this conclusion would require further confirmation and the main objective of this RT-PCR was the confirmation of MS2-RNA construct expression in any quantity. Stably transfected wt EBV transformed B95-8 LCLs for all constructs are being outgrown at the moment to be used in future MBP-MS2 affinity purifications.

Recombinant MBP-MS2 protein was overexpressed in XL1 blue lacI^q strain which expresses the lac repressor to suppress tac promoter activity in the absence of IPTG. This is crucial as pMalc vectors are lethal to *E.coli* once the tac promoter is induced. MBP-MS2 was purified on amylose resin as described in section 2.5.10.1. MBP-MS2 expression was strongly induced 2 h after addition of IPTG (figure 3.18 a, lane I) compared to uninduced cells (figure 3.18 a, lane UI). A part of MBP-MS2 was present in the insoluble protein extract fraction, possibly as a result of protein aggregation due to the high protein expression levels (figure 3.18 a, lane In). The protein was efficiently purified on amylose as shown by the clear increase in MBP-MS2 eluted from amylose with 10 mM maltose in figure 3.18 a, lane E. In contrast, only very small amounts of MBP-MS2 were present in the unbound amylose column flow through and the wash fraction of the column after addition of the cell extract, indicating a strong binding of MBP to the resin (figure 3.18 a, lanes FT and W).

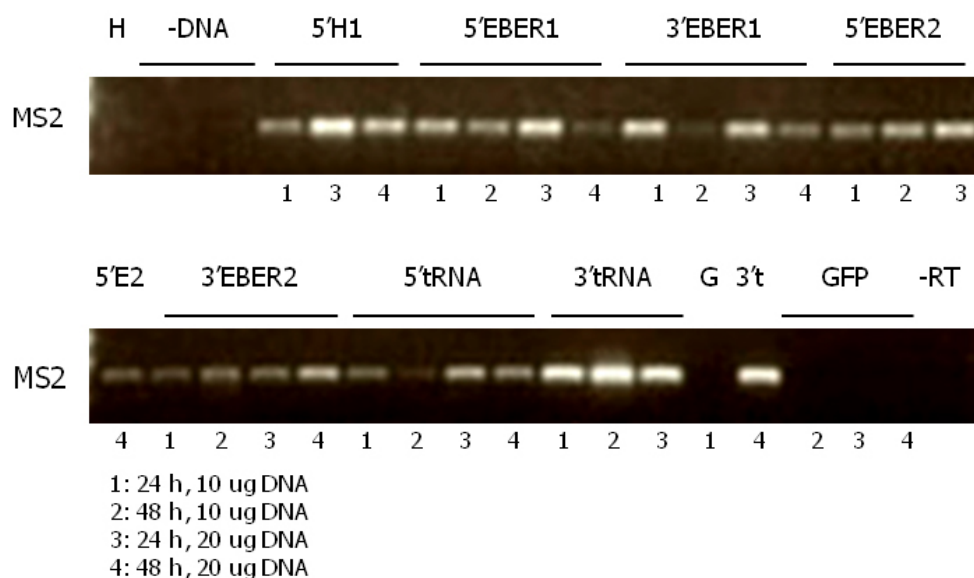


Figure 3.17: Expression of MS2-EBER constructs in LCLs.

LCLs were transfected with 10 or 20 μ g pHEBo-5'MS2-H1 (5'H1), pHEBo-5'MS2-E1 (5'EBER1), pHEBo-3'MS2-E1 (3'EBER1), pHEBo-5'MS2-E2 (5'EBER2, 5'E2), pHEBo-3'MS2-E2 (3'EBER2), pHEBo-5'MS2-tRNA (5'tRNA), pHEBo-3'MS2-tRNA (3'tRNA, 3't) or GFP control (GFP) vector DNA for 24 h and 48 h. MS2-RNA expression was confirmed by RT-PCR. H: H₂O only, -DNA: no cDNA and -RT: no reverse-transcriptase negative controls were included in the RT-PCR reaction. All constructs expressed MS2-EBER RNAs or MS2-tRNA successfully. RT-PCR was performed by Dr Goran Gregorovic.

MS2 is able to bind *E.coli* nucleic acids unspecifically during the recombinant protein expression and purification steps. This decreases the ability of MBP-MS2 to bind its substrate RNA. To remove nucleic acids from MBP-MS2, the amylose purified protein was additionally purified on heparin sepharose (section 2.5.10.1). MBP-MS2 was efficiently bound on heparin sepharose and only a minority of protein was detectable in the heparin flow through and the wash fraction (figure 3.18 b, lanes FT and W). MBP-MS2 was eluted with a step gradient of KCL (figure 3.18 b, lanes Elution).

On average, pMalc recombinant protein products constitute 20-40% of total bacterial cell protein and the typical average yield from pMalc vectors by affinity purification is 2.5-10 mg from 250 ml bacterial culture. A good yield of 7.7 mg of MBP-MS2 from 250 ml XL1 Blue culture was achieved in the experiment shown here.

This MBP-MS2 affinity purification system will be used in our laboratory to seek novel EBER-associated proteins in the future.

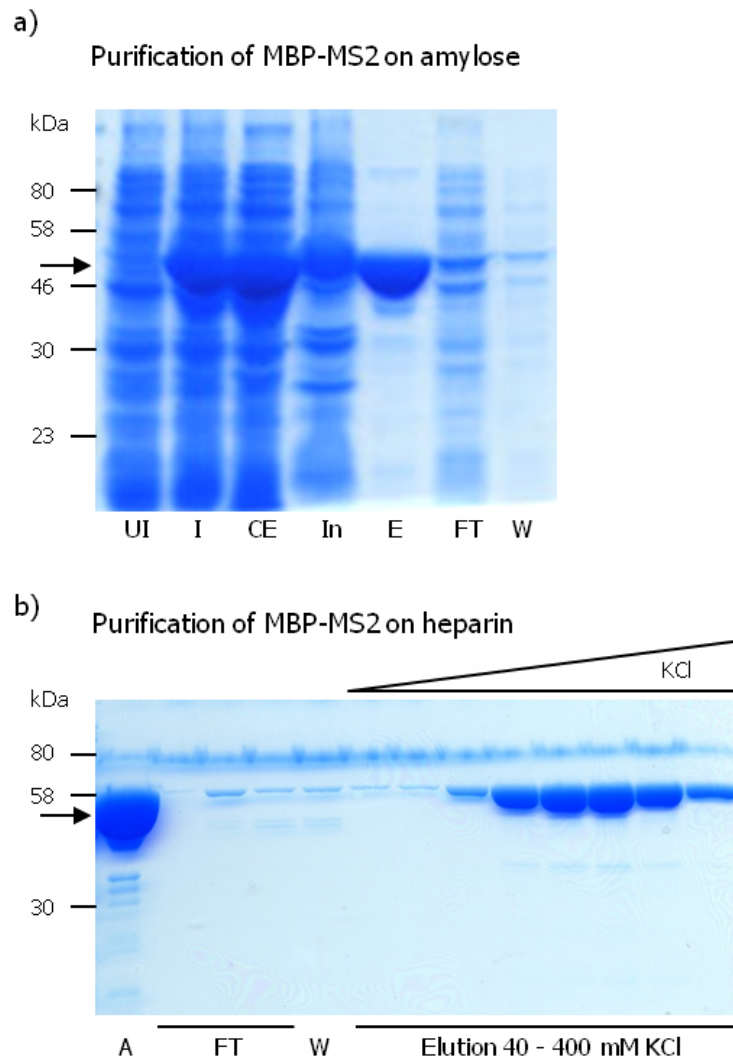


Figure 3.18: Expression and purification of MBP-MS2 protein.

- a) MBP-MS2 (arrow) was highly expressed from pMalc-MBP-MS2 in XL1 blue *E.coli* and efficiently purified on amylose resin. UI: uninduced cells, I: induced cells, CE: crude extract, In: insoluble fraction, E: elution, FT: flow through, W: wash.
- b) MBP-MS2 (arrow) purified on amylose was further purified from nucleic acid contaminants on heparin sepharose. A: amylose purified protein, FT: flow through, W: wash.

3.2.11 Analysis of EBER effects on host cell miRNA expression

To gain a complete understanding of the roles of EBERs during EBV infection and explore options for their mechanism of action, it is essential to determine possible effects of the EBERs not only on gene expression, but also on the regulation of cellular miRNAs. EBERs might regulate miRNAs to mediate changes in host cell gene expression. Herpesvirus saimiri U-rich non-coding RNAs (HSUR) dramatically downregulate miR-27 in transformed T cells [342].

Therefore, RNA sequencing of total RNA from wt EBV, Δ EBER1, Δ EBER2, EBER1-revertant and EBER2-revertant transformed LCLs is being conducted in a collaboration with Prof Bryan R Cullen at the Department of Molecular Genetics and Microbiology, Duke University. Two independent LCLs were grown per virus type and the correct EBER expression was controlled and confirmed by northern blotting (figure 3.19). 18×10^6 cells were sent to our collaborators, where deep sequencing is currently being undertaken. Identification of miRNAs changed with EBERs might enhance our understanding of EBER function and deliver a starting point for the investigating of the mechanism of action of the EBERs.

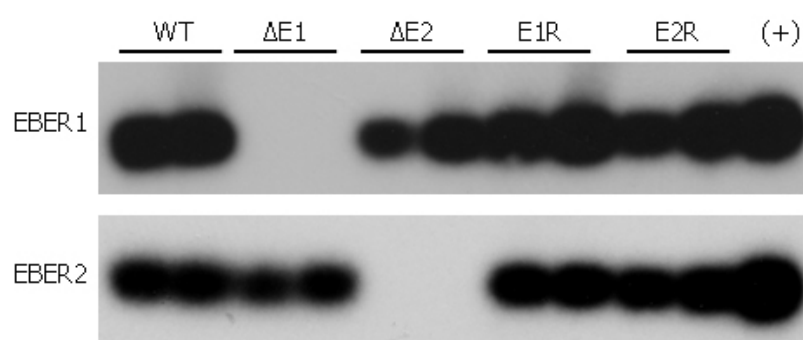


Figure 3.19: EBER expression in LCLs used for RNA sequencing.

Expression of EBER1 and EBER2 was analysed by northern blotting in two wt, Δ EBER1 (Δ E1), Δ EBER2 (Δ E2), EBER1-revertant (E1R) and EBER2-revertant (E2R) transformed LCLs. (+): EBV-positive Akata 2003 cells. All cell lines expressed EBER1 and EBER2 as expected.

3.2.12 Investigation of possible extracellular EBER functions

3.2.12.1 EBER1 and EBER2 are present in the supernatant of LCLs

It has recently been reported that EBER1 is released from infected host cells and stimulates Toll-like receptor 3 (TLR3) signalling in LCLs [259]. This interesting finding raises the question of a possible extracellular function of EBER1 in addition to intracellular effects on host cell gene expression. In our attempt to fully understand EBER functions during EBV infection, the release of EBERs into the supernatant of LCLs was investigated. To this end, 2×10^5 LCLs per ml were cultivated for 4 days as performed by Iwakiri *et al* [259]. The cells were harvested and only the top 70% of supernatant used to extract RNA, while the 30% of supernatant directly above the cell pellet was discarded to prevent a contamination of the supernatant RNA with cell-derived RNA. EBER specific primers were used for reverse transcription and subsequent PCR (table 2.4). Surprisingly, not only EBER1 but also EBER2 was detectable in the supernatant of LCLs, implying a possible extracellular role for both EBERs (figure 3.20).

In this experimental setup it is not possible to know the mechanism by which the EBERs are released into the supernatant. Iwakiri *et al* [259] suggest an active secretion of the EBERs, as only EBER1 but not EBER2 was detected in their study and as EBER1 was

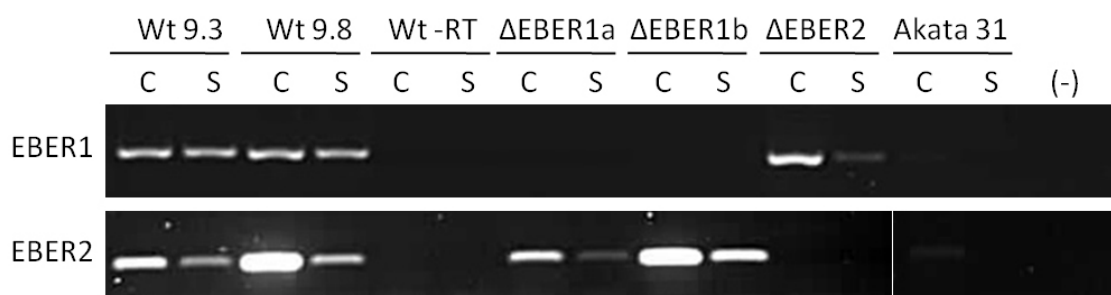


Figure 3.20: RT-PCR of EBERs in LCL supernatant.

Both EBER1 and EBER2 are present in the supernatant of LCLs. C: intracellular RNA control, S: RNA in supernatant, -RT: no reverse transcriptase control, (-): no cDNA control. All RNA extracted from supernatant and 1/10 of RNA extracted from cells was used for reverse transcription. The amounts of EBER1 and EBER2 can not be compared as different numbers of cycles were used for the EBER1 and EBER2 PCR reactions. EBV-negative Akata 31 cells were included as control.

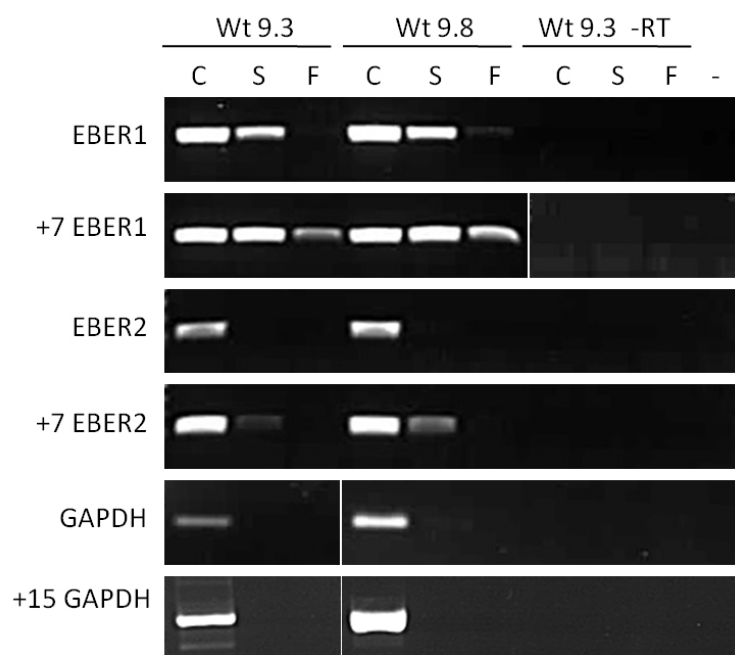


Figure 3.21: RT-PCR of EBERs in filtered LCL supernatant.

Both EBER1 and EBER2 are present in the supernatant of LCLs. EBER levels were strongly reduced in filtered supernatant ($0.22 \mu\text{m}$). More EBER1 than EBER2 was detected. GAPDH was not observed in the LCL supernatant. C: intracellular RNA, S: RNA in supernatant, F: RNA in filtered supernatant. -RT: no reverse transcriptase control, (-): no cDNA control. +7: 7 additional PCR cycles. All RNA extracted from supernatant and 1/10 of RNA extracted from cells was used for reverse transcription.

found in complex with La. However, it is possible that EBERs detected in the supernatant are released from dying LCLs and are not actively exported. The reported lack of EBER2 in the supernatant could be a result of a reduced stability of EBER2 in the supernatant compared to EBER1, in accordance with the faster rate of turnover of EBER2 [211].

In a preliminary effort to understand the release of EBERs from LCLs, the LCL-derived supernatant was filtered using a $0.22 \mu\text{m}$ Nalgene syringe filter. EBER1 was still observed in the filtered supernatant (figure 3.21, top two panels, lanes F), but was reduced significantly compared to unfiltered supernatant (figure 3.21, top two panels, lanes S). EBER2 levels in the supernatant were lower compared to EBER1 and reduced below detection in this experiment after filtration of the supernatant (figure 3.21, middle two panels, lanes S and F). The filter size used allows removal of cell debris. The strong reduction in EBER presence in the supernatant after filtration could suggest cell death as at least a partial reason for EBER release. A small proportion of EBERs or

EBER-protein complexes might also be adsorbed onto the cellulose acetate filter but further investigations will be required to clarify the release of EBERs to the supernatant.

3.2.12.2 EBER1 in LCL supernatant does not induce TLR3 signalling in U-937 cells

EBER1 released from infected cells could significantly contribute to EBV pathogenesis by induction of antiviral responses in adjacent cells. Induction of IFN- β by EBER1 was investigated to test if EBER1 activates TLR3 signalling and might thereby enhance EBV pathogenesis [343]. In collaboration with Dr Ute Meier at Barts and The London School of Medicine and Dentistry, effects of EBERs on U-937 human leukaemic monocyte cells were analysed using a firefly luciferase assay.

pIFN- β vector containing firefly luciferase under the control of the IFN- β promoter was kindly provided by Prof Steve Goodbourn [329]. pGL3 control vector (Promega) containing luciferase under the control of the SV40 promoter was used as positive control for transfection and luciferase assay. All vectors were controlled in a pilot experiment using HEK 293 cells and lipofection. As shown in figure 3.22, co-transfection of pIFN- β with synthetic double-stranded RNA poly(I:C) resulted in a strong induction of the IFN- β promoter and luciferase activity. The relative luminescence was 70% of that of pGL3 positive control reaction, proving functionality of the vector and the luciferase system. It is important to note that poly(I:C) was transfected into the HEK 293 cells. Induction of IFN- β could therefore be a result of intracellular dsRNA sensors such as RIG-I, MDA5 or PKR in addition to TLR3. The primary goal of this experiment was confirmation of the functionality of the vectors used. Transfection of pIFN- β without stimulant led to a luciferase signal of 30% compared to pGL3 control. This might be the result of cellular stress in response to the transfection or leaky expression of firefly luciferase from the vector.

Transfection of pIFN- β and addition of poly(I:C) to U-937 cells induced a modest IFN- β promoter activation (figure 3.23 a). In contrast, supernatant from wt or Δ EBER1 LCLs induced no detectable IFN- β promoter activity and no difference between wt and Δ EBER1 LCL supernatant was observed. The concentration of EBER1 in the LCL

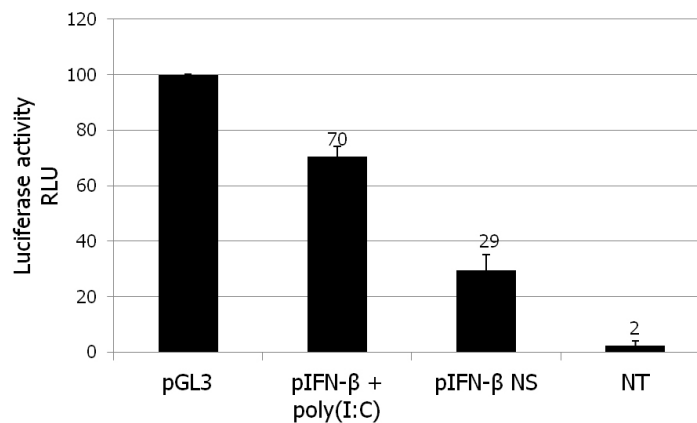


Figure 3.22: Luciferase assay in HEK 293 cells.

A strong IFN- β induction in response to poly(I:C) was observed. pGL3: firefly luciferase control, pIFN- β + poly(I:C): co-transfection of IFN- β promoter firefly luciferase and poly(I:C), pIFN- β NS: IFN- β promoter firefly luciferase without stimulant, NT: non-transfected cells.

supernatant might have been too low to mediate a TLR3 response in U-937 as even the response to the potent poly(I:C) was low. Alternatively, only small amounts of TLR3 might be expressed in U-937 cells.

For future experiments, it would be beneficial to control for TLR3 expression on the U-937 cell surface. However, aided by CD14, dsRNA can be internalised and delivered to TLR3-containing endosomes [344]; addition of poly(I:C) to the cells consistently resulted in IFN- β induction. The importance of EBER1-induced TLR3 signalling in EBV pathogenesis remains debatable and requires further attention to be resolved.

The effects of EBERs released from infected cells on neighbouring cells will be further investigated in collaboration with Dr Michiel Pegtel, VU University Medical Centre Amsterdam.

3.3 Discussion: EBER-regulated host cell genes

In this study, a reliable set of LCL genes regulated with EBER1 or EBER2 expression was identified and published [66] (appendix tables 6.1 and 6.2). Novel EBV BACs containing a deletion of EBER1 or EBER2, or revertants of the EBER1 or EBER2 mutants, were

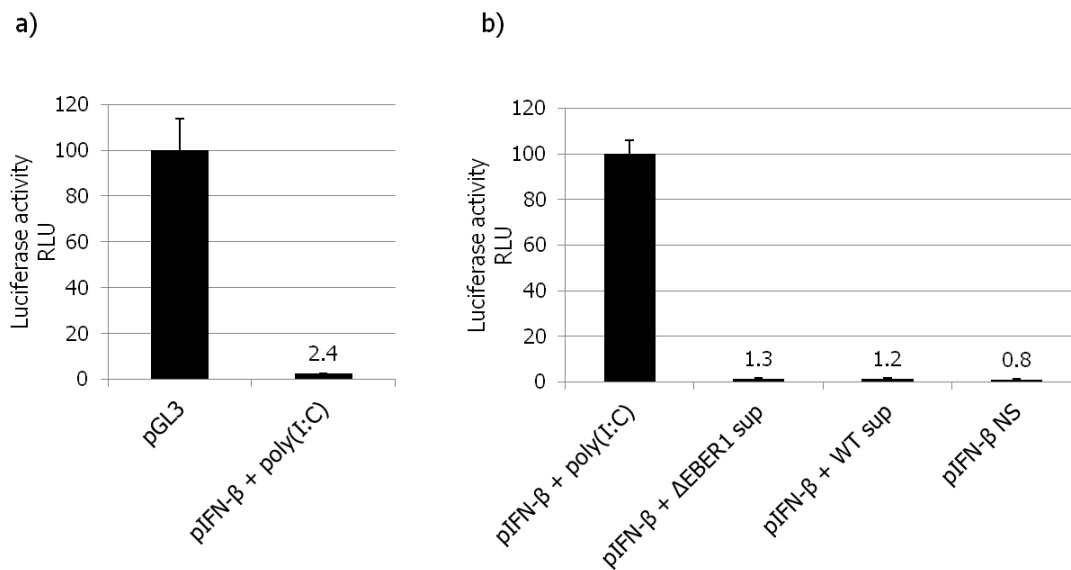


Figure 3.23: Luciferase assay in U-937 cells.

a) A modest IFN- β induction in response to poly(I:C) compared to pGL3 control was observed. pGL3: firefly luciferase control, pIFN- β + poly(I:C): transfection of IFN- β promoter firefly luciferase and addition of poly(I:C). b) No IFN- β induction in response to EBERs in LCL supernatant was observed. pIFN- β + poly(I:C): transfection of IFN- β promoter firefly luciferase and addition of poly(I:C), pIFN- β + Δ EBER1 sup: transfection of IFN- β promoter firefly luciferase and addition of supernatant from Δ EBER1 LCLs, pIFN- β + WT sup: transfection of IFN- β promoter firefly luciferase and addition of supernatant from wt LCLs, pIFN- β NS: transfection of IFN- β promoter firefly luciferase without stimulant.

constructed and transfected into HEK 293 cells alongside wt B95-8 EBV BACs (figure 3.1). Cytoplasmic RNA of LCLs transformed with the different virus types was used for gene expression profiling. The results gained from LCLs are of special importance since B cells are natural host cells of EBV and LCLs resemble the early stages of infected naive B cells *in vivo* (figure 1.3). Cytoplasmic RNA was used for the expression profiling as opposed to total RNA. The analysis of changes in cytoplasmic RNA levels allows the discovery of changes in the mature processed RNA, thereby including transcription, RNA maturation, export to the cytoplasm and RNA stability. Furthermore, omitting the nuclear RNA fraction from the array vastly reduces the complexity of the RNA analysed, which in turn enriches the rarer transcript population and permits the discovery of the differential expression of otherwise missed genes. Moreover, cytoplasmic RNA arrays are more reproducible as the variability of nuclear RNA species is eliminated. This also diminishes the number of false positives ^[345].

The work presented here identified complete, broad range effects of EBERs on host cell gene expression in LCLs for the first time. 54 genes were found to be differentially regulated with EBER1 and 115 genes with EBER2 expression. EBER2 is involved in the regulation of more than double the number of genes regulated by EBER1, which highlights the functional difference between EBER1 and EBER2. Surprisingly, only 9 genes were regulated by both EBER1 and EBER2 (table 3.2). This again emphasises diverse biological roles for EBER1 and EBER2 during EBV infection and stresses the necessity of individual examinations of the two RNAs.

A completely independent microarray experiment was performed in our laboratory in 2008 (^[66] and unpublished data) to compare gene expression in Δ EBER1 and Δ EBER2 LCLs to wt EBV LCLs. Many genes regulated with EBERs identified in this current study were reproduced in the unrelated 2008 experiment, for example IGSF4 and SASH1. The previous microarray missed EBER-revertants and was performed using LCLs derived from tonsil B cells and microarrays in dual-mode with B95-8 LCLs as reference. EBER targets were confirmed despite these variations, thus further increasing the reliability and significance of the data presented here.

Microarray studies in NPC helped to identify EBV-regulated host cell genes which might enhance tumorigenicity and invasion of the carcinoma cells ^[346]. Further investigations showed a correlation of EBER1 hybridisation signals with changes in expression of cell cycle regulators such as pRb, cyclin D1 and CDK4 in NPC epithelial tissue samples ^[347]. None of these genes were found to be regulated with EBER1 in our study. However, the discrepancy is expected, considering the different cell types used and the enormous variation in the experimental technique employed.

A recent microarray study by Eilebrecht *et al* ^[348] used EBER2 expression vectors to show EBER2 dependent regulation of proteins involved in cytoskeletal dynamics in HeLa cells. None of the genes regulated by EBER2 in LCLs were identified in their publication. However, the experiment was performed in non-natural EBV host cells, in the absence of any other EBV components and with a less stringent statistical threshold than our

work, using a p-value of <0.01 in contrast to our p-value of <0.001 . Moreover, in HEK 293 cells, another strongly transformed cell line, no effect of EBER1 or EBER2 was discovered in our study. Comparison of the publications shows the crucial differences of EBER effects in different EBV-infected cells and emphasises the importance of thorough investigations focused on specific and natural EBV host cells.

3.3.1 EBER1 and EBER2 regulate different sets of host cell genes

The microarray studies described in this thesis identified distinct sets of host cell genes to be regulated with EBER1 or EBER2 independently. EBER1 modifies the expression of 54 genes (appendix table 6.1), while EBER2 alters the expression of 115 genes in LCLs (appendix table 6.2). Most importantly, only 9 genes were regulated with the presence of both EBER1 and EBER2 (table 3.2). This finding demonstrates the different effects of EBER1 and EBER2 on the host cell in unprecedented clarity.

Existing knowledge of EBER1 and EBER2 functions during EBV infection had long suggested different roles for the two RNAs. The EBERs clearly differ in their sequence and their average stability which results in a ten-fold higher level of EBER1 compared to EBER2 ^[211]. Moreover, downregulation of EBER1 in response to lytic cycle induction is stronger than downregulation of EBER2, possibly as the result of individual regulation by independent transcription factors from the EBER promoter regions (figure 1.5). Moreover, only EBER1 interacts with RPL22 ^[224] and is also more efficient in the inhibition of PKR activation ^[261]. Finally, autocrine growth factors are induced specifically in response to EBER1 or EBER2 ^[65, 287].

The genes regulated by EBER1 or EBER2 were classified into functional groups using the online Database for Annotation, Visualization and Integrated Discovery (DAVID). The established, very general functional groups were identified as antiviral responses and metabolic processes for EBER1 and regulation of apoptosis and membrane signalling for EBER2 and highlight again different functions of EBER1 and EBER2.

In the following paragraphs, some of the most fascinating genes in the functional groups

identified will be discussed in further detail, speculating how their previously described functions could relate to EBV infection.

3.3.2 EBER1 regulates the expression of 54 host cell genes

EBER1 regulates genes often involved in antiviral responses and several IFN- α species (IFNA4, IFNA5, IFNA6) were found to be upregulated with EBER1 expression (appendix table 6.1). Viral RNA is a potent inducer of IFN in a normal antiviral response. However, IFN- α only inhibits proliferation and outgrowth of EBV-infected B lymphocytes in the first 24 hours after EBV infection [349–351]. EBER expression and thereby EBER1-mediated IFN- α upregulation begins only 36 h post-infection [47] and the growth and survival of established LCLs are no longer sensitive to IFN inhibition [351, 352]. EBERs as well as EBV nuclear antigen 2 confer resistance to the anti-proliferative and anti-apoptotic effects of IFN- α [274, 309, 353]. The induction of IFN- α by EBER1 would therefore not be valuable for the host antiviral defense. On the other hand, enhanced IFN- α/β expression might be beneficial for the establishment of latent EBV infection by preventing superinfection of EBV-positive cells with other viruses and by inhibiting EBV replication and onset of the lytic cycle [354, 355].

Consistent with this idea, murine gammaherpesvirus 68 (MHV68), which is closely related to the human gammaherpesviruses, utilises the cellular IFN response to regulate latency and reactivation [356, 357]. IFN- α/β induced during MHV68 infection upregulates IRF2, which binds interferon-stimulated response elements (ISRE) to repress the transcription of MHV68 M2 protein. M2 is a functional analog of LMP2A and promotes entry of infected B cells into GC reaction and differentiation into plasma cells. Suppression of M2 is essential for the establishment of latency, while overexpression of M2 leads to high rates of lytic cycle induction and uncontrolled viral replication. EBER1-induced IFN- α could suppress viral gene expression and replication to facilitate persistent infection. The LMP1 promoter and EBNA1 Qp promoter can be regulated by IRFs [358, 359].

Additionally, genes regulated with EBER1 were identified to be often important in the regulation of metabolic processes, as for example RNA-specific adenosine deaminase B1 (ADARB1). ADARB1 is important in cell pre-mRNA editing ^[360] and the RNA editing of Hepatitis delta virus antigenome ^[361], proposing a general, as yet undiscovered purpose of ADARB1 in viral infections.

Further interesting examples of genes regulated with EBER1 are CDC42BPA and CHL1 (figure 3.12 a), two proteins implicated in cancer formation and metastasis. CDC42BPA is a serine/threonine protein kinase ^[362] involved in cytoskeleton rearrangement in development and cell invasion ^[363] and was identified as a putative anti-cancer drug target in transformed stem cells ^[364]. In addition, CDC42BP is also upregulated in pancreatic cancer ^[365]. Similarly, CHL1 is commonly deregulated in cancer and upregulated by EBER1. CHL1 expression is decreased in primary tumours but enhanced in invasive metastatic growth ^[366]. Curiously, CHL1 is also implicated in schizophrenia ^[367] and depression ^[368, 369]. Deregulation of these genes could suggest a potential mechanism for EBER1 to support malignant growth in the development of EBV-associated cancers.

3.3.3 EBER2 regulates the expression of 115 host cell genes

Strikingly, EBER2 modifies expression levels of more than double the number of genes regulated with EBER1. Moreover, EBER2 leads to a stronger maximal effect on gene expression levels of a fold-change of 65.4, while the maximal fold-change of a gene regulated with EBER1 is 29.4-fold. Although EBER2 is less abundant than EBER1 ^[211], the stronger effect of EBER2 on the host cell gene expression might suggest a more relevant role of EBER2 than previously assumed.

EBER2 alters the expression of genes involved in the regulation of apoptosis (appendix table 6.2). A member of this group, and one of the most striking target genes of EBER2, is IGF1, which is upregulated 3.6-fold by EBER2. IGF1 is a ligand of cell surface receptor IGF1R, which mediates mitogenic and anti-apoptotic activities and regulates

proliferation, growth and differentiation in normal cells [370]. Consequently, the role of IGFR1 in cancer has been investigated extensively [370, 371] and many tumours display high levels of IGFR1, while IGF1 is essential for the survival of transformed cells. Moreover, cellular oncogene c-MYC induces IGF1 and IGFR, in contrast to tumour suppressors, such as p53, p73 and p63, which suppress IGFR1 expression. As a result, IGFR1 is an established target in cancer therapy. Anti-IGFR1 antibodies and low molecular weight tyrosine kinase inhibitors are used to inhibit growth and enhance apoptosis in cancer cells [370, 371].

The importance of IGF1 regulation in EBV infection is strengthened by findings by Iwakiri *et al* [104, 301], which demonstrated IGF1 induction in NPC and GC cell lines and the potential of secreted IGF1 to act as an autocrine growth factor. This not only confirms our results, but establishes a role for IGF1 in at least three independent EBV host cell types. Both previous studies observed growth promoting abilities of EBER-induced IGF1 under low serum conditions in 0.1% FBS. Analysing the proliferation of LCLs after serum deprivation could potentially reveal enhanced LCL growth dependent on EBER2. Further comparison of different EBV-infected natural EBV host cell types might give additional information about genes consistently regulated with EBER expression in contrast to cell-specific activities of the EBERs.

IGF1 signalling is also altered by other known oncogenic viruses in addition to EBV. Rous sarcoma virus protein pp60sar phosphorylates and thereby activates IGF1R during infection [372]. Hepatitis B virus X protein stimulates IGFR1 transcription and increases IGF1 binding [373].

Work performed in our laboratory found that EBER2 does not enhance B cell transformation [66]. Nevertheless, EBER2 might become essential in later stages of tumour formation, as IGF1 clearly plays an important role in the development of cancer. It is therefore crucial to investigate functions of the EBERs in an *in vivo* model system in addition to LCLs. To this end, humanised mice will be infected with Δ EBER BAC EBV in collaboration with Prof Christian Munz, University of Zurich. This *in vivo* model for EBV infection will deliver novel insights to the roles of EBERs in oncogenesis and

immune evasion as well as possible alternate, as yet unexplored EBER effects.

Interestingly, the reported deregulation of IL-6 in LCLs transformed with EBV Akata was not observed in our microarrays and might explain the difference in the role of EBER2 for the establishment of LCLs between our study [66] and Wu *et al* [65]. The latter study reported that EBER2 improves B cell transformation rates and growth of LCLs at low cell density by increasing IL-6 expression (section 1.2.5.1).

A significant amount of genes regulated with EBER2 are membrane signalling molecules. CXCR3, receptor for chemokine (C-X-C motif) ligands CXCL9, CXCL10 and CXCL11, directs chemotaxis of immune cells and can relay inhibition of angiogenesis in tumour growth [374]. Our observation of the downregulation of CXCR3 in infection was confirmed by Piovan *et al* [375], who reported a reduction of CXCR3 in EBV-transformed LCLs compared to B lymphocytes in blood. Interestingly, CXCR3 levels are not downregulated in B cell lymphomas derived from intraperitoneal injection with peripheral blood mononuclear cells of EBV seropositive donors in SCID mice [375, 376]. This suggests a specific role of CXCR3 in EBV infection during early stages of B cell infection, represented by LCLs. CXCR3 is rarely expressed on naive B cells, but is increased in activated B cells in normal B cell maturation together with enhanced migration to CXCR3 ligands [377]. Likely, this enhanced migration results in an accumulation of B cells in CXCL9-rich B cell follicles [378]. It might therefore be hypothesised that EBER2 downregulates CXCR3 to allow re-circulation of EBV-infected B cells from follicular bodies, following the normal B cell differentiation route.

Another EBER2 upregulated gene is IGSF4 (figure 3.12 b). IGSF4 overexpression is implicated in tumour invasion. On the other hand, IGSF4 can act as a tumour suppressor in cancers of epithelial origin [379]. Interestingly, human T-lymphotropic virus can induce IGSF4 in adult T cell leukaemia, which implies an important role of IGSF4 in the development of oncogenic virus-associated malignancies.

3.3.4 EBER1 and EBER2 regulate 9 host cell genes in common

Only nine host cell genes are regulated by both EBER1 and EBER2. The regulation of these genes could be a consequence of a cell response to viral RNA or might suggest a special importance of the regulation of these genes during EBV infection. Some of the genes regulated by both EBER1 and EBER2 have previously been implicated in very interesting biological functions, which gives room for speculation about the role of the genes during EBV infection. Strikingly, a large fraction of the nine genes regulated by both EBERs are deregulated in cancer to support cell proliferation and prevent apoptosis. RNF43 is a HAP95 binding ubiquitin ligase that promotes cell growth and is frequently upregulated in colon cancer [380, 381]. Supporting an involvement of the EBERs in oncogenesis, RNF43 is upregulated 4.8- and 3.1-fold in wt LCLs compared to Δ EBER1 or Δ EBER2 LCLs, respectively.

Moreover, EBERs downregulate HHEX (4.8-fold by EBER1 and 6.5-fold by EBER2) a transcription factor able to reduce growth, colony-forming ability and malignancy of hepatoma cell lines [382]. The regulation of HHEX and other transcription factors potentially leads to downstream control of numerous host cell genes.

Further, IL12RB2 is strongly downregulated by EBER1 (17.1-fold) and EBER2 (30.5-fold). IL12RB2 is known for its ability to promote proliferation of T cells and natural killer (NK) cells and enhances IFN- γ production [383–385]. It is also normally expressed on naive, germinal centre and memory B cells, where it induces B cells to proliferate and differentiate, but not on pro- and pre-B cells. Strikingly, IL12RB2 knockout mice develop autoimmune lymphoproliferative disorder and lung tumours. Moreover, it had previously been described that LCLs, BL-derived cell lines and B cell malignancies, such as mantle-cell lymphoma and follicular lymphoma, often silence expression of IL12RB2 by promoter hyper-methylation [386, 387]. Interestingly, IL12 inhibits proliferation and increases apoptosis if IL12RB2 is induced in these tumours.

Our findings not only confirm the previous observation of IL12RB2 downregulation in LCLs, but also identify both EBER1 and EBER2 as mediating EBV components.

Downregulation of a tumour suppressor strongly links EBERs to oncogenesis. The methylation status of the IL12RB2 promoter depending on EBERs could easily be explored by comparing wt and Δ EBER LCLs by methylation-specific PCR and might reveal an as yet unknown role of EBERs in epigenetic regulation. It could be hoped that suppression of EBER action in EBV-associated tumours would upregulate IL12RB and render the tumour cells sensitive to IL12 signalled apoptosis.

To analyse whether EBER1 and EBER2 have accumulative effects on the regulation of the nine genes regulated by both EBERs, EBER1-EBER2 double deletion mutant LCLs would have to be compared to single EBER deletion mutants and wt EBV LCLs. Double EBER deletion mutants might also reveal additional EBER targets regulated synergistically by EBER1 and EBER2. However, this current project used individual deletions of EBER1 and EBER2 to focus on the unique effects as well as common functions of the EBERs.

3.3.5 Possible mechanisms of action for EBER-mediated gene regulation

The mechanism by which EBERs regulate host cell gene expression has so far remained unclear and can only be speculated upon. Several possibilities seem reasonable and might explain how EBERs regulate genes on the transcriptional level identified here as well as suggest additional roles for EBERs in post-transcriptional control.

3.3.5.1 EBERs could regulate host cell gene expression via cellular interaction partners

Firstly, EBERs might induce changes in host cell gene expression by interaction with known interaction partners such as La, PKR, RIG-I and RPL22 ^[192, 223, 260, 284] (section 1.2.4).

EBER1 might regulate host cell genes via its interaction with RPL22. RPL22 has been shown to deregulate more than 1000 genes in *Drosophila*, possibly by its association

with linker histone H1 and colocalisation on condensed chromatin [244]. EBERs could be involved in epigenetic modifications such as hypermethylation of certain cellular promoters, histone modification, chromatin remodeling or expression of histone variants. Epigenetic changes take place during reactivation of EBV [388, 389] and might be a way for EBERs to sustain latency.

Alternatively, EBERs might alter translation of RNA transcripts in favor of internal ribosomal entry sites (IRES) since many mRNAs containing an IRES have crucial functions in proliferation, differentiation and apoptosis [390]. RPL22 binds to the conserved 98 nt 3'X untranslated region of Hepatitis C virus and stimulates IRES-mediated translation [248]. EBER interaction partner La protein is also extensively linked to IRES-mediated translation in cellular and viral translation. La mediates IRES translation in poliovirus, human immunodeficiency virus and Hepatitis C virus [252–254, 391]. Interaction with EBERs might sequester La from this function or EBERs might direct La to an EBER target IRES, if present in the cytoplasm. However, La is generally associated with all RNA polymerase III transcripts, including 5S rRNA and tRNA, to facilitate processing, folding and subcellular localisation of these regulatory non-coding RNAs [392]. This is potentially the only reason for the interaction of EBERs with La. Alternatively, recombinant La has been found to inhibit activation of interferon-inducible protein kinase PKR by sequestering double-stranded RNA [255].

EBER1 interacts with PKR, a key mediator of antiviral effects and prevents its dimerisation *in vitro*, which is required for autophosphorylation and activation, thereby preventing IFN-induced apoptosis and abrogation of protein translation [260, 261, 273, 274, 276]. PKR is part of a complex signalling network not only involved in the regulation of the translation machinery but also in the control of cell growth, apoptosis and cell differentiation. Numerous cellular proteins, including the stress activated JNK, p38 MAPK, p53, NF- κ B and the transcription factors STAT1 and STAT3, are regulated by PKR [270, 393] (figure 1.8). Further work is needed to determine which downstream signalling is activated by EBER1 and to establish detailed putative modification of the roles of PKR in its signalling network caused by EBER1 binding *in*

vivo.

EBERs are detected by several immune modulators. RIG-I leads to the induction of IFN- α , IFN- β and IL-10 expression in BL-derived cell lines via NF- κ B and interferon regulatory factor 3 (IRF3) in response to EBER binding [284, 287]. IL-10 acts as an autocrine growth factor in BL-derived cell lines and assists survival of transformed cells [288]. RIG-I signalling might explain the induction of some antiviral EBER-induced host cell genes identified in this study, such as IFNA4, IFNA5 and IFNA6, which are induced by EBER1. The assignment of RIG-I could be further investigated by expressing a dominant-negative form of RIG-I in cells containing EBERs and analysis of the degree of IFN- α induction in comparison to cells expressing wt RIG-I.

Furthermore, EBER1 activates antiviral 2'-5'-OAS and as consequence the RNase L RNA degradation pathway *in vitro* [289, 394]. Recently it has become evident that at least two RNase L-independent 2'-5'-OAS-induced antiviral pathways exist [395]. 2'-5'-OAS generally recognises virus-derived dsRNA. However, controlled activation of 2'-5'-OAS and selected degradation of mRNAs by EBERs would be a powerful tool for EBV to inhibit antiviral effects.

Additionally, EBERs could influence host cell gene expression by interaction with novel EBER interaction partners. So far unidentified EBER binding partners might be cellular and viral transcription factors. To explore this avenue, MS2-EBER constructs were designed and recombinant MBP-MS2 protein purified as presented in section 3.2.10. The MS2-EBER constructs are now being expressed in LCLs and purified MBP-MS2 will be used to purify MS2-EBER-protein complexes. The proteins which interact with EBERs will subsequently be identified by mass spectrometry and might significantly advance our understanding of the modification of host cell gene expression by EBER1 or EBER2.

3.3.5.2 EBERs could regulate host cell gene expression via cellular miRNAs

Secondly, EBERs might regulate cellular miRNA to change host cell gene expression or inhibit antiviral actions of miRNAs. Virus-miRNA interactions are an integral part

of virus-host cell interaction and regulation of miRNAs induced by the innate immune response might enable immune evasion and establishment of latent infection for persistent viruses [396].

In line with this idea, herpesvirus saimiri U-rich non-coding RNA 1 (HSUR1) binds to miR-27 and leads to degradation of the mature miRNA and regulation of miR-27 targets such as transcription factor forkhead box 1 [342], thus showcasing a potent mechanism for a virus to influence a large transcriptional network to its benefits. Consistently, miR-27 is also downregulated in murine CMV infection and overexpression of miR-27 reduces viral replication [397]. Similar to EBERs, HSURs are strongly conserved but not essential for transformation [398, 399], implying an important function in *in vivo* infection. These findings support the importance of the investigation of previously discussed *in vivo* effects of EBERs in humanised mice, as well as the investigation of changes in host cell miRNA expression with EBER expression (section 3.2.11). Therefore, deep RNA sequencing of total RNA from wt EBV, Δ EBER1, Δ EBER2, EBER1-revertant and EBER2-revertant transformed LCLs is being performed in a collaboration with Prof Bryan R Cullen at the Department of Molecular Genetics and Microbiology, Duke University (section 3.2.11). Besides identifying differences in miRNA levels correlating with EBERs, deep sequencing might discover novel EBV miRNAs in LCLs, as demonstrated in NPC [400, 401].

3.3.5.3 EBERs could regulate host cell gene expression via viral miRNAs

Naturally, it is also feasible that EBERs regulate viral miRNAs, which in turn modulate both cellular and viral genes relevant to oncogenesis and immunomodulation. LMP1 [168], LMP2A [169], EBNA2 [170] and EBV DNA polymerase BALF5 [171] are all regulated by viral miRNAs. The regulation of cellular miRNA targets, such as MICB, CXCL11, PUMA and BIM, enables EBV to suppress antiviral immune responses or to prevent apoptosis [172–175]. Interestingly, miR-BART5, which regulates PUMA, is completely conserved in rhesus lymphocryptovirus [189].

EBERs themselves could theoretically be processed into miRNAs. Adenovirus VAI is

exported from the nucleus by exportin 5 (Exp5) ^[402], serves as substrate for dicer and eventually acts as miRNA ^[403, 404]. A stem loop similar to the minihelix stem region essential for Exp5-mediated export in VAI is present in EBER1 ^[402, 405]. However, EBER1 does not interact with Exp5 ^[198] and is not processed by dicer ^[404]. A possible processing of EBER2 into miRNA still needs to be investigated. It would also be interesting to compare EBER sequences with known EBV miRNA sequences.

3.3.5.4 EBERs could regulate host cell gene expression via mRNAs

Potentially, EBERs could directly regulate gene expression by binding to target mRNA to alter stability, shuttling to the cytoplasm or initiation of translation. Comparison of the EBER sequences with target gene mRNA sequences might identify potential complementary binding sites. One already identified significant feature of the EBER sequence is an RNA sequence found in the splicing site of the plasma cell specific transcription factor XBP1. XBP1 needs to be spliced into an active transcription factor in the cytoplasm ^[215] to be able to activate transcription from promoter Zp of lytic cycle regulator BZLF1 ^[214]. The splicing site sequence of the EBERs could sequester splicing enzymes from XBP1 and prevent activation of XBP1, the Zp promoter and induction of the lytic cycle.

3.3.6 EBERs have potential extracellular functions

Besides the proven effect of EBERs on the cell they are expressed in, EBER1 has also been found to be released from BL-derived Mutu and Akata cell lines as well as LCLs ^[259]. This opens interesting possibilities for EBER1 effects on neighbouring cells. Release of EBER1 was confirmed in our LCLs (figure 3.20). Additionally, EBER2 was present in the cell supernatant in addition to EBER1, but at lower levels (figure 3.21). However, TLR3 signalling was not induced by EBERs in LCL supernatant in U-937 cells used in our study (figure 3.23) in contrast to observations in LCLs by Iwakiri *et al* ^[259]. This difference might be explained by differences in the sensitivity to EBERs of

the cells tested, different amounts of EBERs released from the LCLs or technical variations. The role of EBER1 in EBV pathogenesis via TLR3 signalling requires additional in depth research.

It still needs to be elucidated by which mechanism EBERs are released into the supernatant. Filtration of LCL supernatant before RT-PCR led to a significant reduction in the amount of EBERs detected (figure 3.21). This might suggest that cell death is at least partially responsible for the presence of EBERs in the supernatant. It is unclear how EBERs detected in filtered supernatant gain access to the extracellular periphery. Possibilities are active secretion as small nucleoprotein complexes, release in exosomes (size range typically 40-100 nm) or microvesicles (size range typically 0.1-1 μ m) [315, 406]. RNase treatment of the supernatant could clarify the release of EBERs further, as lipid-enclosed RNAs are protected from RNases, while free EBERs would be degraded. Additionally, specific secretion pathways or intracellular vesicle transport, such as the RAB GTPase system or ESCRT, could be inhibited to elucidate possible roles in the secretion of EBERs. Zomer *et al* [407] reported a continuous secretion and transfer of exosomes from EBV-infected LCLs to co-cultured, non-infected cells. Exosomes can contain EBERs and are able to deliver functional miRNAs, such as BARF1, resulting in a repression of target gene expression in the recipient cell, for example CXCL11/I-TAC [407, 408].

Effects of EBERs in exosomes on adjacent cells, specifically in respect to IFN induction, are now being further investigated in a collaboration with Dr Michiel Pegtel, VU University Medical Centre Amsterdam. It would be extremely interesting to establish whether EBERs found in exosomes are able to regulate gene expression in recipient cells in a co-culturing system comparable to the gene regulation in EBER-positive donor cells. EBER1 might be able to influence cells surrounding EBV-infected cells without actual infection and thereby establish an environment favourable for EBV with minimal viral product expression and exposure to host cell immune responses.

3.3.7 EBER-regulated host cell genes: future directions

In this thesis, the overall changes in host cell gene expression with EBER expression were defined. All EBER targets, primary and secondary, were identified in the microarray performed in this study. To further elucidate the mechanism of action of EBERs, it will be crucial to define primary EBER targets in contrast to secondary effects. To this end, EBERs will be reintroduced into deletion LCLs using EBER expression vectors. Restoration of gene expression levels to wt expression levels will then be investigated in a time course to define potentially more primary target genes whose expression is restored faster.

To complement EBER deletion in the LCLs, a novel lentivirus system will be established in our laboratory, which will allow a reliable, stable and highly efficient reintroduction of EBER expression in Δ EBER EBV transformed cells. The lentivirus system will overcome restrictions of other transfection methods, such as limitations of transfection efficiency and reduction of EBER expression in stable cultures.

Additionally, it is necessary to confirm changes of EBER target gene expression on the protein level. Poor correlation is often observed between protein and RNA regulation in eukaryotic systems. Up to 30-fold changes in mRNA level can be seen without corresponding alteration of the protein level and 20-fold changes in protein level without reflecting change in mRNA level ^[409].

Furthermore, several research directions are being actively pursued to understand the mechanism of action of the EBERs. As discussed above, novel EBER interaction partners are being identified (section 3.2.10). Further, effects of the EBERs on immune response and tumour formation *in vivo* are being investigated in humanised mice (section 3.3.3). The role of extracellular EBERs and the potential modification of miRNAs by the EBERs are also currently under investigation (sections 3.2.11 and 3.3.6).

In the long term, understanding the functions and mechanism of action of EBERs during EBV infection and the establishment of EBV-associated cancers might allow the identification of novel anti-cancer therapeutic targets by identifying cell components

essential for oncogenesis. If EBERs prove to be crucial for EBV tumour formation *in vivo*, EBERs might be an ideal therapeutic target themselves. EBERs are not essential for EBV infection, and inhibition of their action might therefore not be prone to severe selective pressure towards transforming virus mutants. Moreover, EBERs are virus-specific and targeting EBERs might result in minimal side-effects for patients, potentially presenting a prophylactic therapy to prevent EBV-associated cancers.

4 Interaction of EBER1 with RPL22

4.1 Introduction

EBER1 interacts strongly with ribosomal protein L22 (RPL22) ^[223, 224], a constituent of the large ribosomal subunit ^[224, 226]. Even though the role of RPL22 in uninfected cells is still unclear ^[230, 231], RPL22 is a protein with many diverse proposed functions in a variety of cellular processes (reviewed in section 1.2.4.1), which might be crucially influenced by EBER1 during EBV infection.

Most strikingly, EBV ^[224], and EBER1 specifically ^[228], delocalise RPL22 from the nucleoli and the cytoplasm into the nucleoplasm in Raji, EBV-positive BJAB-B1 and HeLa cells. The delocalisation of RPL22 was confirmed in this study and analysed in a range of natural EBV-host cells to elucidate the physiological role of the delocalisation. Moreover, the translocation of RPL22 was investigated for the first time using EBER deletion EBV.

RPL22 is not essential for the survival or the overall development of RPL22 knockout mice, but deletion of the protein leads to a selective induction of p53-mediated apoptosis in α/β -T lymphocyte precursors, while γ/δ -T cell precursors are spared and develop as normal ^[231]. In addition to frequent roles of ribosomal proteins in p53 signalling, these findings suggest RPL22 as a potential candidate in the regulation of p53-mediated stress responses, which was investigated in section 4.3.

To fully understand the functions both of EBER1 during EBV infection and of the interaction of EBER1 with RPL22, a possible regulatory role of EBER1 in host cell translation was also explored (section 4.4).

EBV is not the only virus that interacts with RPL22 [246–249] and the numerous examples of viral proteins that bind to RPL22 suggest a common mechanism of viruses to influence their hosts or imply a general role of RPL22 in the cellular response to viral infections, likely different from its function as a ribosomal protein.

4.2 Results: RPL22 localisation

The aim of this section is to confirm the delocalisation of RPL22 from nucleoli to the nucleoplasm upon EBV infection and more detailed analysis of the significance of EBER1 for the translocation process.

4.2.1 Expression of FLAG-RPL22 in HEK 293 cells

To confirm the interaction of EBER1 with RPL22, commercially available anti-RPL22 antibodies from Santa Cruz Biotechnology were tested initially for immunoprecipitation of RPL22 from EBV BAC-transfected HEK 293 cell lysates. Unfortunately, these antibodies were not able to pull down RPL22 in four different cell lysis conditions tested (not shown). Thus, FLAG-RPL22-pBK2-CMV expression vectors were generated to overcome the limitations of the commercial antibodies (section 2.3.12).

Expression of FLAG-RPL22 from FLAG-RPL22-pBK2-CMV expression vector was analysed in transiently transfected HEK 293 cells containing wt, Δ EBER1 and Δ EBER2 EBV BACs by western blotting using M2 Mouse monoclonal anti-FLAG antibody. All cells showed good expression of the recombinant protein and different EBV backgrounds did not reproducibly influence RPL22 protein expression (figure 4.1).

4.2.2 EBER1 but not EBER2 interacts with RPL22 in HEK 293 cells

To investigate effects of EBER1 interaction with RPL22 on host cell physiology, it was necessary to confirm the interaction of the two components in our HEK 293 system. Puro/oriP-pSUPER-EBER1 or Puro/oriP-pSUPER-EBER2 were co-transfected with

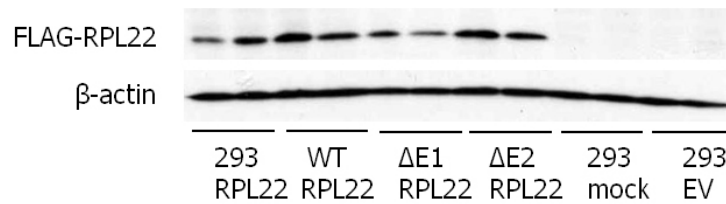


Figure 4.1: FLAG-RPL22 expression from FLAG-RPL22-pBK2-CMV in HEK 293 cells.

EBV-negative HEK 293 (293) and HEK 293 cells containing wt, ΔEBER1 (ΔE1) and ΔEBER2 (ΔE2) EBV BACs were transiently transfected with FLAG-RPL22-pBK2-CMV (RPL22) by lipofection for 24 h in duplicate. Expression of FLAG-RPL22 was analysed by anti-FLAG western blot. Protein extracts of mock transfected and empty vector pBK2-CMV (EV) transfected HEK 293 cells were included as controls. FLAG-RPL22 was expressed at good levels in all 293 cells, independent of EBV BAC background.

FLAG-RPL22-pBK2-CMV into HEK 293 cells for 24 h (sections 2.5 and 2.2.6). Camelpox virus FLAG-Schlafen protein expressed from pcDNA3.1 FLAG-Schlafen was included as negative control. Protein extracts were pulled down with anti-FLAG antibody on protein G sepharose beads and the binding of FLAG-RPL22, EBER1 or EBER2 to the beads was determined by western blot and northern blot analysis, respectively. FLAG-RPL22 or FLAG-Schlafen were expressed in the transfected cells and were efficiently immunoprecipitated using anti-FLAG antibodies (figure 4.2, section 2.5.7).

Subsequently, a northern blot was performed using RNA from the immunoprecipitated protein samples to establish if EBER1 or EBER2 interacted with FLAG-RPL22 or FLAG-Schlafen (figure 4.3). Both EBER1 and EBER2 were strongly expressed in the HEK 293 cells and detected in the unbound fraction of the immunoprecipitation (figure 4.3, sup). RPL22 specifically co-immunoprecipitated EBER1 but not EBER2 (figure 4.3, pull down). Neither EBER1 nor EBER2 interacted with FLAG-Schlafen control protein. Consequently, the result verified the specific interaction of EBER1 with RPL22.

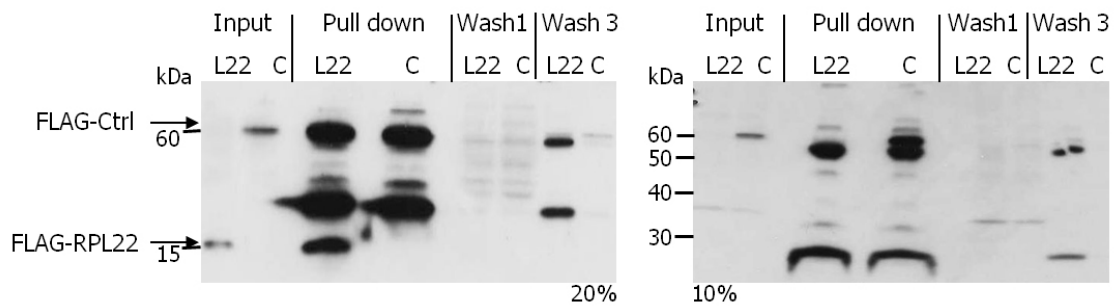


Figure 4.2: Immunoprecipitation of FLAG-RPL22 and FLAG-Schlafen protein from HEK 293 cells.

EBV-negative HEK 293 cells were transiently transfected with FLAG-RPL22-pBK2-CMV or pcDNA3.1 FLAG-Schlafen control plus Puro/oriP-pSUPER-EBER1 or Puro/oriP-pSUPER-EBER2 for 24 h. FLAG-tagged proteins were immunoprecipitated using anti-FLAG antibodies and protein G sepharose beads. Both FLAG-RPL22 (L22) and FLAG-Schlafen control (C) were expressed (input) and precipitated efficiently (pull down), as almost no expressed protein remained in the unbound wash fractions (wash 1 and wash 3). The same samples are shown on a 20% polyacrylamide gel on the left and a 10% polyacrylamide gel on the right to allow optimal visualisation of the different-sized proteins. RPL22 is not visible on the 10% gel on the right which allows separation of FLAG-Schlafen and anti-FLAG antibody heavy chain in the immunoprecipitate (pull down).

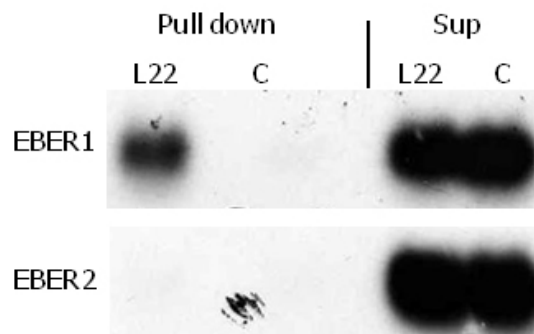


Figure 4.3: EBER1 is co-immunoprecipitated with FLAG-RPL22 in HEK 293 cells.

Northern blot analysis of EBER1 and EBER2 co-immunoprecipitation with FLAG-RPL22 or FLAG-Schlafen protein. Top panel: EBER1 was co-immunoprecipitated with FLAG-RPL22 (L22) but not FLAG-Schlafen control (C). Lower panel: EBER2 was not bound by FLAG-RPL22 (L22) or FLAG-Schlafen control. Both EBER1 and EBER2 were present in the unbound immunoprecipitation fraction (Sup).

4.2.3 EBER1 but not EBER2 interacts with RPL22 in LCLs

The interaction of EBER1 with RPL22 was also verified in LCLs, which are the most important model system used in this study. For this, FLAG-RPL22 or FLAG-Schlafen control protein were expressed transiently from p294-CMV/W-FLAG-RPL22 or pcDNA3.1 FLAG-Schlafen in LCLs by Neon transfection for 48 h (section 2.2.6). FLAG-tagged proteins were immunoprecipitated from LCL lysates using anti-FLAG antibodies and protein G sepharose beads, as described in method section 2.5.7. Protein expression and immunoprecipitation were confirmed by western blotting (figure 4.4 a), followed by northern blotting for EBER1 and EBER2 (figure 4.4 b). Consistent with the results in other cell lines, EBER1 interacts with RPL22 but EBER2 does not bind RPL22 in LCLs.

4.2.4 EBV delocalises FLAG-RPL22 from the nucleoli to the nucleoplasm in HEK 293 cells

Previous studies reported a delocalisation of RPL22 from the nucleoli to the nucleoplasm upon EBV infection in BL-cells ^[224]. To confirm this observation and further clarify the role of EBER1 in the delocalisation of RPL22 with our recombinant virus system, the distribution of RPL22 was investigated in further detail. For the first time, RPL22 interaction was analysed in respect to EBER presence in an otherwise wt EBV background.

To test the importance of EBER1 for the delocalisation of RPL22, FLAG-RPL22 was expressed in non-infected HEK 293 cells and HEK 293 cells stably transfected with wt, Δ EBER1, Δ EBER2 or EBER1-revertant EBV BACs. The expression and localisation of FLAG-RPL22 was evaluated by confocal microscopy 24 h post-transfection. The distribution of FLAG-RPL22 in the different EBV backgrounds is shown in figure 4.5. In non-infected cells, FLAG-RPL22 was frequently present in the nucleoli. Nucleoli were visible as dark, dense structures in the nucleus in the phase contrast images. Upon EBV infection, RPL22 was translocated from the nucleoli to the nucleoplasm with

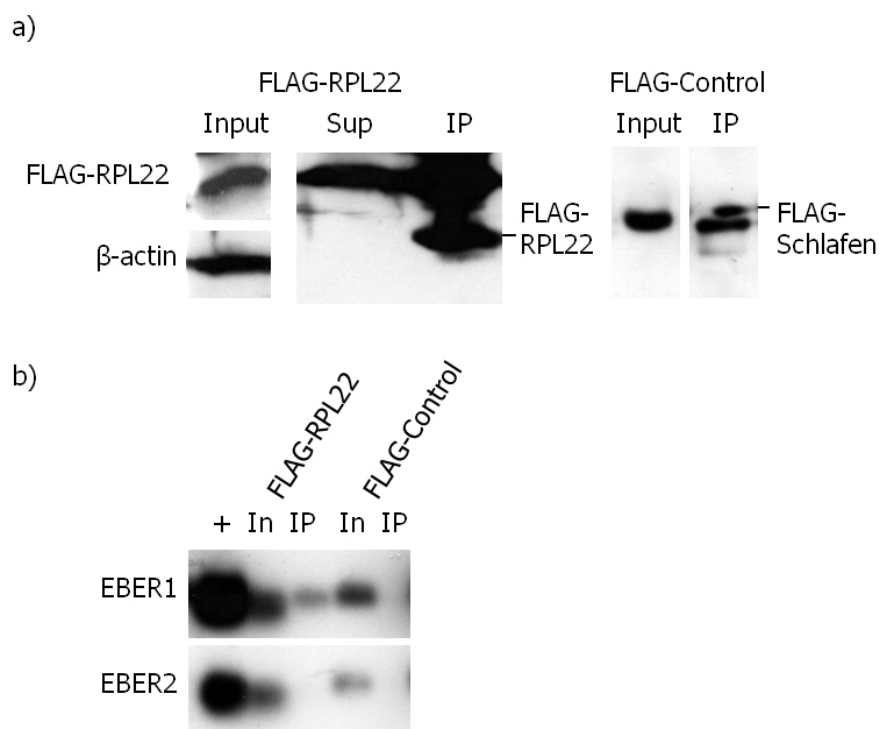


Figure 4.4: EBER1 interacts with RPL22 in LCLs.

a) FLAG-RPL22 and FLAG-Schlafen control were expressed transiently from p294-CMV/W-FLAG-RPL22 or pcDNA3.1 FLAG-Schlafen, respectively (Input). FLAG-RPL22 was pulled down efficiently using anti-FLAG antibody and protein G sepharose beads (IP), as no protein was detected in the unbound supernatant (Sup). b) Northern blot analysis of EBER1 and EBER2 co-immunoprecipitation with FLAG-RPL22 or FLAG-Schlafen protein. Both EBER1 and EBER2 were detected in the unbound immunoprecipitation fraction (In). EBER1 co-immunoprecipitated with FLAG-RPL22 but not with the FLAG-control protein (IP), while EBER2 did not bind to either of the two proteins. RNA extracted from EBV-positive Akata 2003 cells was included as positive control (+).

partial exclusion from nucleolar structures. Interestingly, EBV infection in the absence of EBER1 did not lead to a redistribution of RPL22. RPL22 remained in the nucleoli, comparable to non-infected cells. Δ EBER2-infected cells display wt delocalisation of RPL22 to the nucleoplasm. A subset of cells maintained FLAG-RPL22 in the cytoplasm in non-infected and all EBV-infected cell lines tested (not shown). The effect of EBER1 on FLAG-RPL22 delocalisation was confirmed in EBER1-revertant BAC-transfected cells which showed wt localisation of RPL22 in the nucleoplasm (figure 4.5). Hence, RPL22 delocalisation in dependence of EBV infection was demonstrated and a clear role of EBER1 in the process shown.

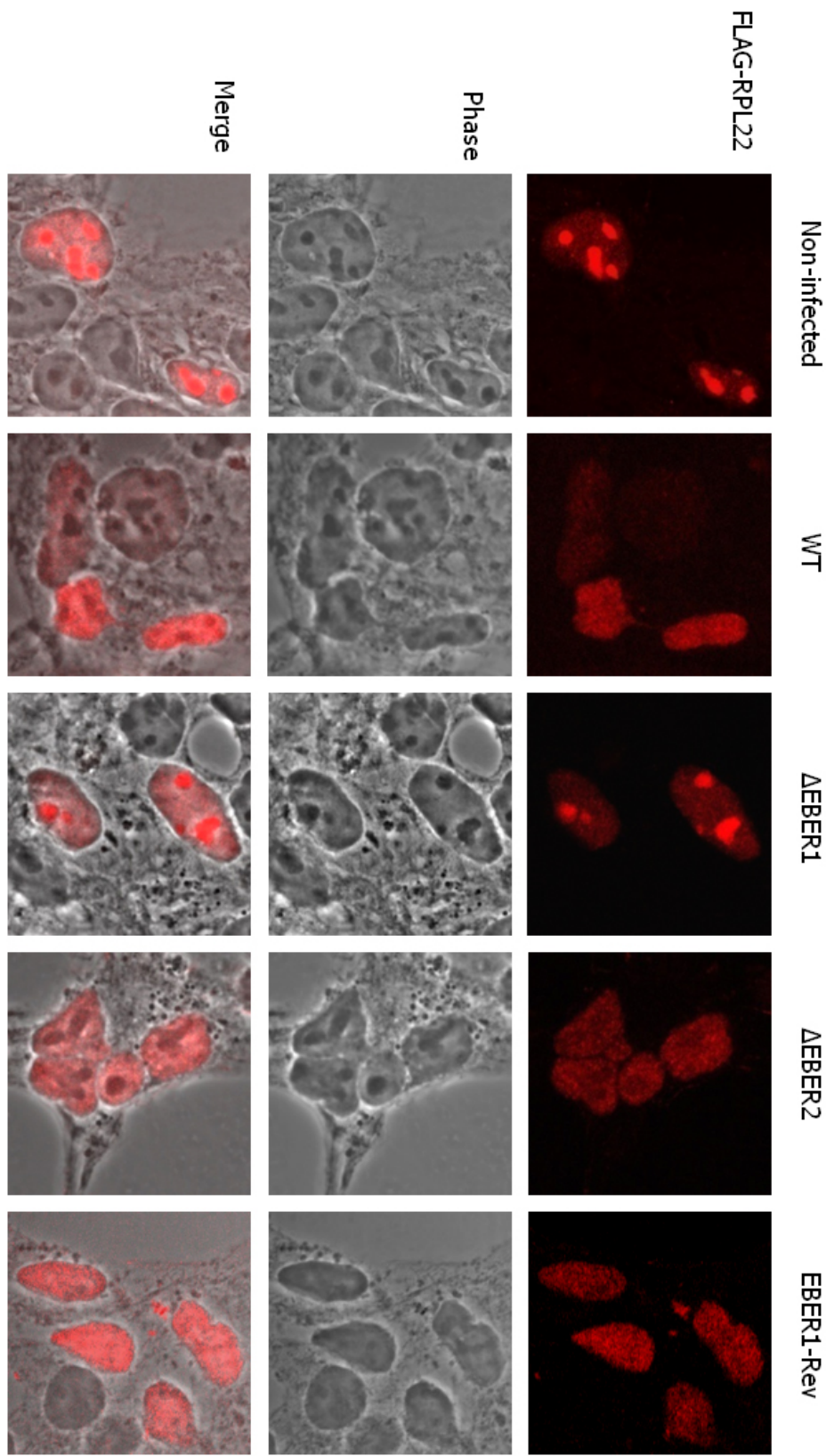


Figure 4.5: FLAG-RPL22 localisation in EBV BAC-transfected HEK 293 cells is dependent on EBER1.

a) FLAG-RPL22 location was analysed in non-infected, wt, ΔEBER1, ΔEBER2 and EBER1-revertant (EBER1-Rev) EBV-infected HEK 293 cells transiently expressing FLAG-RPL22 from FLAG-RPL22-pBK2-CMV for 24 h. RPL22 was detected using anti-FLAG antibody. RPL22 was mainly localised in the nucleoli of non-infected cells. RPL22 was translocated from the nucleoli in non-infected cells to the nucleoplasm in wt EBV-infected cells. EBER1 is essential for the delocalisation of RPL22 and absence of EBER1 restrained RPL22 in the nucleoli comparable to non-infected cells. ΔEBER2 and EBER1-revertant EBV-infected cells displayed wt delocalisation of RPL22 to the nucleoplasm.

4.2.5 The distribution of FLAG-RPL22 is representative of endogenous RPL22 in HEK 293 cells

Localisation of endogenous RPL22 was compared to FLAG-RPL22 using antibody kindly provided by Prof J Steitz, Yale University, in order to confirm a proper physiological distribution of FLAG-RPL22 (table 2.9). Firstly, detection of RPL22 was confirmed by western blot (figure 4.6).

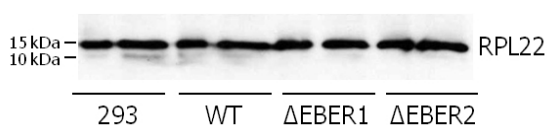


Figure 4.6: Detection of endogenous RPL22.

Protein of the correct size was detected in protein lysates of non-infected, wt, Δ EBER1 and Δ EBER2 EBV-infected HEK 293 cells using polyclonal rabbit anti-RPL22 antibody.

Subsequently, the delocalisation of endogenous RPL22 was analysed by confocal microscopy and compared to the earlier results for FLAG-RPL22 (figure 4.7). Localisation of endogenous RPL22 was found to be identical to the distribution of FLAG-RPL22 and to be changed from the nucleoli to the nucleoplasm by EBER1. Thus, FLAG-tagged RPL22 is considered an appropriate substitute for endogenous RPL22 in the investigation of the function of RPL22 during EBV infection.

4.2.6 EBER1 mediates FLAG-RPL22 delocalisation in HEK 293 and BJAB cells

To further assess the role of EBER1 in the delocalisation of RPL22, Δ EBER1 EBV-infected HEK 293 cells were transfected with Puro/oriP-pSUPER-EBER plasmids for 24 h to complement EBER deletion from EBV BACs. As shown in figure 4.8, RPL22 was localised in the nucleoli in Δ EBER1 HEK 293 cells transfected with empty control vector, comparable to non-infected cells. Expression of EBER1 led to a redistribution of RPL22 to the nucleoplasm in 24-50% of infected cells, while EBER2 expression did not influence RPL22 localisation. This experiment not only verified the data obtained using

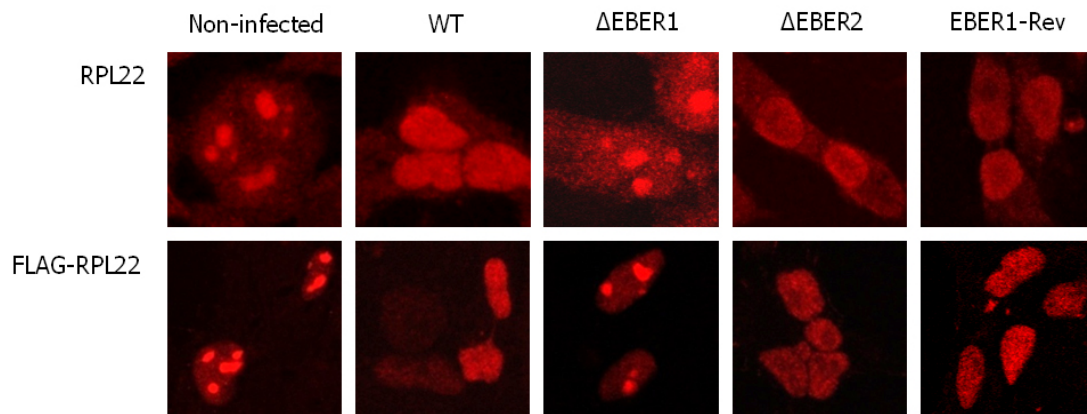


Figure 4.7: EBER1 redistributes endogenous RPL22.

RPL22 location was analysed in non-infected, wt, Δ EBER1, Δ EBER2 and EBER1-revertant EBV-infected HEK 293 (top row) and compared to FLAG-RPL22 distribution (bottom row). RPL22 was translocated from nucleoli in non-infected cells to the nucleoplasm in cells containing EBER1. Delocalisation of endogenous and FLAG-RPL22 was identical.

EBER1-revertant BACs (figure 4.5), but also demonstrated the functionality of EBER1 expressed from the Puro/oriP-pSUPER-EBER1 plasmid.

The fact that not all cells showed delocalisation of RPL22 after expression of EBER1 might have been due to several reasons. First of all, not all cells transfected with FLAG-RPL22 might have been successfully transfected with Puro/oriP-pSUPER-EBER plasmids. Secondly, the amounts of EBER1 expressed from the vector were lower than EBER levels expressed from BACs (figure 3.13). Therefore, EBER1 might not have been able to bind sufficient RPL22 to expose the relocation phenotype in every cell and thirdly, delocalisation of RPL22 might require more than 24 h for expression and protein redistribution.

Next, the exclusive responsibility of EBER1 for RPL22 delocalisation was demonstrated. To this end, non-infected cells were transfected with Puro/oriP-pSUPER-EBER1 or EBER2 plasmids. RPL22 was localised in the nucleoli in cells expressing EBER2 or containing empty vector control (figure 4.9), which corresponds to a RPL22 distribution pattern of non-infected cells. In contrast, expression of EBER1 led to the delocalisation of RPL22 into the nucleoplasm without the presence of any other viral component in 61-76% of the cells, demonstrating that EBER1 alone is sufficient to

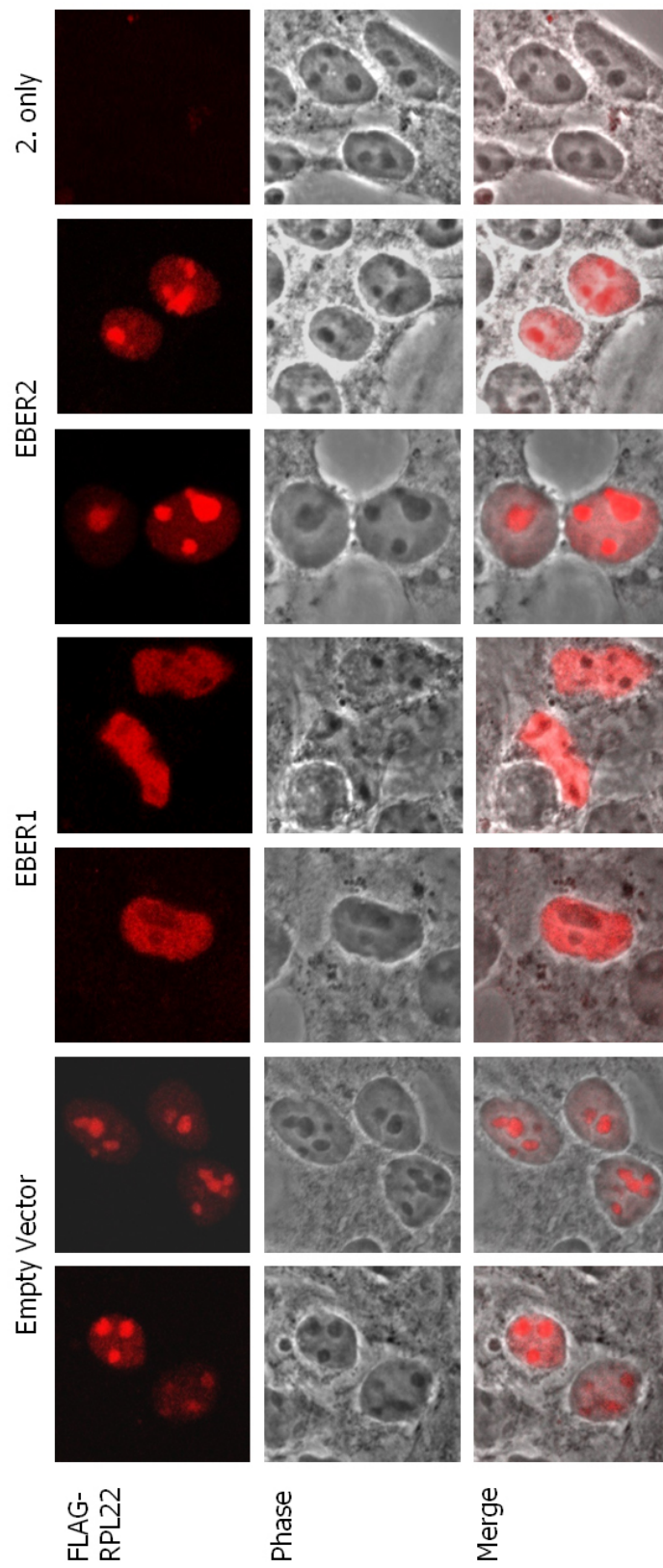


Figure 4.8: Expression of EBER1 delocalises FLAG-RPL22 in Δ EBER1 EBV-infected HEK 293 cells.

FLAG-RPL22 location was analysed in Δ EBER1 EBV-infected cells expressing oriP-SUPER-EBER1 (EBER1), Puro/oriP-pSUPER-EBER2 (EBER2) or Puro/oriP-pSUPER-HI promoter plasmid (Empty vector) as control. EBER1 expression resulted in the delocalisation of FLAG-RPL22 from the nucleoli into the nucleoplasm. EBER2 expression or empty vector control had no effect on FLAG-RPL22 relocation. 2. only: secondary antibody only control.

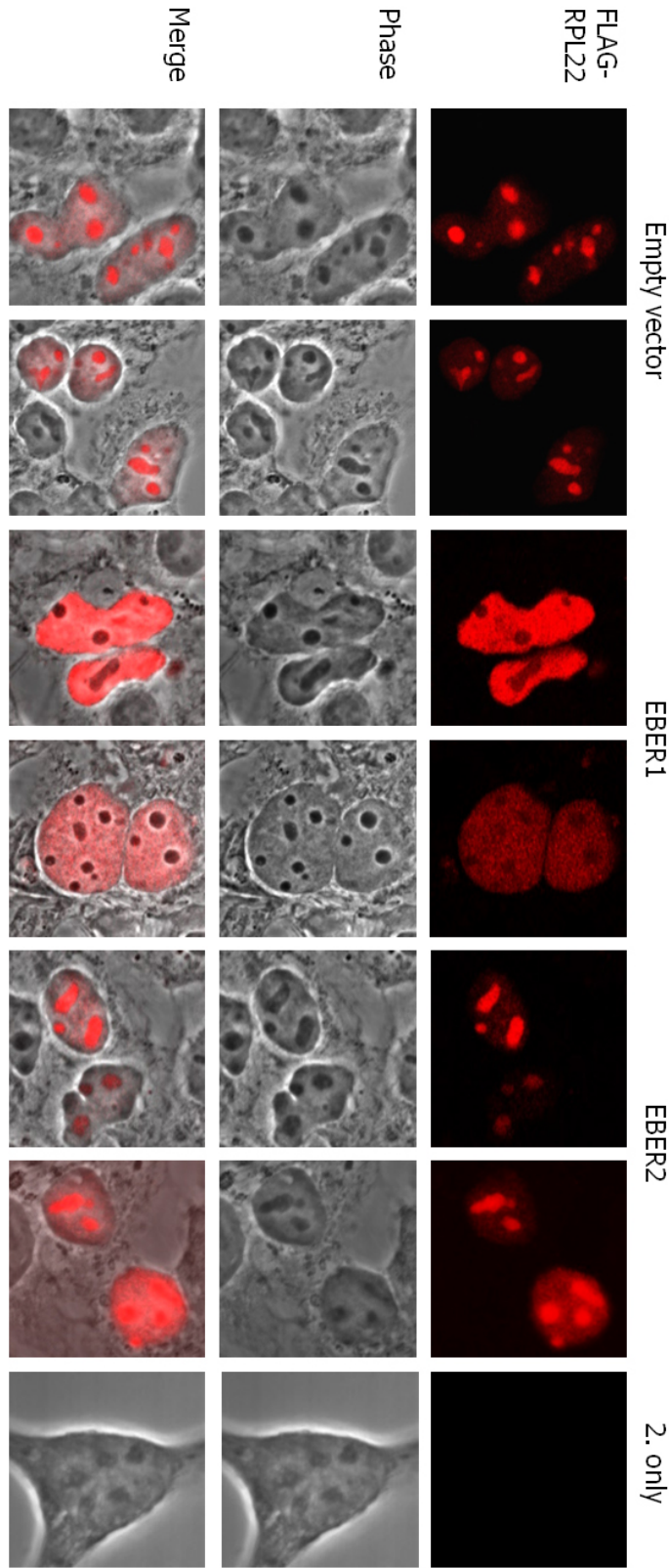


Figure 4.9: EBER1 delocalises FLAG-RPL22 independent of other viral factors in HEK 293 cells. FLAG-RPL22 location was analysed in non-infected cells expressing Puro/oriP-pSUPER-EBER1 (EBER1), Puro/oriP-pSUPER-EBER2 (EBER2) or Puro/oriP-pSUPER-HI promoter plasmid (Empty vector) as control. EBER1 expression resulted in the delocalisation of FLAG-RPL22 from the nucleoli into the nucleoplasm. Expression of EBER2 or empty vector control had no effect on FLAG-RPL22 relocation. 2. only: secondary antibody only control.

cause the translocation of RPL22 described in EBV-infected cells. In addition, EBER1 and EBER2 were expressed in the EBV-negative BL-lymphoma cell line BJAB and RPL22 localisation was analysed by confocal microscopy (figure 4.10). Cells expressing EBER2 or no EBER contained RPL22 in the nucleoli in 5-6% of the cells. Expression of EBER1 led to a reduction of cells displaying nucleolar RPL22 to 2%. Additionally, RPL22 was delocalised to the nucleoplasm in 0.5% of cells expressing EBER1. The limitation in RPL22 delocalisation from nucleoli to the nucleoplasm in EBER1-expressing cells might be the consequence of the modest transfection rates achieved with Neon transfection. RPL22 was not observed in the nucleoplasm in BJAB cells transfected with Puro/oriP-pSUPER-EBER2 or Puro/oriP-pSUPER-HI. This result demonstrates that EBER1 is able to delocalise RPL22 in a B cell background.

4.2.7 EBV delocalises RPL22 from the nucleoli to the nucleoplasm in HONE-1 cells derived from NPC and AGS cells derived from GC

Delocalisation of RPL22 by EBER1 in B-lymphoma BJAB and HEK 293 cells implies a possible role for the delocalisation of the protein in various EBV-infected cells. Therefore, the location of RPL22 in cell lines derived from natural EBV host cells was analysed to determine the importance of RPL22 redistribution in different EBV tumour backgrounds. RPL22 in EBV-positive and EBV-negative versions of the GC-derived cell line AGS and the NPC-derived cell line HONE-1 was analysed by confocal microscopy (figure 4.11). RPL22 was present in the nucleoli of non-infected cells and was translocated to the nucleoplasm in a subset of EBV-infected cells, consistent with the results for HEK 293 cells. The detailed quantification of RPL22 delocalisation is discussed later in section 4.2.10.

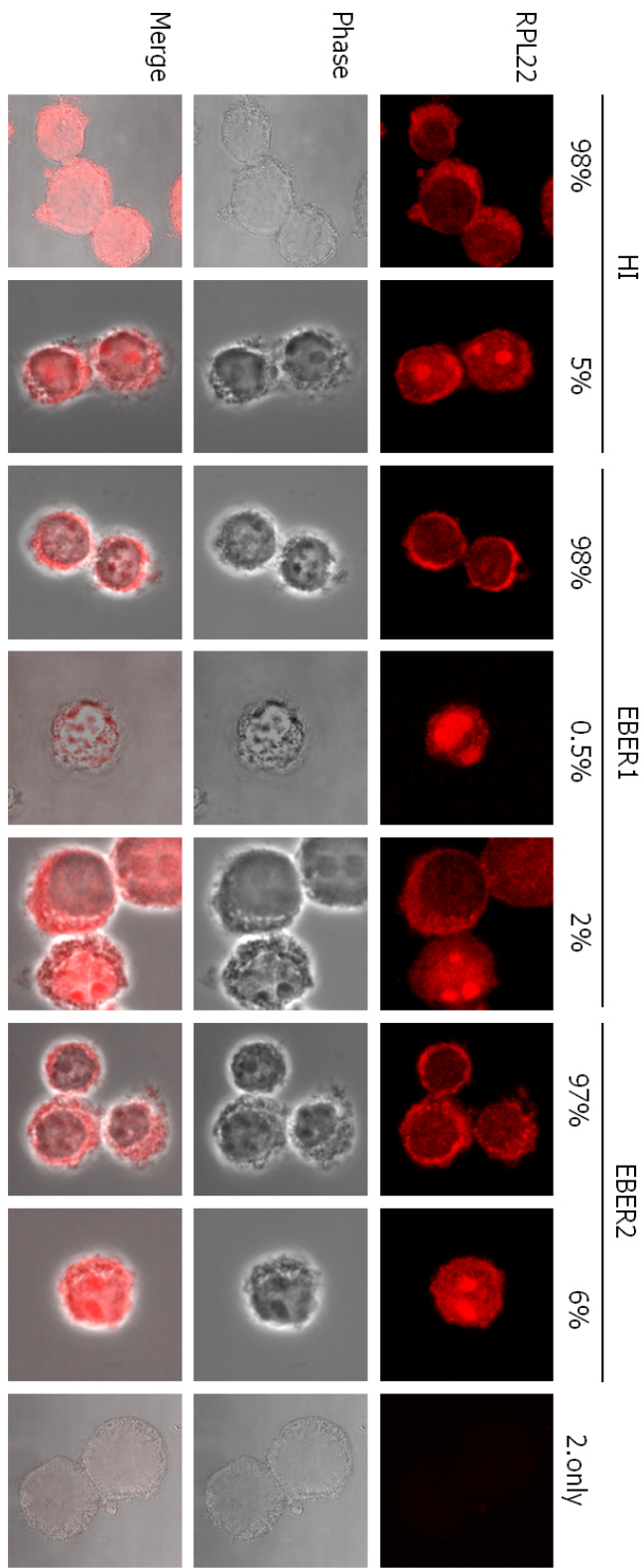


Figure 4.10: EBER1 delocalises RPL22 independent of other viral factors in BJAB cells. RPL22 location was analysed in EBV-negative cells transfected with Puro/oriP-pSUPER-EBER1 (EBER1), Puro/oriP-pSUPER-EBER2 (EBER2) or Puro/oriP-pSUPER-HI promoter plasmid (HI) as control. RPL22 was present in the nucleoli of 5% and 6% of cells expressing EBER2 or HI control, respectively. Expression of EBER1 resulted in a delocalisation of RPL22 to the nucleoplasm in approximately 0.5% of cells and a reduction of nucleolar RPL22 to 2% of the cells. 2. only: secondary antibody only control.

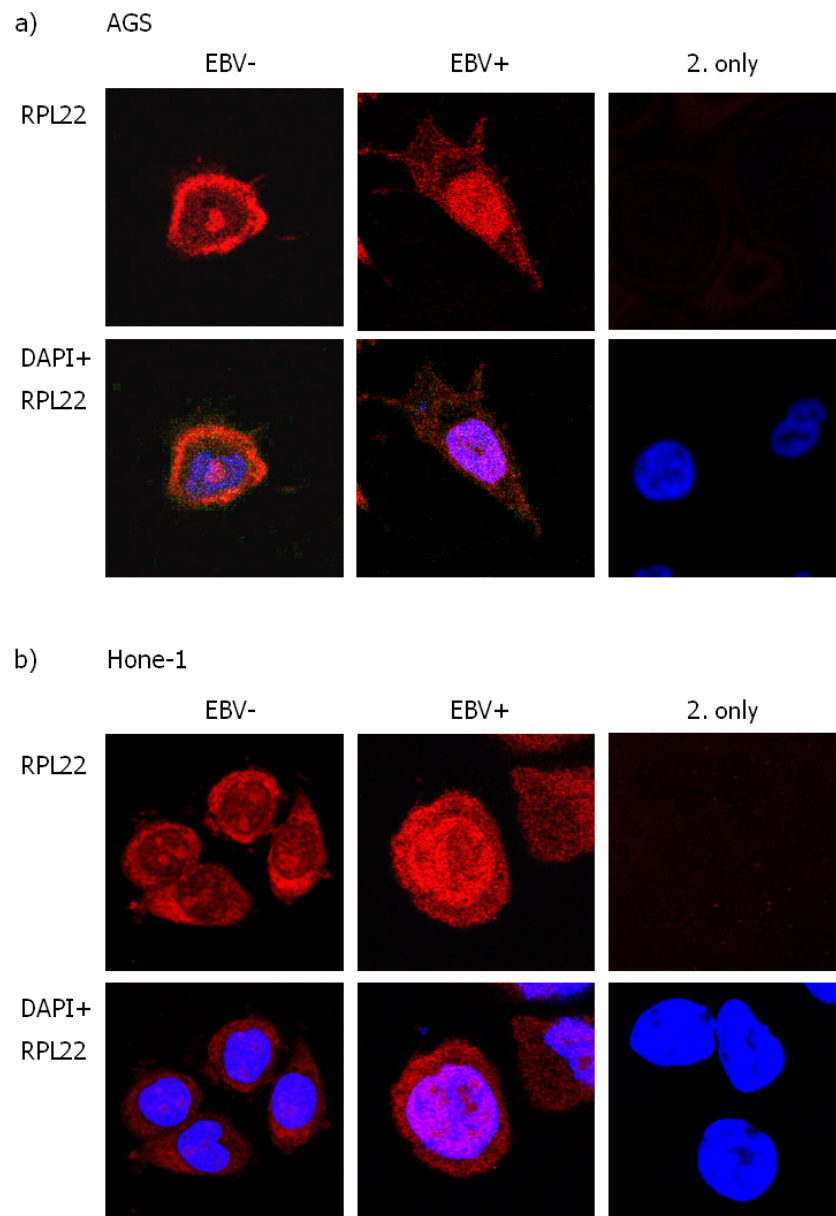


Figure 4.11: RPL22 is delocalised in GC-derived AGS and NPC-derived HONE-1 cells upon EBV infection.

Endogenous RPL22 distribution in EBV-positive (EBV+) and EBV-negative (EBV-) counterparts of a) GC-derived AGS and b) NPC-derived HONE-1 cells was analysed by confocal microscopy. RPL22 was delocalised from the nucleoli to the nucleoplasm in EBV-positive cells. 2. only: secondary antibody only control.

4.2.8 Localisation of RPL22 in NPC tumour biopsies

In order to address possible effects of EBER1 on RPL22 in EBV-associated cancer cells, RPL22 distribution in NPC tumour sections was analysed in the laboratory of Prof Alan K S Chiang, Hong Kong University, during a student exchange summer school programme in 2009. Figure 4.12 a shows fluorescence microscopy images of two NPC biopsy sections successfully stained for RPL22. The dense, well ordered epithelial cells formed a typical NPC nest accompanied by infiltrating lymphocytes. RPL22 localisation was determined in several sections of two NPC biopsies derived from independent patients (figure 4.12 b). Surprisingly, RPL22 was found to be present in the cytoplasm of all cells and in the nucleoli of 35% of the cells in NPC tumours, which corresponds to an RPL22 distribution pattern usually observed in EBV-negative cell lines (figure 4.11). Even though NPC cells are generally EBV-positive ^[99], further investigations are needed to clarify EBV and EBER1 status of the NPC tumours used in this study to confirm the lack of RPL22 delocalisation despite the presence of EBER1. Unfortunately, the resolution of hematoxylin & eosin staining and EBER ISH on the NPC tissues performed by the Department of Pathology at Hong Kong University was not sufficient to conclusively clarify EBER status. EBV is expected in the transformed epithelial cells but not in the surrounding lymphocytes ^[410]. It would also be advantageous to increase the number of NPC biopsies analysed, in case there are patient-specific differences in the translocation of RPL22.

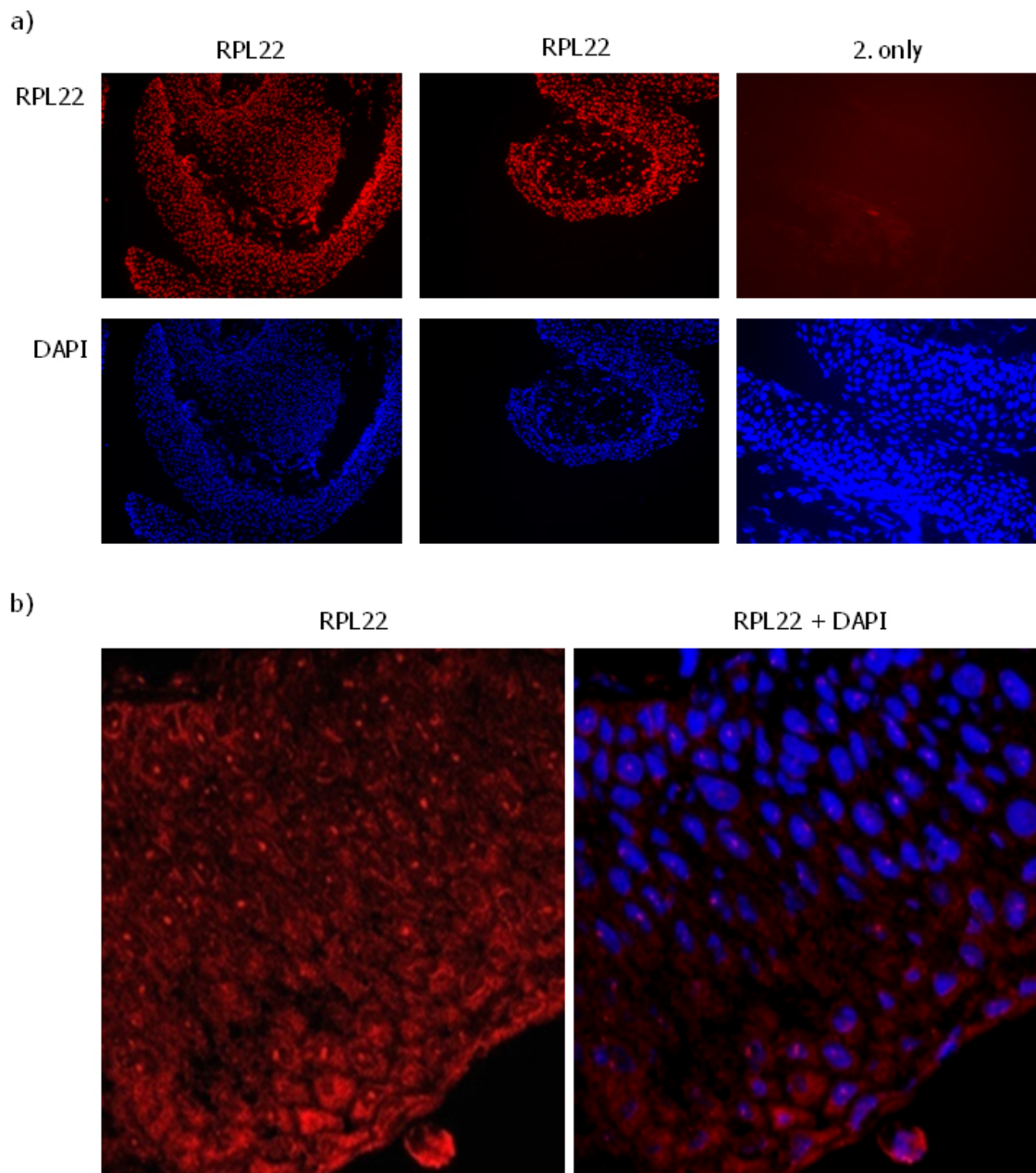


Figure 4.12: RPL22 distribution in NPC biopsies.

a) Overview of two NPC tumour biopsy sections stained for RPL22 and secondary antibody only control (2. only). b) Magnified NPC biopsy section. RPL22 is located in the cytoplasm of all cells and the nucleoli of 35% of the NPC tumour cells.

4.2.9 Localisation of RPL22 in LCLs differs from cancer-derived cell lines

RPL22 location in LCLs transformed with wt or Δ EBER1 EBV was investigated to further expand the set of cell lines analysed. Surprisingly, LCLs showed an RPL22 distribution distinct from the previously observed patterns in HEK 293, BJAB, AGS and HONE-1 cells. In 99.5% of LCLs, transformed either by wt EBV or by Δ EBER1 EBV, RPL22 was localised in the cytoplasm (figure 4.13, panel 1 and 3), as might be expected for a ribosomal protein. Only 0.5% of Δ EBER1 LCLs displayed RPL22 in the nucleoli (figure 4.13, panel 4). Consistent with a delocalisation of RPL22 by EBER1, RPL22 was never detected in the nucleoli of wt EBV-transformed LCLs, while RPL22 resided in the nucleoplasm in 0.5% of the cells (figure 4.13, panel 2).

The biogenesis of ribosomes is a tightly regulated process ^[239, 411] and deregulation of many ribosomal proteins can lead to cell cycle arrest or apoptosis ^[412]. Consequently, LCLs might not accumulate or be able to tolerate accumulation of RPL22 in the nucleoli due to a less transformed state compared to HEK 293, BJAB, AGS and HONE-1 cells. The delocalisation of RPL22 might be more pronounced or of greater relevance in cancer-derived cell lines or RPL22 delocalisation might not be the main purpose of EBER1 interaction with the ribosomal protein. The latter would be supported by the fact that RPL22 was not delocalised in NPC biopsies (figure 4.12).

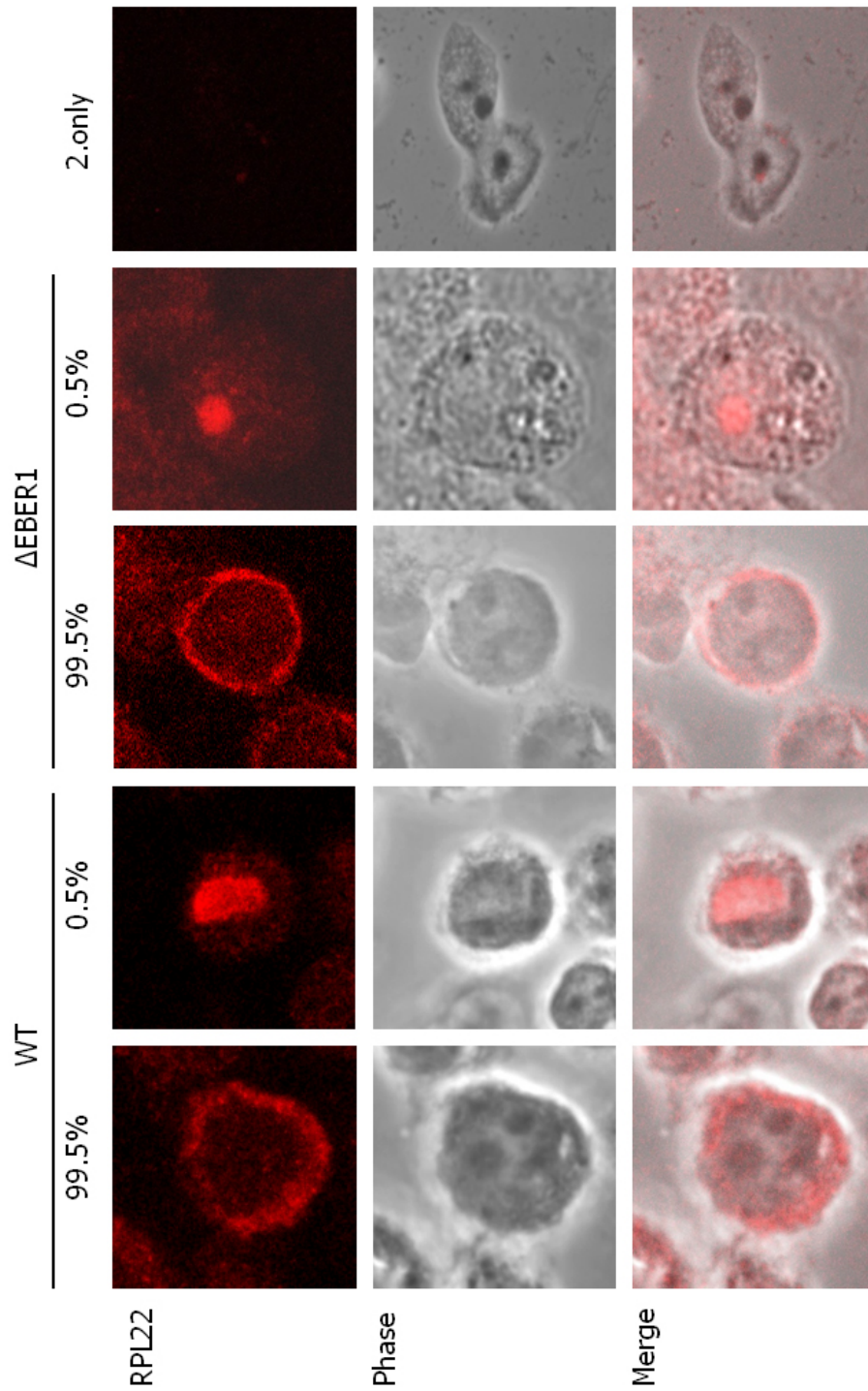


Figure 4.13: RPL22 is mainly localised in the cytoplasm in LCLs. RPL22 was predominately localised in the cytoplasm in 99.5% of wt or Δ EBER1 EBV-transformed LCLs. Only 0.5% of Δ EBER1 LCLs showed nucleolar RPL22. RPL22 was delocalised to the nucleoplasm in 0.5% of wt LCLs. 2.only: secondary antibody only control.

4.2.10 Quantification of RPL22 localisation

The delocalisation of endogenous RPL22 in different EBV-infected cells was quantified by counting 50-100 cells in stochastically chosen fields by confocal microscopy. The cells were appointed nuclear, nucleolar or cytoplasmic RPL22 location, or combinations thereof, according to the strongest RPL22 signal visible. Only stably EBV-transfected or transformed cells were used in this experiment and the location of endogenous RPL22 was analysed. The distribution of RPL22 as a percentage of the total number of cells classified is given in table 4.1.

Table 4.1: Endogenous RPL22 localisation. Cells were classified as nucleolar, nucleoplasmic, cytoplasmic or combinations thereof according to the staining of endogenous RPL22 by confocal fluorescence microscopy.

Cell line	Cytoplasmic %	Nucleoplasmic %	Nucleolar %
HEK 293	95	0	5
HEK 293 EBV+	60	40	0
LCL Δ EBER1 EBV	99.5	0	0.5
LCL wt EBV	99.5	0.5	0
AGS	100	0	5
AGS EBV+	90	10	0
HONE-1	100	0	25
HONE-1 EBV+	98	2	0

The distribution of RPL22 in non-infected and EBV-infected cells varied between the different cell types analysed. Nevertheless, common RPL22 distribution patterns emerged. RPL22 was never observed in the nucleoplasm in non-infected cells, while nucleolar accumulation of RPL22 was never detected in wt EBV-positive cells (table 4.1).

RPL22 clearly accumulated in the nucleoli of 5% of HEK 293 cells, whereas the other 95% of HEK 293 cells presented RPL22 in the cytoplasm. Notably, RPL22 was delocalised to the nucleoplasm in 40% of cells stably transfected with wt EBV BAC. In AGS and HONE-1 cells, RPL22 was present in the nucleoli and the cytoplasm simultaneously in a subset of cells, in addition to an only cytoplasmic location. Thus, the

distribution of RPL22 in AGS and HONE-1 cells differed slightly from HEK 293 cells. 5% of non-infected AGS and 25% of non-infected HONE-1 cells accumulated RPL22 in the nucleoli. Upon EBV infection, RPL22 was delocalised significantly in both cell lines. In AGS, RPL22 was observed in the nucleoplasm of 10% of the cells. In HONE-1 cells, RPL22 was detected in the nucleoplasm of a more modest 2% of cells.

LCLs displayed an alternative RPL22 pattern in which RPL22 was almost exclusively cytoplasmic. Only 0.5% of LCLs transformed with Δ EBER1 EBV presented RPL22 in the nucleoli. Consistently, only 0.5% of wt LCLs showed a delocalisation of RPL22 to the nucleoplasm.

RPL22 is assumed to be transported into the nucleoli for incorporation into the ribosomes by binding to 28S rRNA ^[226]. Competition of EBER1 with 28S rRNA for binding of RPL22 might inhibit co-transport into the nucleoli and retain RPL22 in the nucleoplasm ^[229]. Consequently, aggregation of RPL22 in the nucleoplasm of EBV-positive cells might only be observable in cells which accumulate RPL22 in their nucleoli. Accordingly, delocalisation would only be expected to a very limited extent in LCLs, as observed.

4.2.11 EBV infection does not disrupt nucleoli in host cells

EBER1 might retain RPL22 in the nucleoplasm by competing with 28S rRNA [226]. Alternatively, EBER1 could generally disrupt nucleoli during EBV infection. In order to confirm a specific delocalisation of RPL22 by EBER1 as opposed to a general disruption of the nucleoli, the state of the nucleoli during EBV infection was investigated in HEK 293 cells. To this end, the distribution of the nucleolar marker protein fibrillarin was examined by confocal microscopy. Fibrillarin is involved in pre-rRNA processing and ribosome assembly [413]. Firstly, the delocalisation of FLAG-RPL22, expressed from FLAG-RPL22-pBK2-CMV, was confirmed (figure 4.14 a). As described previously in this study, FLAG-RPL22 was localised in the nucleoli of non-infected cells (figure 4.14 a, panel 1) and Δ EBER1 EBV-infected HEK 293 cells (figure 4.14 a, panel 3). Infection of the cells with wt EBV (figure 4.14 a, panel 2) or expression of EBER1 from Puro/oriP-pSUPER-EBER1 (figure 4.14 a, panel 4), resulted in a delocalisation of FLAG-RPL22 from the nucleoli to the nucleoplasm and exclusion of FLAG-RPL22 from the nucleoli. Fibrillarin staining was performed in the same transfections (figure 4.14 b). All cells investigated contained fibrillarin clearly in the nucleoli, as expected. Nucleoli were intact in cells that expressed EBER1 and had RPL22 translocated to the nucleoplasm (figure 4.14 b, panel 2 and 4). Thus, EBV infection does not generally disrupt nucleoli, as determined by fibrillarin staining and as confirmed in the phase contrast images, which show intact nucleoli. The delocalisation of RPL22 by EBER1 is therefore specific for RPL22.

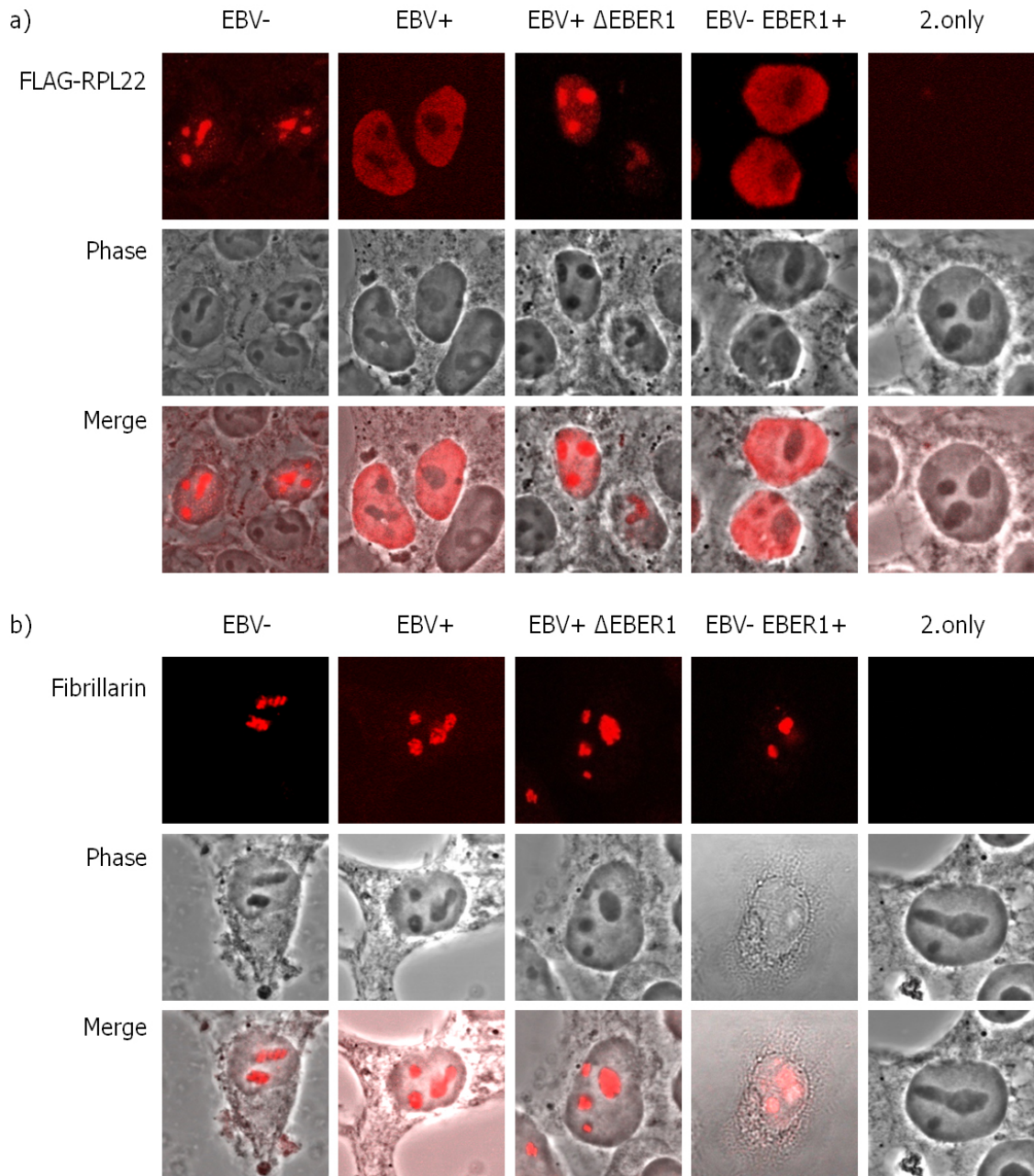


Figure 4.14: Nucleoli are intact in EBV-infected cells.

a) Delocalisation of FLAG-RPL22 from the nucleoli in non-infected cells (EBV-) and cells lacking EBER1 (EBV+ ΔEBER1) to the nucleoplasm of wt EBV-infected cells (EBV+) and cells expressing EBER1 from Puro/oriP-pSUPER-EBER1 (EBV- EBER1+) was confirmed in HEK 293 cells. b) No difference in fibrillarin staining was observed in non-infected cells (EBV-), cells lacking EBER1 (EBV+ ΔEBER1), wt EBV-infected cells (EBV+) or cells expressing EBER1 from Puro/oriP-pSUPER-EBER1 (EBV- EBER1+). Nucleoli were not disrupted by EBV infection or EBER1. 2.only: secondary antibody only control.

4.3 Results: RPL22 and cell stress responses

The aim of this section is the investigation of a potential role of RPL22 and of the interaction of EBER1 with RPL22 in cell stress responses.

4.3.1 Expression of FLAG-RPL22 in LCLs

FLAG-RPL22 was expressed in LCLs to simplify the investigation of RPL22 and to overcome restrictions due to the limited availability of antibody against endogenous RPL22. Surprisingly, the previously employed FLAG-RPL22-pBK2-CMV (section 2.3.12.1) did not express FLAG-RPL22 in LCLs using the Neon transfection system. Transfection of FLAG-RPL22-pBK2-CMV was optimised using the Neon optimisation programme, but expression of FLAG-RPL22 was not detected by western blot (not shown). Successful transfection of LCLs with the Neon system was confirmed by expression of a control protein (not shown). Additionally, expression of FLAG-RPL22 from FLAG-RPL22-pBK2-CMV was checked again in HEK 293 cells to ensure the integrity of the expression vector.

FLAG-RPL22 expression in LCLs might have been deficient due to cell type specific differences in protein expression from the CMV promoter or due to toxic effects of RPL22 overexpression in LCLs. The latter would be consistent with a lack of RPL22 accumulation in the nucleoli, as was observed previously (figure 4.13). Novel FLAG-RPL22 expression vectors were therefore established, including FLAG-RPL22-p294-CMV/W (section 2.3.12.2) and FLAG-RPL22-p3.1W (section 2.3.12.3). The new expression vectors enabled the investigation of the expression of FLAG-RPL22 under control of the CMV or the W promoter.

Expression of FLAG-RPL22 in LCLs still proved to be difficult. No expression was achieved from FLAG-RPL22-p3.1W. FLAG-RPL22 was expressed sporadically from FLAG-RPL22-p294-CMV/W, but the expression was unreliable. However, expression of FLAG-RPL22 from the p294-CMV/W vector demonstrated the functionality of the vector in the LCLs. Expression of RPL22 was analysed after 0, 6, 12, 16, 24, 27 and 48

h. Additionally, 0.01, 0.1, 1, 4 or 10 μ g DNA were used for the transfections. Despite the variation in both time of expression and DNA concentration, expression of the protein was not improved or reliably reproduced.

During the course of the optimisation, no reasons for the limited FLAG-RPL22 expression in LCLs have become apparent. FLAG-RPL22 was expressed consistently in HEK 293 cells. The analysis of RPL22 delocalisation in LCLs showed RPL22 mainly in the cytoplasm but very rarely accumulated in the nucleoli (figure 4.13). Together with the difficulty to express FLAG-RPL22 in LCLs, in contrast to HEK 293 cells, this suggested that LCLs might not be able to tolerate RPL22 accumulation and as a consequence overexpression of the protein.

4.3.2 Expression of FLAG-RPL22 does not reduce protein expression in LCLs

To understand a possible toxic effect of RPL22 overexpression in LCLs, protein expression in LCLs transfected with FLAG-RPL22-p294-CMV/W was analysed. To this end, LCLs were co-transfected with pGL3 firefly luciferase vector and either FLAG-RPL22-p294-CMV/W, p294-CMV/W or pcDNA3.1 FLAG-Schlafen. Expression of firefly luciferase was determined by luciferase assay as a representation of protein expression in LCLs. Luciferase activity was not reduced in LCLs expressing FLAG-RPL22 (figure 4.15 a), suggesting that FLAG-RPL22 expression does not inhibit protein translation in general. Thus, the difficulties to overexpress RPL22 were not the result of inhibitory effects of RPL22 on protein expression.

Further, the survival of the LCLs was assessed 27 h post-transfection (figure 4.15 b). No difference in the survival of cells expressing FLAG-RPL22 and cells transfected with empty vector control was observed, whereas cell survival was reduced significantly by the transfection process compared to non-transfected control cells. Successful expression of FLAG-RPL22 in this experiment was confirmed by western blotting (figure 4.15 c). The experiment was performed repeatedly to ensure proper expression of FLAG-RPL22

despite the challenges described in the section above (section 4.3.1).

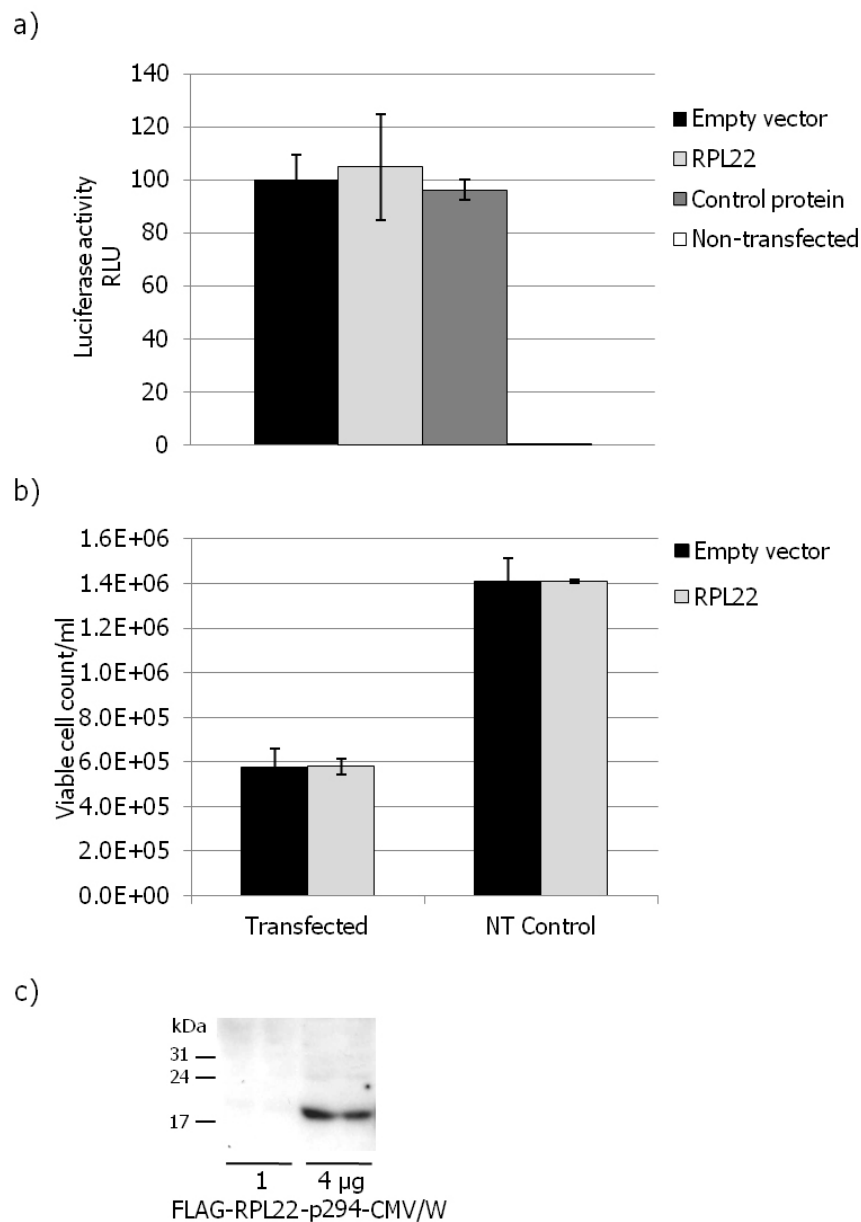


Figure 4.15: Expression of FLAG-RPL22 does not inhibit protein expression or LCL survival.

a) Expression of firefly luciferase from pGL3 vector was not repressed by FLAG-RPL22 from FLAG-RPL22-p294-CMV/W (RPL22) in comparison to empty p294-CMV/W vector control (empty vector) or FLAG-Schlafen from pcDNA3.1-FLAG-Schlafen (control protein). b) Survival of LCLs was assessed by trypan-blue staining 27 h post-transfection. Cell survival of LCLs transfected with FLAG-RPL22-p294-CMV/W (RPL22) was not reduced compared with p294-CMV/W (empty vector) control. Non-transfected cells (NT) are included as control. c) Expression of FLAG-RPL22 was confirmed by western blot in the LCLs analysed in a) and b) transfected with 4 µg FLAG-RPL22-p294-CMV/W. No FLAG-RPL22 was detected in cells transfection with 1 µg DNA.

4.3.3 Comparison of FLAG-RPL22 expression in wt and Δ EBER1

LCLs

Possible effects of RPL22 on host cell survival and protein expression could be altered by its interaction with EBER1. The survival of cells that overexpress FLAG-RPL22 was therefore investigated in wt and Δ EBER1 LCLs. Two independent cell lines were co-transfected with pGL3 firefly luciferase plus FLAG-RPL22-p294-CMV/W or p294-CMV/W for 24 h and 48 h in duplicate. Viable cells were quantified by trypan blue staining. No difference in cell survival was observed between wt and Δ EBER1 LCLs or between cells expressing FLAG-RPL22 and cells transfected with empty vector control 24 h and 48 h post-transfection (figure 4.16 a and b). Transfection rates of the different cells were controlled using luciferase assay (figure 4.16 c). The expression of FLAG-RPL22 was confirmed in all cell lines transfected with FLAG-RPL22-p294-CMV/W (figure 4.16 d) by western blotting against FLAG. Δ EBER1 LCLs 2 expressed slightly higher levels of FLAG-RPL22, probably as a consequence of a somewhat higher transfection rate in these cells (figure 4.16 c).

Taken together the results presented in figure 4.15 and figure 4.16 indicate that overexpression of RPL22 does not inhibit translation or cell growth in LCLs.

4.3.4 Effects of inducible FLAG-RPL22 expression in stably

transfected IB4 cells

In a parallel approach to investigate possible consequences of the overexpression of FLAG-RPL22 and to establish a more reliable tool for the investigation of RPL22 overexpression, an inducible FLAG-RPL22 system was established. To this end, FLAG-RPL22-MEP4 (section 2.3.12.4) was constructed, which contains FLAG-RPL22 under the control of the inducible metallothionein promoter.

IB4 LCLs were transfected with the constructed vector and stable cell lines expanded by Dr Gareth Brady. Expression of FLAG-RPL22 after induction with 5 μ M CdCl₂ was monitored in two independent cell lines in triplicate by western blotting for FLAG (figure

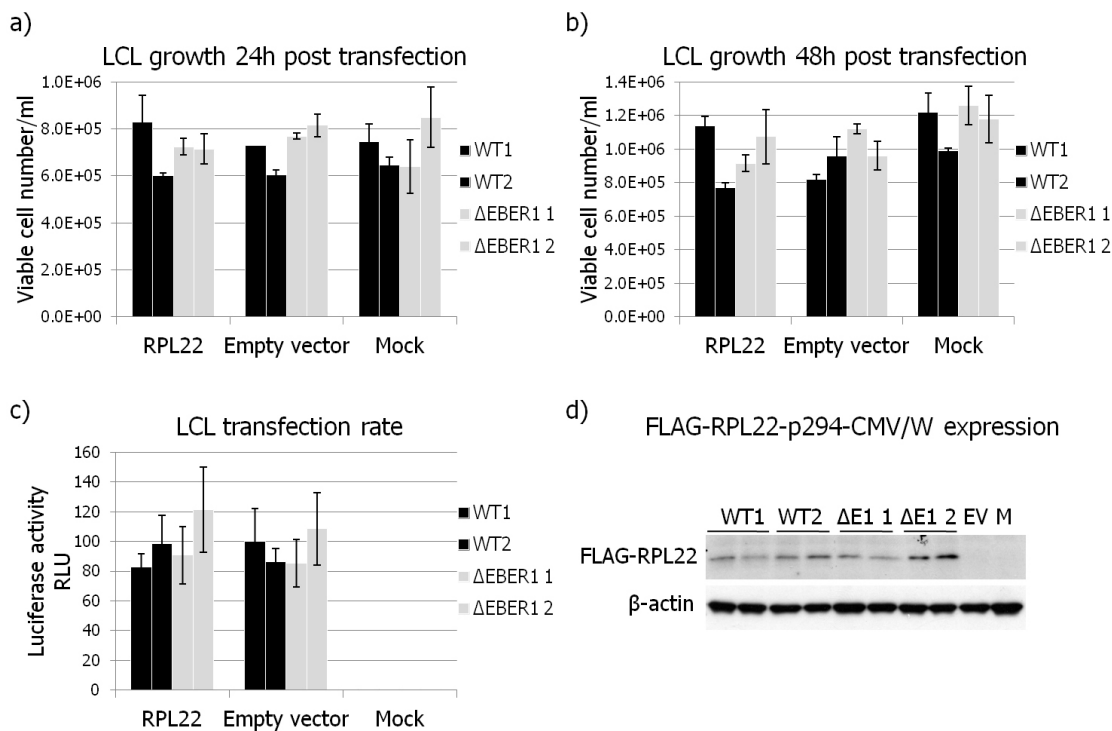


Figure 4.16: Survival of wt and Δ EBER1 LCLs expressing FLAG-RPL22.

No difference in the survival of wt and Δ EBER1 LCLs expressing FLAG-RPL22 was observed by trypan blue stain a) 24 h and b) 48 h post-transfection. Two independent cell lines (1 and 2) per virus type were tested in duplicate and FLAG-RPL22 expression from FLAG-RPL22-p294-CMV/W (RPL22) compared to p294-CMV/W (empty vector) and mock transfected cells. c) Expression vectors were co-transfected with pGL3 firefly luciferase and transfection rates analysed by luciferase assay to compare transfection rates of individual cell lines. No significant differences in the transfection rates were observed. d) Western blot analysis of FLAG-RPL22 expression. FLAG-RPL22 was expressed in duplicate of two wt EBV-transformed LCLs (WT1, WT2) and Δ EBER1 transformed LCLs (Δ E1 1, Δ E1 2). RPL22 levels were slightly elevated in Δ E1 2. No FLAG signal was detected in p294-CMV/W (EV) and mock transfected LCLs (M).

4.17). Both cell lines expressed FLAG-RPL22 at a good level and FLAG-RPL22 was not expressed in non-induced cells.

Western blotting does not give any indication of the amount of FLAG-RPL22 expressed per individual cell or of the percentage of cells that express the protein. A limited number of cells expressing a large amount of protein or a large number of cells expressing very little protein can lead to the same western blotting result. To establish if a sufficient proportion of IB4 cells expressed FLAG-RPL22 to display possible effects on cell growth, confocal microscopy analysis of stably transfected cells was performed. Expression of

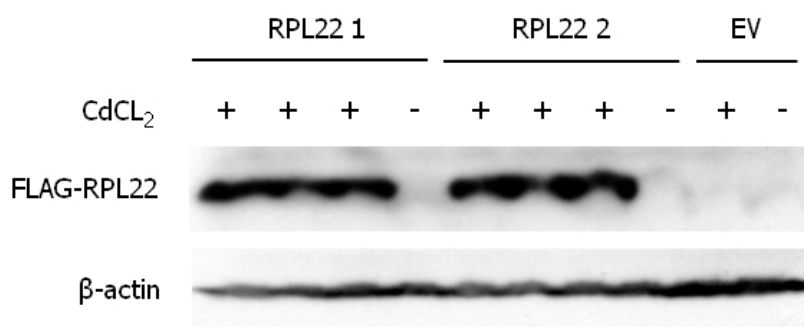


Figure 4.17: Expression of FLAG-RPL22 in IB4 cells.

Expression of FLAG-RPL22 was confirmed by western blotting in two independent IB4 cell lines stably transfected with FLAG-RPL22-MEP4 (RPL22 1 and RPL22 2) after induction with 5 μ M CdCl₂ (+) in triplicate for 48 h. No FLAG-RPL22 was expressed in non-induced cell lines (-). IB4 cells stably transfected with pMEP4 (EV) were included as control.

FLAG-RPL22 was induced with 5 μ M CdCl₂ in two independent cell lines for 48 h and FLAG-RPL22 detected using anti-FLAG antibody. A clear induction of FLAG-RPL22 was observed in induced cells compared to non-induced control cells (figure 4.18).

Subsequently, the induction of FLAG-RPL22 in IB4 cells was analysed in more detail. Different levels of FLAG-RPL22 induction were identified, which ranged from a weak induction to a very strong induction of expression of FLAG-RPL22, as shown in figure 4.19. Expression of FLAG-RPL22 was classified into non-induced, induced (+), strongly induced (++) and very strongly induced (+++) FLAG-RPL22 expression by judging the intensity of the microscopy signal in 100 randomly chosen cells. Usage of a lower detector gain for cells with very strong FLAG-RPL22 expression (+++) revealed a localisation of FLAG-RPL22 in the cytoplasm and the nucleoli.

An average of 36.7% of all cells from two independent IB4 cell lines stably transfected with FLAG-RPL22-MEP4 showed a strong expression (++) of FLAG-RPL22. Strong FLAG-RPL22 induction was found in 26.3% of all cells in the first and 47% of all cells in the second cell line. Furthermore, FLAG-RPL22 was very strongly expressed (+++) in 5.2% and 3.6% of all cells in the first or in the second cell line, respectively, resulting in an average of 4.4% of all cells. The number of cells with weak expression of FLAG-RPL22 was not quantified as it was difficult to distinguish between the background staining in non-induced cells due to leaky expression or unspecific binding of the antibodies (figure

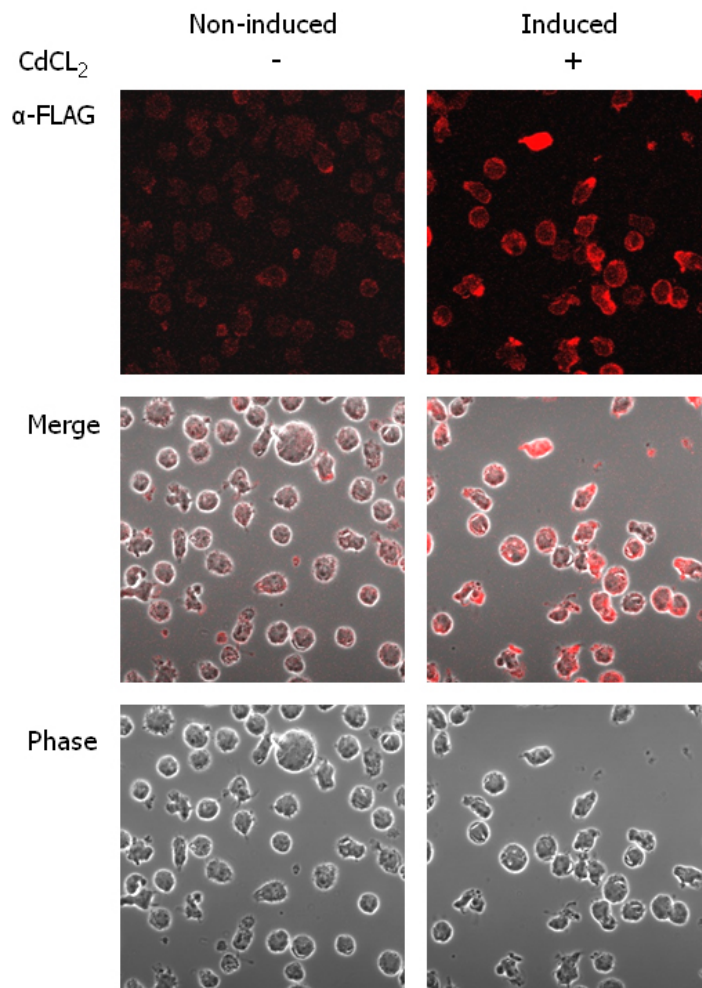


Figure 4.18: Overview of the expression of FLAG-RPL22 in IB4 cells.

IB4 cells stably transfected with FLAG-RPL22-MEP4 were induced with 5 μ M CdCl₂ (+) and FLAG-RPL22 expression analysed by confocal microscopy 48 h post-induction. Expression of FLAG-RPL22 was clearly induced in a large proportion of IB4 cells.

4.18 left panel) and weak, specific induction of FLAG-RPL22 by CdCl₂ (figure 4.18 right panel). In summary, more than 40% of IB4 cells displayed a clear expression of FLAG-RPL22.

The effect of expression of RPL22 on cell survival was subsequently investigated by induction of FLAG-RPL22 and trypan blue staining 24 h and 48 h post-induction (figure 4.20). No difference in IB4 cell survival between the cell lines expressing FLAG-RPL22 (RPL22 1 and RPL22 2) or the cell lines transfected with pMEP4 control was observed. The survival of induced cells was determined both as a total viable cell number and as a percentage of the uninduced cell control, as addition of CdCl₂ reduced the survival of the

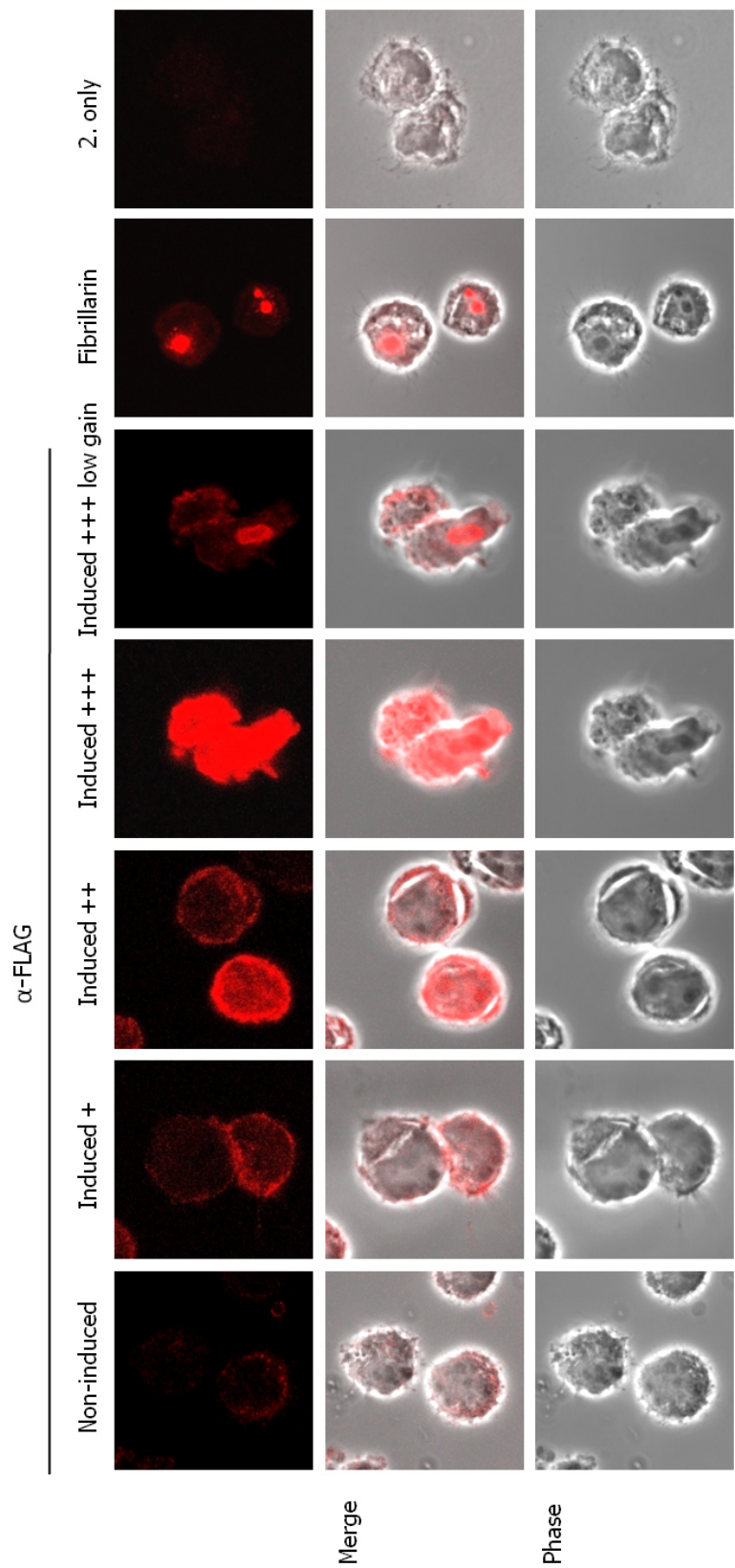


Figure 4.19: Analysis of the induction of FLAG-RPL22 expression in IB4 cells.

IB4 cells stably transfected with FLAG-RPL22-MEP4 were induced with 5 μ M CdCl₂ and FLAG-RPL22 expression analysed by confocal microscopy after 48 h. Induction of FLAG-RPL22 was classified into non-induced, induced (+), strongly induced (++) and very strongly induced (+++). Strongly induced cells were additionally imaged using a reduced detector gain. Fibrillarlin staining and secondary antibody only (2. only) were included as controls.

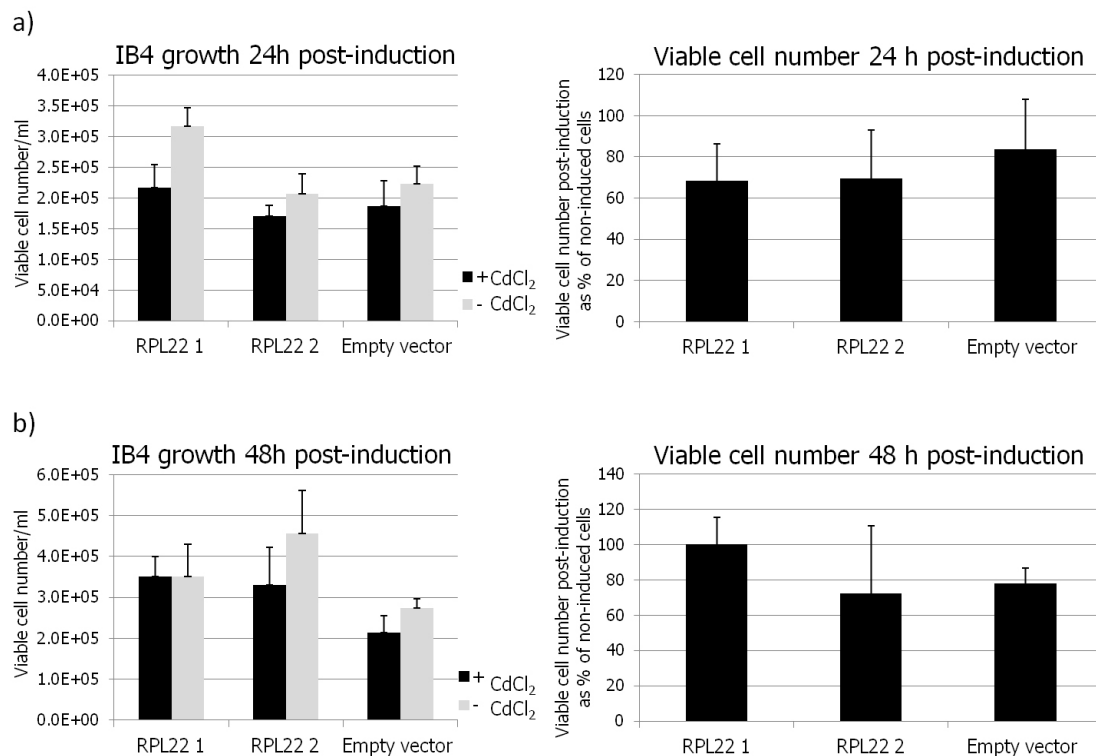


Figure 4.20: Survival of IB4 cells expressing FLAG-RPL22.

Cell viability was not altered by the expression of FLAG-RPL22. No difference in IB4 cell survival of two cell lines expressing FLAG-RPL22 (RPL22 1 and RPL22 2) or cells transfected with pMEP4 (empty vector) was observed by trypan blue staining a) 24 h and b) 48 h post-induction with 5 μ M CdCl₂. Cell survival of the induced cells is presented as total viable cell number (left) and as a percentage of the uninduced cell control (right).

cells slightly even in empty vector transfected cells in which no protein was expressed.

In the quantification by confocal microscopy, 50.6% of all cells in the second IB4 cell line clearly expressed FLAG-RPL22 in contrast to only 31.5% of all cells in the first cell line. This higher expression of FLAG-RPL22 in the second cell line was also found by western blot (figure 4.21). Additionally, the amount of FLAG-RPL22 in comparison to endogenous RPL22 was compared by western blotting using anti-RPL22 antibody and quantification with densitometry software. The intensity of the FLAG-RPL22 band was approximately 55% or 70% of the intensity of the endogenous RPL22 band in the two IB4 cell lines. Assuming that only 31.5% and 50.6% of the cells expressed FLAG-RPL22 (figure 4.19), the relative amount of RPL22 in cells that successfully express the recombinant protein can be estimated as 170% and 140% of the amount of

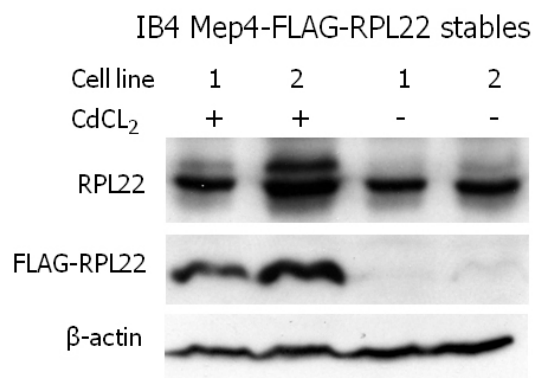


Figure 4.21: Expression levels of endogenous RPL22 and FLAG-RPL22 in IB4 cells stably transfected with FLAG-RPL22-MEP4 were analysed by western blotting for FLAG or endogenous RPL22 48 h post-induction with 5 μ M CdCl₂ (+) and in non-induced cells (-). β -actin was included as loading control.

endogenous RPL22. Hence, the levels of FLAG-RPL22 in individual cells are significant and any effects of RPL22 overexpression on the survival of the IB4 cells should have become apparent.

In conclusion, FLAG-RPL22 expression was successfully induced in more than 40% of the cells in two independent IB4 cell lines stably transfected with FLAG-RPL22-MEP4 using CdCl₂ for 48 h. Taking the transfection rate into account, it can be estimated that 1.4-1.7 times more FLAG-RPL22 than endogenous RPL22 is expressed in induced cells (figure 4.21). FLAG-RPL22 expression was confirmed by western blotting (figure 4.17) and confocal microscopy (figure 4.19).

Despite the clear induction of FLAG-RPL22, no effect of RPL22 overexpression on IB4 cell survival was observed (figure 4.20). These results suggest that RPL22 has no toxic effect on IB4 cells, in agreement with findings indicating that overexpression of RPL22 does not inhibit translation or cell growth in freshly transformed LCLs (figure 4.15 and figure 4.16). However, strongly FLAG-RPL22 expressing IB4 cells accumulated FLAG-RPL22 in the nucleoli (figure 4.19), which freshly transformed LCLs might not be able to tolerate (table 4.1). IB4 cells are a long established cell line ^[323] and might have acquired unknown mutations of ribosomal stress pathways. In addition, IB4s contain EBV integrated into the genome but no episomal viral DNA ^[414] and the C promoter is deleted from the EBV sequence ^[48]. RPL22 might have effects on LCLs which remained undiscovered due to these unusual characteristics of IB4s.

It would be interesting to investigate effects of inducible FLAG-RPL22 overexpression in freshly transformed LCLs. The FLAG-RPL22-MEP4 vector constructed here was

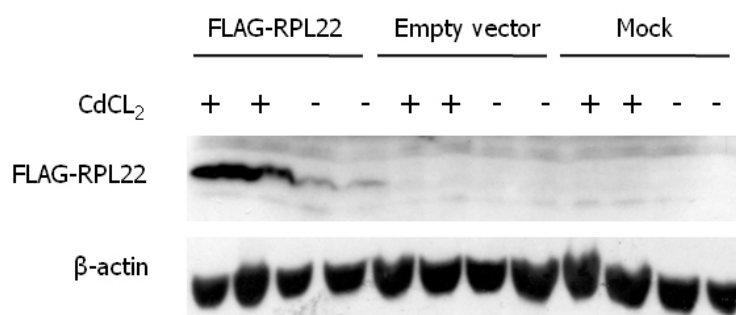


Figure 4.22: Expression of FLAG-RPL22 from FLAG-RPL22-MEP4 in LCLs.

FLAG-RPL22 was expressed from FLAG-RPL22-MEP4 in LCLs after induction with 5 μ M CdCl₂ for 48 h. Protein extracts of non-induced cells, pMEP4 empty vector and mock transfected cells were included as controls in the western blot.

successfully transfected and expressed in LCLs (figure 4.22) in transient transfection. To establish stably transfected cell lines, the hygromycin resistance cassette of FLAG-RPL22-MEP4 will have to be replaced by a different resistance marker to allow selection in LCLs, as BAC transformed LCLs already contain hygromycin resistance genes on the BAC.

4.3.5 Analysis of cell survival in response to chemotherapeutic agents in LCLs

Many ribosomal proteins play key roles in cell survival, especially via p53-mediated signalling pathways ^[412] and EBER1 had previously been implicated in the prevention of apoptosis ^[100, 101, 298, 308, 309]. To examine an involvement of the interaction of EBER1 with RPL22 in p53-dependent stress responses, LCLs were exposed to a variety of chemotherapeutic agents. To do so, 2.5×10^5 cells/ml were incubated with the toxin of choice or no toxin as control for 16 h. The number of viable cells was then determined by trypan blue exclusion staining and protein samples were collected.

Cisplatin is a platinum-containing, broad activity alkylating agent effective against a variety of cancers. Cisplatin leads to DNA cross-links by replacing its chloride for nitrogen atoms of adjacent guanines, thereby stalling RNA polymerase II and triggering transcription-coupled DNA repair. Moreover, cisplatin treatment results in inhibition of

rRNA synthesis and ribosome biogenesis as well as nucleolar disruption ^[415].

Three wt or Δ EBER1 EBV LCLs were treated with 20 μ g/ml cisplatin for 16 h in triplicate. Cisplatin strongly diminished cell viability and reduced LCL numbers from approximately 3×10^5 cells/ml to 1×10^5 cells/ml within 16 hours (figure 4.23, top left) while control cells increased in number (figure 4.23, top right), consistent with earlier reports on the response of LCLs to cisplatin ^[416]. Cisplatin affected the growth of wt or Δ EBER1 EBV LCLs to a similar extent, suggesting that EBER1 or the interaction of EBER1 with RPL22 does not influence this stress response of EBV-positive cells.

Additionally, induction of p53 and p73 by cisplatin was examined by western blotting (figure 4.23, bottom). p53 expression was clearly increased by cisplatin in both wt and Δ EBER1 EBV LCLs after 16 h. Variations in the amount of p53 detected in the protein extracts of the different cell lines were largely the result of variations in the amount of protein loaded, represented by β -actin. There was no significant and reproducible difference in p53 expression between wt and Δ EBER1 EBV BAC transformed LCLs. As expected, only very low levels of p53 were observed in the control cells.

One form of p73, most likely p73 β , was present at various extents in toxin-treated and control cells at the start of the experiment (0 h). Cisplatin resulted in a weak induction of expression of p73 α and a reduction of expression of p73 β , while p73 δ remained constant in toxin-treated cells. No correlation between the expression of p73 and the presence of EBER1 was discovered. Currently, it is controversial if cisplatin induces p73 ^[417, 418] and the effects are most possibly both concentration and cell type specific.

The proteins detected in the p73 western blot are likely to correspond to the 69.6 kDa p73 α , the 54.3 kDa p73 β and the 44.5 kDa p73 δ isoform of p73 (UniProtKB/Swiss-Prot). However, the p73 gene comprises 15 exons and multiple alternative splicing variants. Together with transcription from alternative promoters, this results in at least 35 mRNA variants, which encode theoretically 29 different p73 protein isoforms ^[419]. In addition, p73 is regulated through post-translational modifications. Thus, the detected protein bands for p73 cannot be identified with certainty.

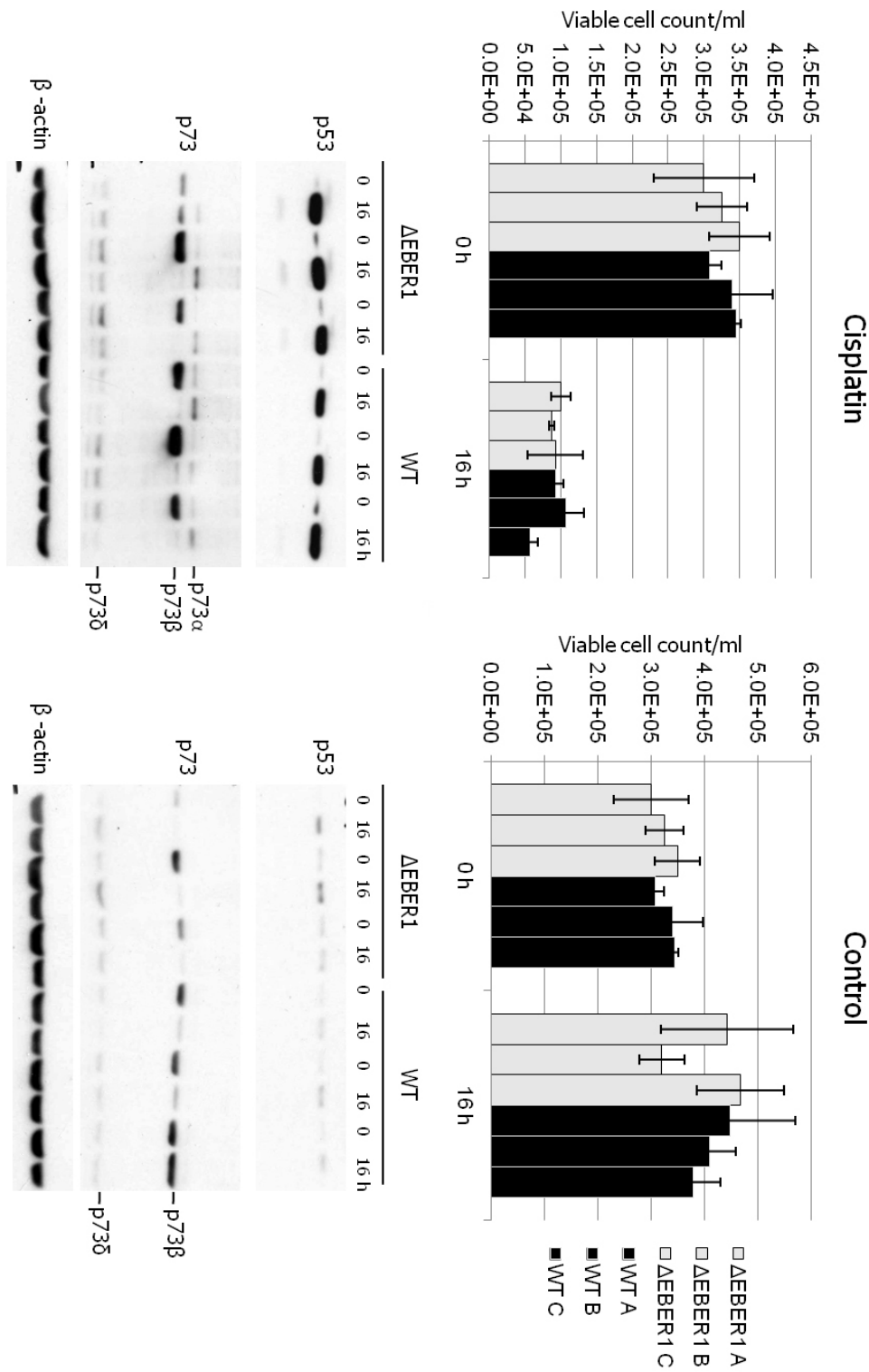


Figure 4.23: Effects of cisplatin treatment on the survival of LCLs. Three ΔEBER1 EBV LCLs (ΔEBER1 A-C, grey) or wt EBV LCLs (WT A-C, black) were exposed to 20 μ g/ml cisplatin for 16 h in triplicate. The number of viable cells was quantified by trypan blue staining and induction of p53 and p73 was analysed by western blotting. No difference in the response to cisplatin was found between wt and ΔEBER1 EBV LCLs. Control cells without toxin treatment were included for comparison (control).

Next, wt and Δ EBER1 EBV BAC LCLs were challenged with 5 nM actinomycin D for 16 h. Actinomycin D is a potent inhibitor of RNA polymerases, specifically of RNA polymerase I at low concentrations as used here. Similar to cisplatin, actinomycin D inhibits ribosome biogenesis and leads to a disruption of the nucleoli [415, 420].

Actinomycin D had a cytostatic effect on the LCLs and the number of viable cells after 16 h was at the level of the cell input number (figure 4.24, top left). No difference in cell growth between wt and Δ EBER1 EBV BAC LCLs was noticed. Nevertheless, induction of p53 and p73 by the chemotherapeutic agent was investigated by western blotting. A strong induction of p53 was observed in response to actinomycin D, consistent with the growth arrest (figure 4.24, bottom left). However, there was no significant difference in p53 induction in wt and Δ EBER1 EBV BAC LCLs, suggesting that the interaction of EBER1 with RPL22 does not alter the cell stress response. In agreement with previous studies [421], p73 was not induced by actinomycin D. Interestingly, amounts and isoforms of p73 expressed strongly varied between the cell lines, independent of EBER1 status. Expression of p73 α was increased after 16 h in both the actinomycin-treated cells and the untreated control cells, implying that p73 α was induced in response to an uncontrolled factor in the cell environment or cell density.

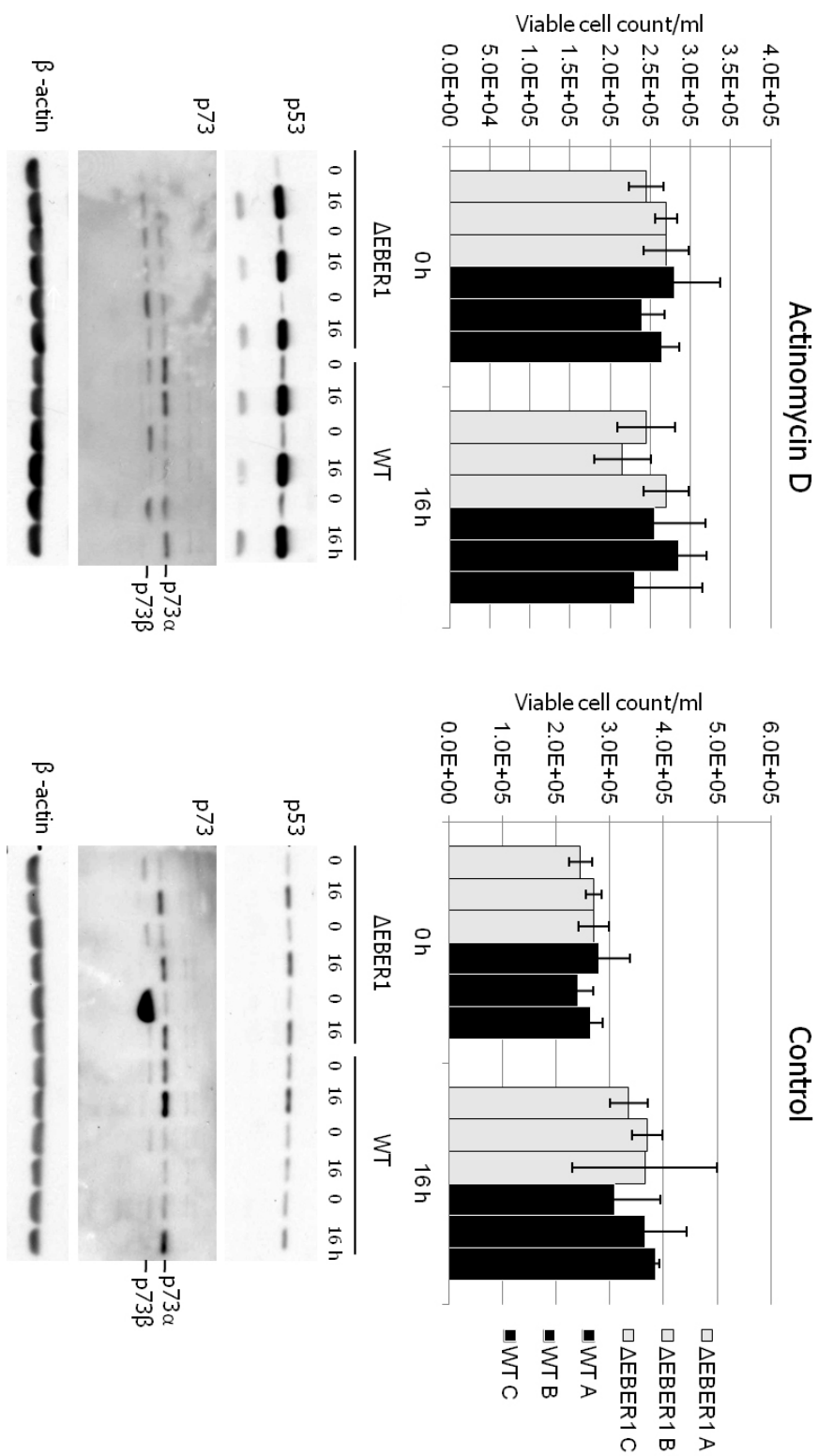


Figure 4.24: Effects of actinomycin D treatment on the survival of LCLs. Three ΔEBER1 EBV LCLs (ΔEBER1 A-C, grey) or wt EBV LCLs (WT A-C, black) were treated with 5 nM actinomycin D for 16 h in triplicate. Viable cell counts were determined by trypan blue staining and expression of p53 and p73 was analysed by western blotting. No difference in the response to actinomycin D was found between wt and ΔEBER1 EBV LCLs. Control cells grown without toxin were included for comparison (control).

Finally, LCLs were exposed to 20 $\mu\text{g/ml}$ etoposide for 16 h, a topoisomerase inhibitor which also constrains ribosome biogenesis by arresting late rRNA processing ^[415]. In contrast to the chemotherapeutic agents investigated above, etoposide does not disrupt nucleolar structures. For the LCLs, the number of viable cells was clearly reduced in etoposide-treated cells from 2.5×10^5 cells/ml to 1.5×10^5 cells/ml within 16 h, while control cells grew up to 3×10^5 cells/ml in the same time (figure 4.25, top). Wt and ΔEBER1 EBV BAC LCLs showed the same growth inhibition in response to etoposide and also the same growth rate in control cells. Etoposide increased expression of p53 very efficiently but the presence or absence of EBER1 in the cells did not lead to an observable difference in the induction of the protein, while non-treated control cells expressed only low levels of p53, as expected (figure 4.25, bottom). Again, p73 expression was analysed by western blotting and a diverse set of isoforms and protein levels was observed in the LCLs; these were not related to EBV strain. As observed in LCLs treated with actinomycin D (figure 4.24), expression of p73 α was enhanced after 16 h in both the etoposide-treated cells and the untreated control LCLs.

Subsequently, the survival of wt, ΔEBER1 and EBER1-revertant LCLs in response to 20 $\mu\text{g/ml}$ etoposide or cisplatin was measured in a time course 0, 8, 24 and 32 h post-addition of the toxin to explore distinct characteristics in the kinetics of cell death depending on EBV type. Figure 4.26 illustrates the number of viable cells as determined by trypan blue exclusion at the given time points. Cell growth was inhibited in cells treated with cisplatin (figure 4.26, top) and etoposide (figure 4.26, middle) in contrast to non-treated control LCLs (figure 4.26, bottom), as anticipated. Consistent with the observations after 16 h (figure 4.23 and figure 4.25), no differences between viable cell count of wt, ΔEBER1 and EBER1-revertant LCLs were identified.

Overall, the presence of EBER1 in the LCLs did not alter stress responses of the cells with respect to cell survival or expression of p53 and p73.

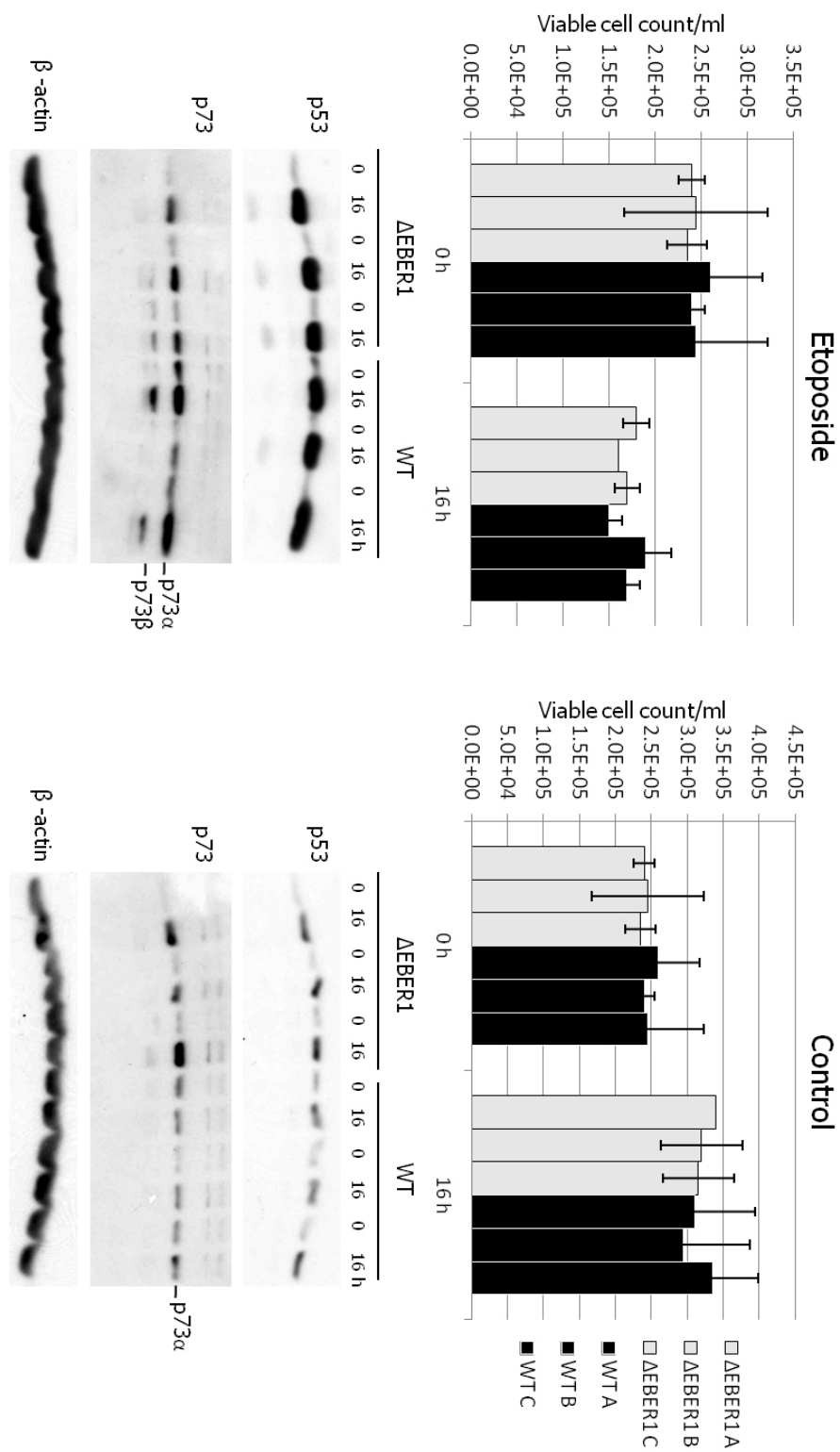


Figure 4.25: Effects of etoposide treatment on the survival of LCLs.

Three ΔEBER1 EBV LCLs (ΔEBER1 A-C, grey) or wt EBV LCLs (WT A-C, black) were treated with 20 μ g/ml etoposide for 16 h in triplicate. The number of viable cells was quantified by trypan blue exclusion staining and expression of p53 and p73 was analysed by western blotting. No difference in the response to etoposide was found between wt and ΔEBER1 EBV LCLs. Control cells grown without etoposide were included as control (control).

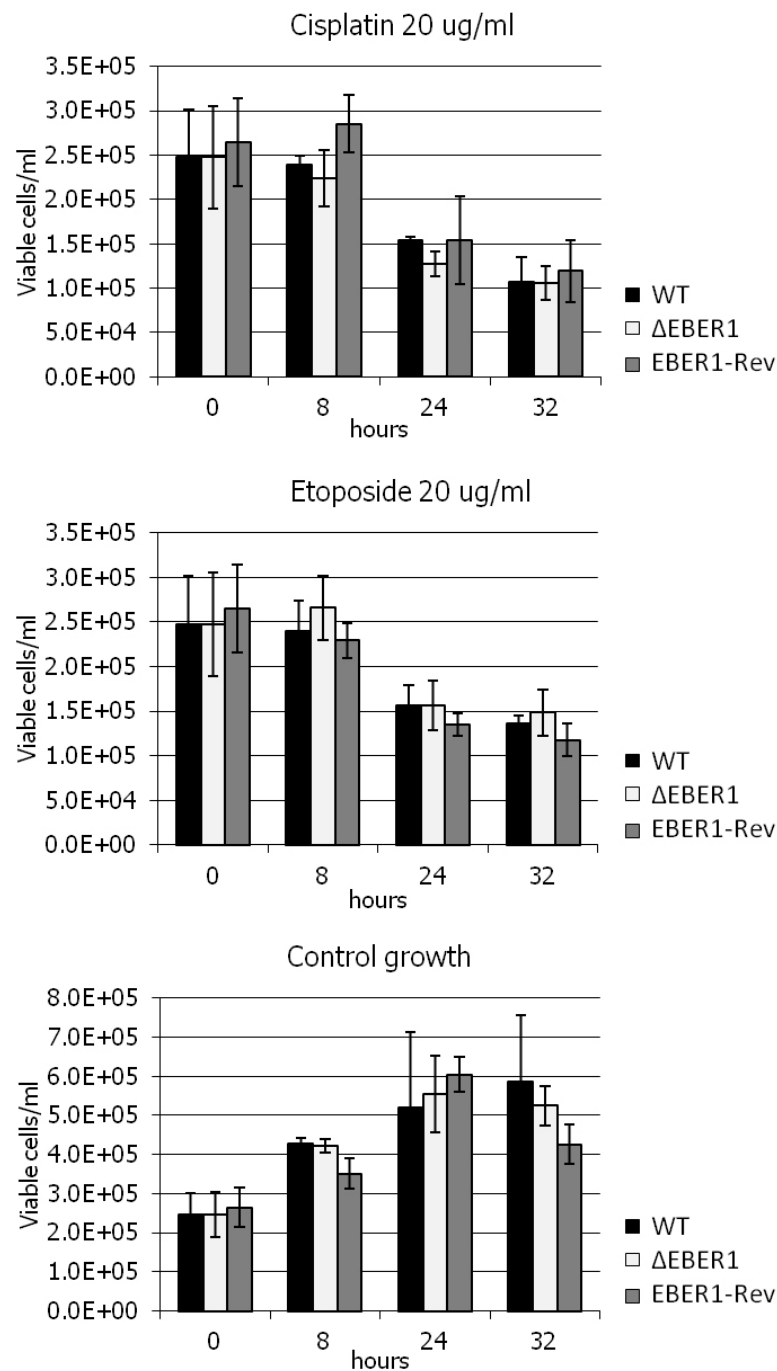


Figure 4.26: Effects of cisplatin or etoposide on the survival of LCLs.

Wt (black), Δ EBER1 (bright grey) and EBER1-revertant LCLs (dark grey) were treated with 20 μ g/ml cisplatin (top) or etoposide (middle) in quadruplicate. Viable cell counts were quantified by trypan blue staining at time points 0, 8, 24 and 32 h. Cell survival of all LCLs was similar. Control cells without toxin treatment were included for comparison (control growth).

4.3.6 Establishment of wt and Δ RPL22 MEF cell lines

To investigate the function of the interaction of EBER1 with RPL22, it would be necessary to first understand the physiological role of RPL22 in uninfected cells. For this purpose, a new cellular system consisting of immortalised mouse embryonic fibroblasts (MEF) with wt or RPL22 double deletion genotype was created. Primary Δ RPL22 MEFs, kindly provided by Prof David L Wiest, Fox Chase Cancer Center, Philadelphia, were immortalised according to the 3T3 protocol (section 2.2.8). Characteristically, the growth rate of the primary wt and Δ RPL22 MEFs was reduced during the early passaging of the cells, depicted as a multiple of cell number doublings within 3 days (figure 4.27 a). Subsequently, cells became immortalised gradually and cell growth increased, as shown at the top of figure 4.27. Deletion of RPL22 was confirmed in the established cell lines by RT-PCR and western blotting (figure 4.27 b and c).

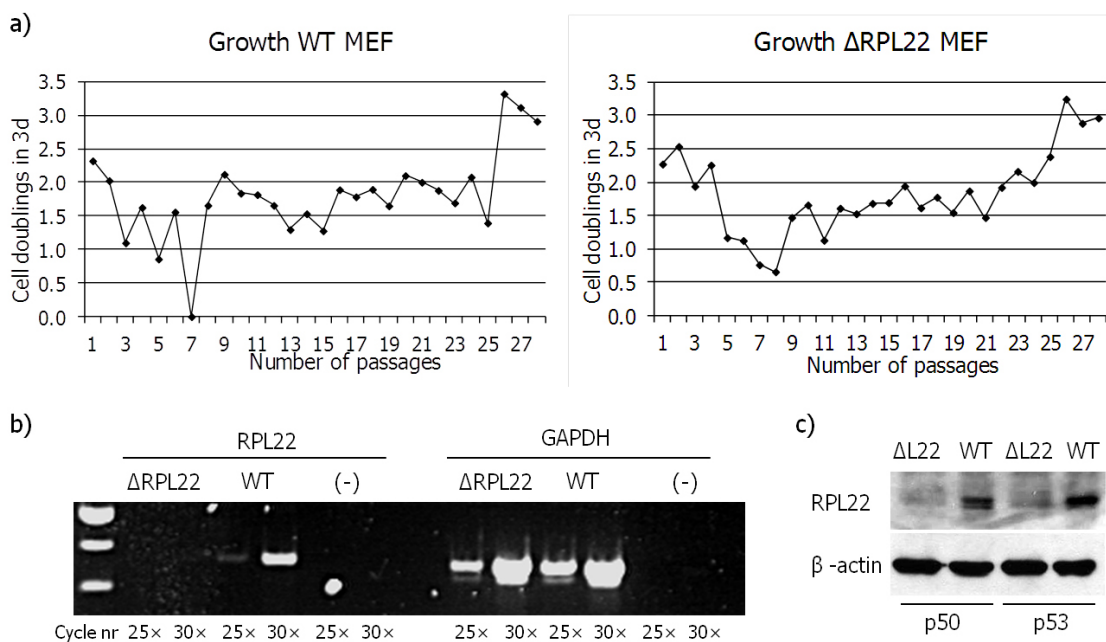


Figure 4.27: Establishment of immortalised Δ RPL22 mouse embryonic fibroblasts.

a) Growth properties of wt and Δ RPL22 MEFs in the 3T3 cultivation protocol. Growth rates increased after 20-25 passages. Deletion of RPL22 was confirmed in Δ RPL22 MEFs on b) RNA and c) protein level by RT-PCR and western blot, respectively, in passages 50 and 53 (p50, p53).

4.3.7 Analysis of cell survival in response to chemotherapeutic agents in wt and Δ RPL22 MEF cells

The newly established wt and Δ RPL22 MEFs allowed the investigation of the role of RPL22 in stress responses in cell culture for the first time. RPL22 is known to mediate a p53-dependent checkpoint in α/β -T cell development [231]. Together with well described functions of many ribosomal proteins in p53-mediated stress responses, this makes RPL22 a promising candidate for an involvement in p53 signalling. Influencing a possible role of RPL22 in p53 signalling via its interaction with EBER1 would be an interesting way for EBV to alter the responses of its host cells to EBV infection.

Survival and growth of wt and Δ RPL22 MEFs were examined extensively by culturing 5×10^4 MEFs per well in 6-well plates for 24 h. Subsequently the cells were exposed to cisplatin, etoposide, actinomycin D, mitomycin C or no toxin as control and viable cell numbers were quantified by trypan blue exclusion staining in time courses. Different concentrations of toxins were used and MEFs from different passage numbers assayed.

Firstly, well established wt and Δ RPL22 MEFs were challenged by addition of 10 μ g/ml or 50 μ g/ml cisplatin for 0, 8, 24 and 32 h in duplicate. Viable cell counts were assessed and are displayed as a percentage of the control cells to normalise for variations in the growth of untreated cells, thereby emphasising the difference between toxin-treated and non-treated cells (figure 4.28). Wt and Δ RPL22 MEFs showed a similar growth rate in the control cells (figure 4.28 a). Addition of cisplatin reduced the viable cell number significantly in a concentration dependent manner. Within the first 8 hours of the experiment, 50 μ g/ml cisplatin lowered the viable cell number to below 70% of the control cells (69.5% for wt and 68% for Δ RPL22 MEFs), while 97.5% and 93.5% of cells were still alive in wt or Δ RPL22 MEFs, respectively, when 10 μ g/ml cisplatin was administered to the cells (figure 4.28 b and c). The percentage of viable cells was further reduced after 24 h and 32 h, leaving less than 10% viable cells in 50 μ g/ml cisplatin samples.

Cisplatin treatment of the cells led to similar cell death in wt and Δ RPL22 MEFs after 8 h

and 24 h both with 10 $\mu\text{g/ml}$ and with 50 $\mu\text{g/ml}$ cisplatin. Slightly more viable wt MEFs than ΔRPL22 MEFs were found in cells treated with 10 $\mu\text{g/ml}$ cisplatin for 32 h (17.1% for wt and 4.7% for ΔRPL22 MEFs). However, very few viable cells were present in these samples, which potentially makes small differences in cell counts more significant than is physiologically relevant. Stronger and reproducible effects of RPL22 were looked for to allow the subsequent investigation of a potential modification of the function of RPL22 by EBV and EBER1 specifically.

Cell survival in response to 10 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$ etoposide was therefore explored in an identical experimental set up. Similar numbers of viable cells were found in MEFs treated with 10 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ etoposide after 8 hours of cultivation of the cells with the drug. 90.4% and 87.3% of cells were viable compared to control cells in wt MEFs and 76.1% and 72.5% in ΔRPL22 MEF samples (figure 4.29). The slightly more pronounced reduction in cell survival observed in ΔRPL22 MEFs compared to the wt cells was not statistically significant. Addition of etoposide reduced the viable cell number in a concentration dependent manner at later points of the time course. Cultivation of the cells in the presence of 10 $\mu\text{g/ml}$ etoposide for 24 h decreased the number of viable cells to approximately 40% of the untreated control cell number, while only around 30% viable cells were detected in samples treated with 50 $\mu\text{g/ml}$ etoposide. A further reduction of viable cell numbers was noticed at time point 32 h. Most importantly, ΔRPL22 MEFs manifested no distinct reaction to the anti-cancer agent.

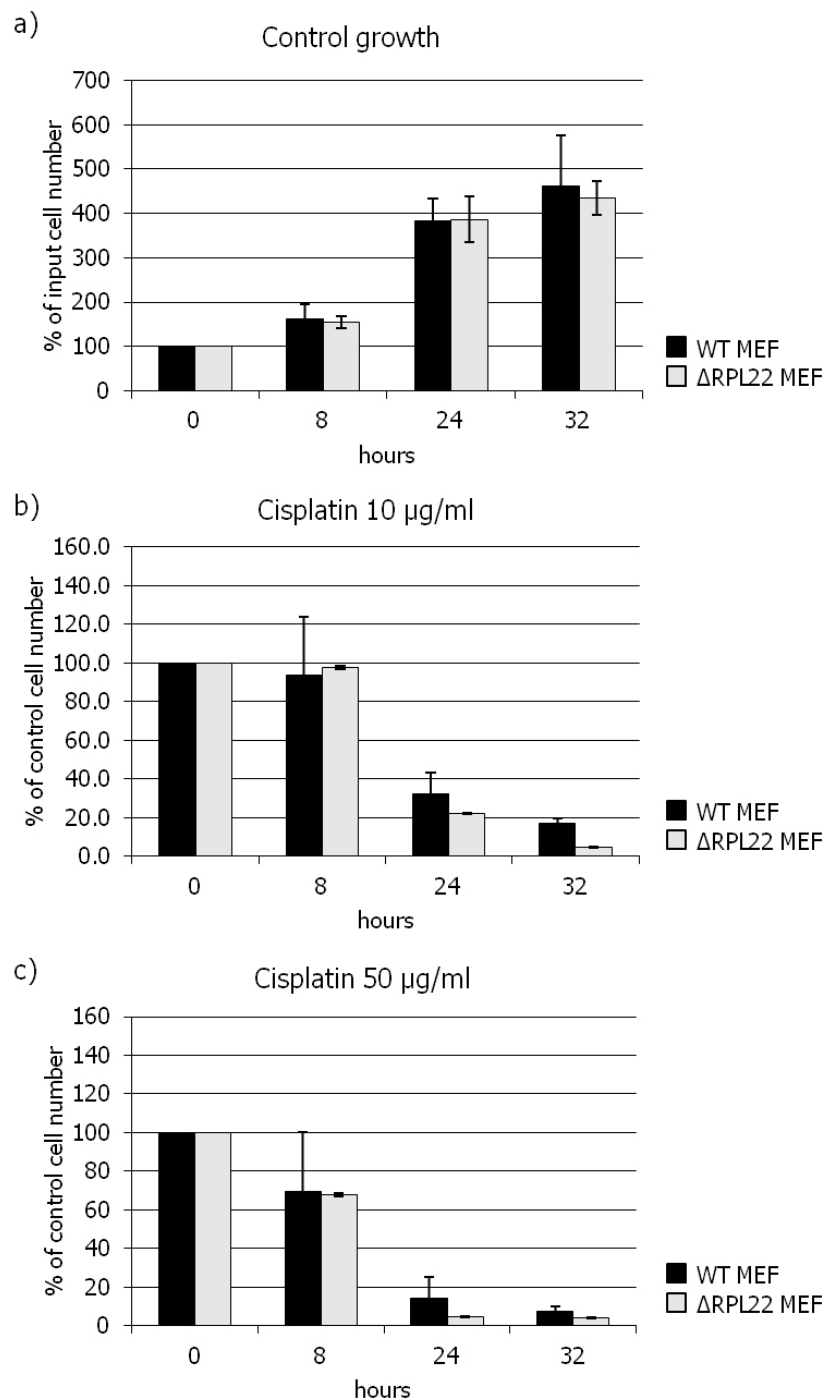


Figure 4.28: Effects of cisplatin on the survival of wt and Δ RPL22 MEFs.

Viable cell counts were determined by trypan blue exclusion for wt (black) and Δ RPL22 MEFs (grey) in a) untreated control cells and cells treated with b) 10 μ g/ml cisplatin or c) 50 μ g/ml cisplatin for 0, 8, 24 and 32 h.

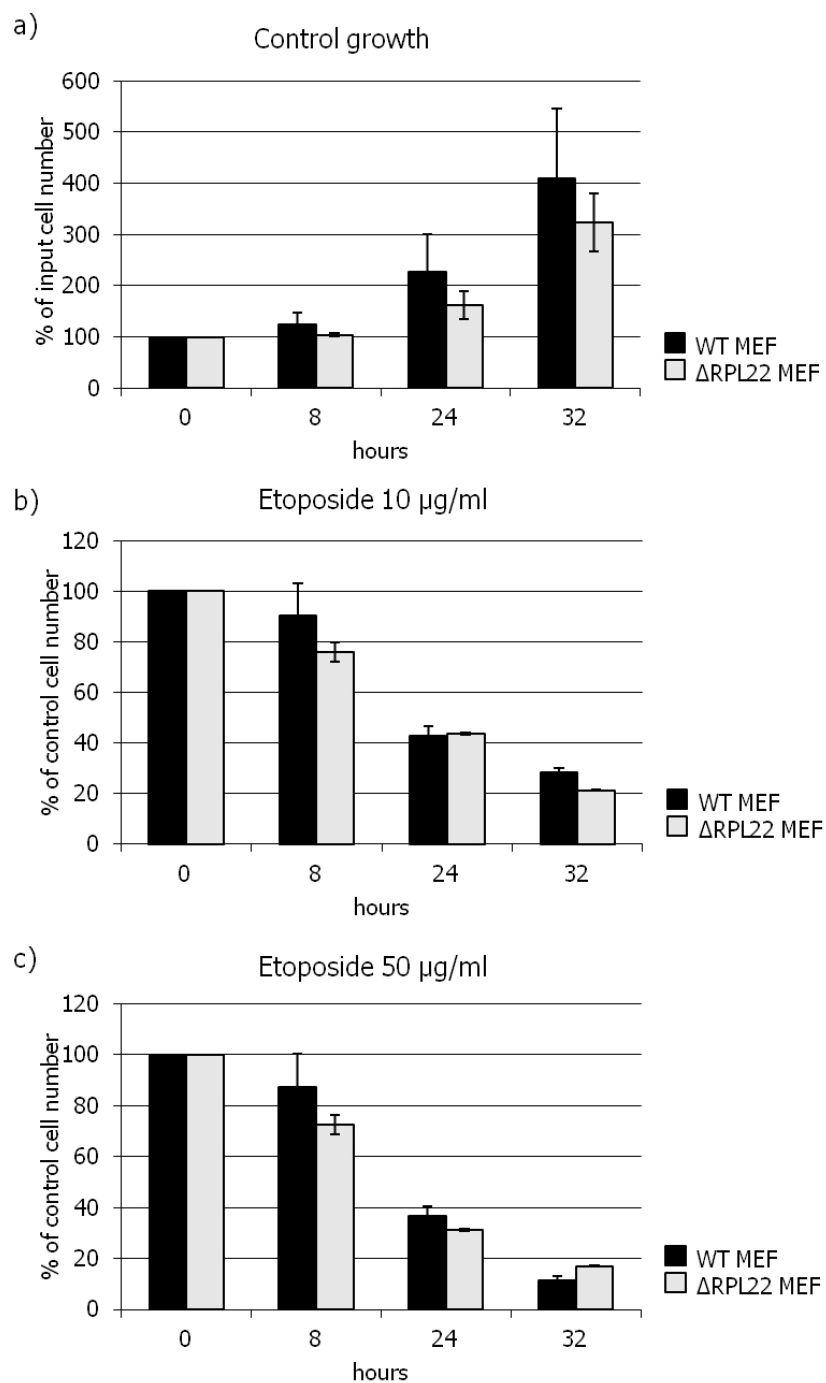


Figure 4.29: Effects of etoposide on the survival of wt and ΔRPL22 MEFs.

Viable cell counts were determined by trypan blue exclusion for wt (black) and ΔRPL22 MEFs (grey) in a) untreated control cells and cells exposed to b) 10 μg/ml etoposide or c) 50 μg/ml etoposide for 0, 8, 24 and 32 h.

No differences in cell survival in response to the anti-cancer drugs cisplatin and etoposide were detected between wt and Δ RPL22 MEFs in time courses (figure 4.28 and figure 4.29). These experiments were performed using fairly high passage number MEFs, which might have acquired mutations in their stress response pathways during the immortalisation process. Thus, effects of 20 μ g/ml cisplatin, 20 μ g/ml etoposide or 5 nM actinomycin D on cell survival were studied in MEFs from passage 12 (not shown) and passage 5 (figure 4.30) for 24 h in triplicate. Wt and Δ RPL22 MEFs behaved identically in response to all toxins in both passage 5 and passage 12 MEFs. For passage 5, control cells grew out from 2.75×10^4 cells/ml to around 4×10^4 cells/ml (figure 4.30 a). Addition of etoposide reduced cell number to close to 2×10^4 cells/ml (figure 4.30 b), while cisplatin strongly diminished viable cells to less than 6×10^3 cells/ml (figure 4.30 c). Actinomycin displayed a more cytostatic effect on the MEF cells and viable cell number increased marginally to 3×10^4 cells/ml (figure 4.30 d).

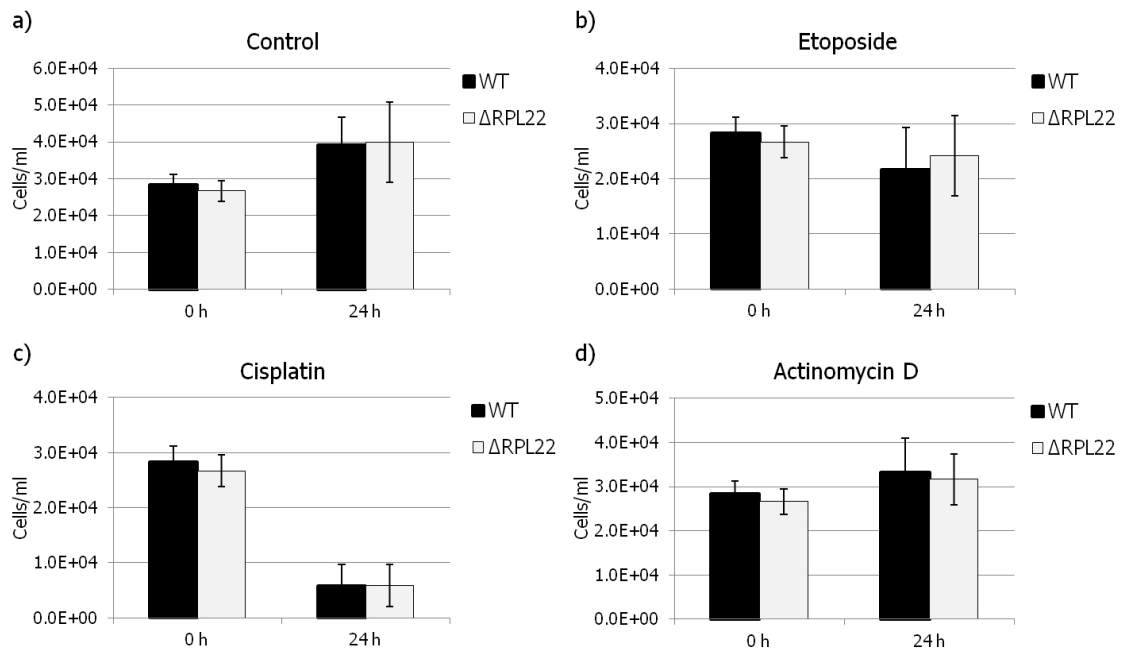


Figure 4.30: Effects of etoposide, cisplatin and actinomycin D on the survival of passage 5 wt and Δ RPL22 MEFs.

Viable cell counts were determined for wt (black) and Δ RPL22 MEFs (grey) in a) untreated control cells and cells treated with b) 20 μ g/ml etoposide, c) 20 μ g/ml cisplatin or d) 5 nM actinomycin D for 24 h.

Although the results obtained from the Δ RPL22 MEFs suggest that RPL22 does not play a significant role in cell death induced by chemotherapeutic agents, expression levels of p53 and p73 were examined by western blotting (figure 4.31). p73 was clearly increased to a similar extent in wt and Δ RPL22 MEFs in all cells after 24 h, including non-treated control cells, suggesting that p73 expression is enhanced due to cell density or unknown stress inducers, as observed in LCLs (section 4.3.5). Only very low levels of p53 were found in all the samples, indicating that growth arrest or cell death occurs in a p53-independent manner in the MEFs, possibly due to already mutated p53 signalling in passage 5 MEFs. Of note is that the passage number was assigned upon arrival of the primary cells in our laboratory and does not include a possible limited number of passages in the originating laboratory.

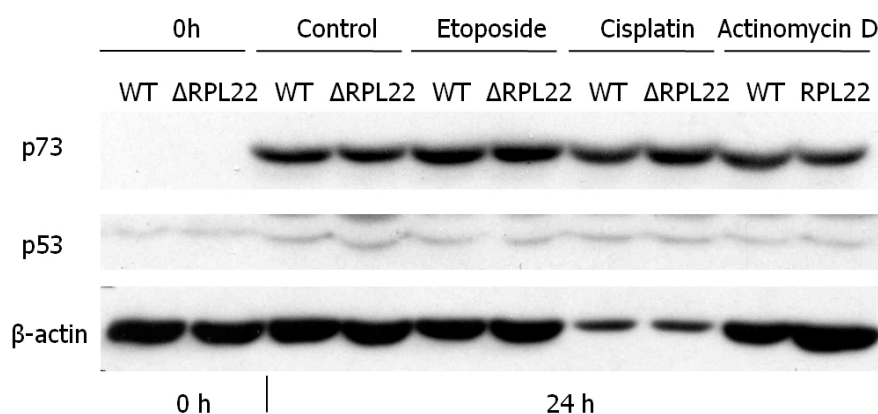


Figure 4.31: Expression of p53 and p73 in response to etoposide, cisplatin and actinomycin D in passage 5 wt and Δ RPL22 MEFs.

Passage 5 wt and Δ RPL22 MEFs were treated with 20 μ g/ml cisplatin, 20 μ g/ml etoposide or 5 nM actinomycin D for 24 h and expression of p53 and p73 was analysed by western blotting. β -actin levels were included as loading control. Less protein was loaded in cisplatin samples as a consequence of the limited availability of viable cells.

Lastly, wt and Δ RPL22 MEFs were exposed to 10 μ g/ml mitomycin C for 0, 8, 16, 24, 32 and 40 h as final anti-cancer drug to be tested. Mitomycin C strongly limited the number of viable cells and led to the complete death of the cell culture within 40 hours, but RPL22 presence did not alter the cell response, as wt and Δ RPL22 MEFs reacted analogously to the toxin (figure 4.32).

In summary, deletion of RPL22 from MEFs has no effect on cell survival and expression of p53 and p73 in response to a series of anti-cancer drugs, indicating that RPL22 is not essential for cell stress responses to these specific chemotherapeutic agents in MEFs in these experimental conditions. The established wt and Δ RPL22 MEF cell lines will allow further investigation of RPL22 functions in stress responses or other cell processes in our research group in the future.

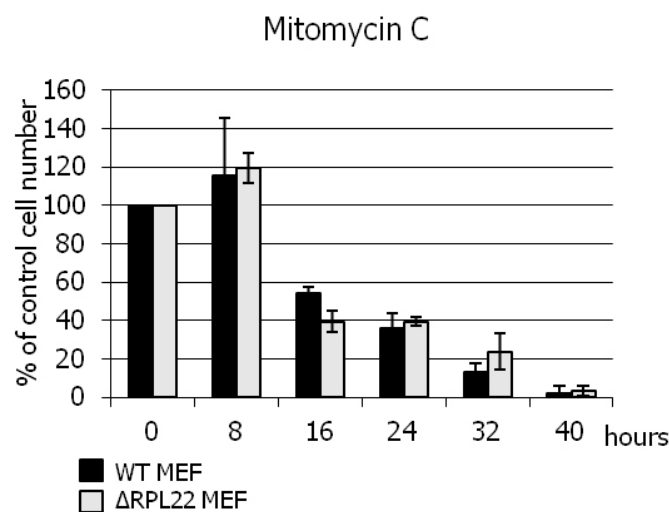


Figure 4.32: Effects of mitomycin C on the survival of wt and Δ RPL22 MEFs.

Viable cell counts were determined for wt (black) and Δ RPL22 MEFs (grey) cultivated with 10 μ g/ml mitomycin C for 0, 8, 24, 32 and 40 h.

4.4 Results: potential effects of EBER1 on host cell translation

The aim of this section is to develop an experimental system to investigate a possible role of RPL22 in host cell translation, which could potentially be altered by the interaction of the ribosomal protein with EBER1.

4.4.1 EBV infection does not alter RPL22 expression levels

RPL22 is clearly redistributed from the nucleoli to the nucleoplasm in HEK 293, AGS and HONE-1 cells infected with EBV (figure 4.7 and figure 4.11). This phenotype strongly implies sequestration of the protein from the nucleoli or its purposeful accumulation in the nucleoplasm as a potential mechanism of EBER1 action in host cells. To clarify this point, total RPL22 levels in EBV-infected or non-infected HEK 293 cells (figure 4.33 a and b) and wt or Δ EBER1 EBV-transformed LCLs (figure 4.33 c) were examined at the protein and RNA level. RPL22 levels were not reproducibly altered by EBER1.

4.4.2 EBV infection does not deplete RPL22 from ribosomes in HEK 293 cells

Ribosomal proteins are incorporated into ribosomal subunits in the nucleoli. Retention of RPL22 in the nucleoplasm by EBER1 might therefore lead to sequestration of the protein from ribosomes, as total RPL22 levels are constant in EBV-infected cells (figure 4.33). The association of RPL22 with ribosomes was therefore investigated by ribosomal fractionation on sucrose density gradients (section 2.5.8) and western blotting for RPL22 in HEK 293 cells stably transfected with wt, Δ EBER1 or EBER1-revertant BACs (figure 4.34). Good separation of ribosomal subunits and polyribosomal fractions was achieved in the fractionation and the observed peaks for 80S, 60S, 40S ribosomal subunits and polyribosomes are indicated in figure 4.34. RPL22 was found to be present in fractions containing polyribosomes, 80S and 60S ribosomal subunits, as expected for a component

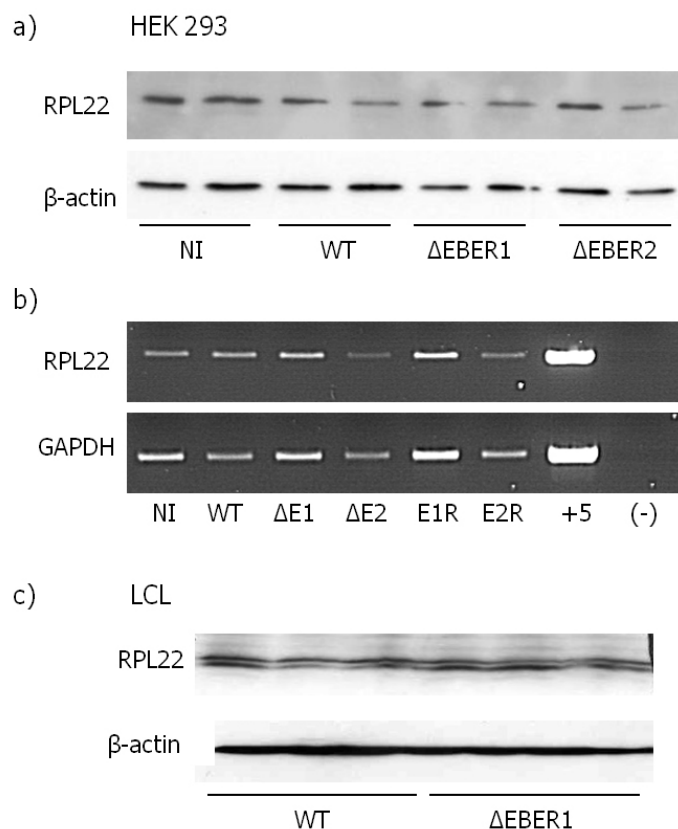


Figure 4.33: Expression levels of RPL22 are not altered by EBER1.

a) RPL22 protein levels in non-infected (NI), wt, Δ EBER1 and Δ EBER2 EBV-infected HEK 293 cells. b) RPL22 mRNA levels in non-infected (NI), wt, Δ EBER1 (Δ E1), Δ EBER2 (Δ E2), EBER1-revertant (E1R) and EBER2-revertant (E2R) EBV-infected HEK 293 cells. GAPDH is included as loading control. +5: 5 additional PCR cycles, (-): no DNA control. c) RPL22 protein levels in wt and Δ EBER1 transformed LCLs. The expression levels of RPL22 were not changed by EBER1.

of the large ribosomal subunit, while only minimal amounts of RPL22 were detected in fractions containing the 40S ribosomal subunit in all HEK 293 cells analysed, proving a reliable separation of the ribosomal fractions. No significant and reproducible difference in RPL22 distribution in dependence of EBER1 was observed (figure 4.34). The same number of cells was used in all fractionations and equal amounts of protein sample were employed for western blotting. Differences in the fractionation spectra could account for minor differences observed in the amounts of RPL22. Slightly more RPL22 is visible in Δ EBER1 polysomal fractions (figure 4.34, middle), as the ratio of polysomes to ribosomes was higher in this specific fractionation. Minor changes in the ratio of ribosomal subunits to polysomes is part of the variation commonly seen between fractionations independent

of EBER status. In general conclusion, RPL22 was found in ribosomes of wt, Δ EBER1 and EBER1-revertant HEK 293 cells (figure 4.34).

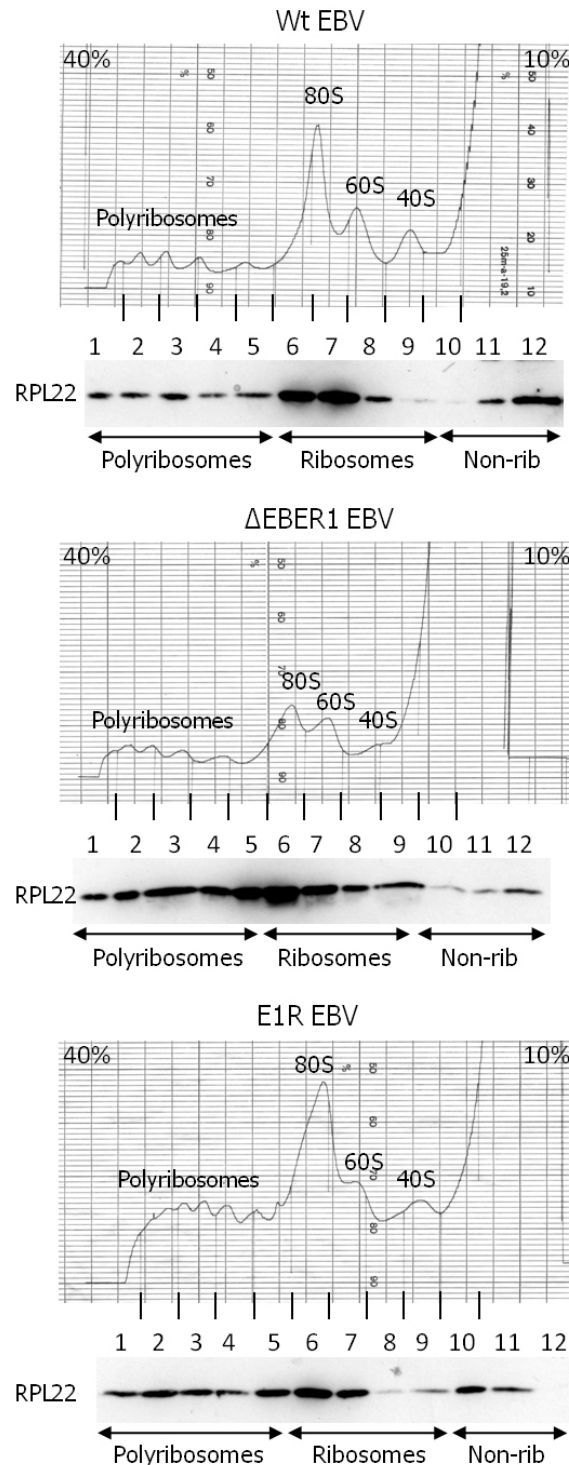


Figure 4.34: RPL22 is not depleted from ribosomes in EBV-positive HEK 293 cells. The fractionation spectra and distribution of RPL22 is shown for wt (top), Δ EBER1 (middle) and EBER1-revertant (bottom) EBV BAC-transfected HEK 293 cells. RPL22 was detected in ribosomes of all cells. Non-rib: non-ribosomal fractions.

4.4.3 EBV infection does not deplete RPL22 from ribosomes in LCLs or Akata cells

Earlier studies by Toczyski *et al* [224] observed normal incorporation of RPL22 into ribosomes in EBV-positive Raji cells; in contrast, more recent investigations showed a reduction of RPL22 from ribosomes upon EBV infection in Akata cells [228]. In an attempt to clarify whether depletion of RPL22 from ribosomes might be cell type dependent, association of RPL22 with ribosomes was investigated in LCLs transformed with wt or Δ EBER1 EBV. Examples of RPL22 western blots of two independent wt or Δ EBER1 LCLs are presented in figure 4.35 a. Fractionation of LCLs was technically challenging and only low levels of protein were detected, but RPL22 was equally found in ribosomes of both wt and Δ EBER1 LCLs.

Additionally, EBV-positive BL-derived Akata 2003 cells were investigated by sucrose gradient fractionation analysis. As seen for LCLs, RPL22 was present in ribosomal subunits and polysomal fractions of sucrose gradient fractionations (figure 4.35 b).

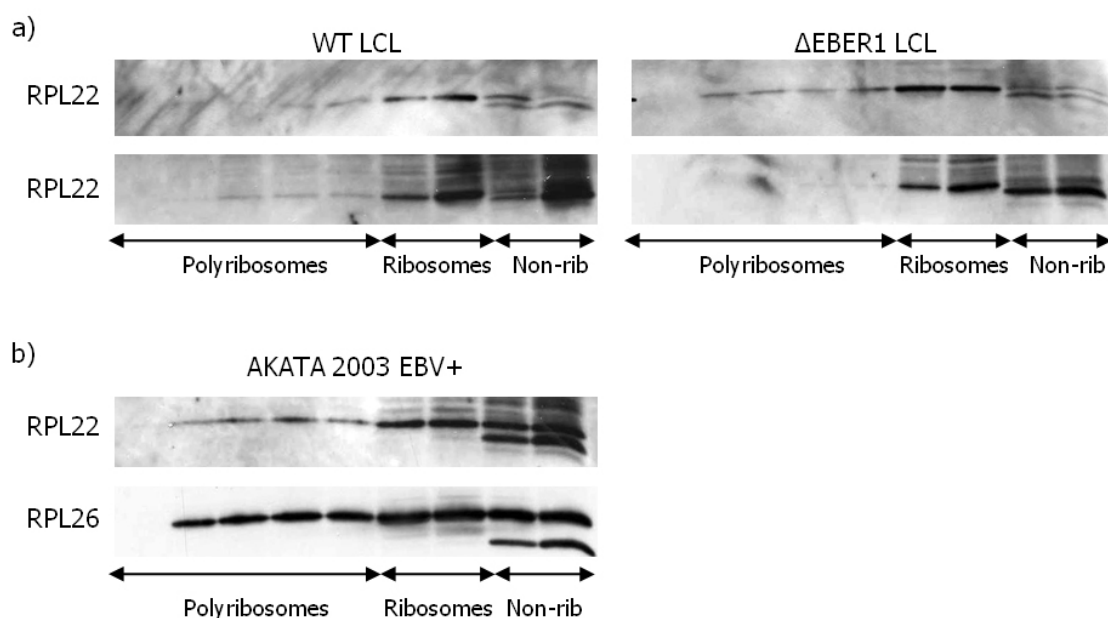


Figure 4.35: RPL22 is not depleted from ribosomes in EBV-positive LCLs and Akata cells.

The distribution of RPL22 is shown for a) two wt or Δ EBER1 LCLs and b) Akata 2003 cells. RPL22 was detected in ribosomes of all cells by western blotting. RPL26 was included as control. Fractions are indicated. Non-rib: non-ribosomal fractions.

4.4.4 Attempt to identify differences in host cell translation between wt and Δ EBER1 LCLs

4.4.4.1 Experimental design for the analysis of effects of EBER1 on host cell translation

Hypothetically, EBER1 might regulate translation via its strong interaction with RPL22. The association of RPL22 with the large ribosomal subunit without being essential for translation implies a regulatory role of the protein in translation [230]. In addition, EBER1 might influence host cell translation by an unknown mechanism directly or in cooperation with other cellular and viral factors independent of RPL22. Many ncRNAs are involved in post-transcriptional regulation [146, 147] and regulating host cell translation would offer a way for EBV to control its host cells in addition to the EBER-mediated changes in host cell gene expression at transcriptional level discovered earlier in this study (chapter 3). The two crucial parameters for the evaluation of translational efficiency of individual mRNAs are the proportion of mRNA that is ribosome-bound and the ribosome density on the mRNA [239].

To develop a system to investigate differences in the association of specific mRNAs with the translation machinery for our laboratory, the suitability of sucrose gradient fractionation followed by microarray analysis was assessed. To this end, a limited number of EBV immortalised LCLs were fractionated by sucrose gradient fractionation and polysome-associated RNA was extracted and profiled to determine changes in mRNA abundance (figure 4.36). The preliminary data obtained is presented in the sections below. As for host genes regulated on the transcriptional level, translationally regulated genes would be expected to influence cytokine production, cell immortalisation or establishment and maintenance of latency in general.

4.4.4.2 Microarray analysis and identification of EBER1-regulated target genes

Sucrose gradient fractionations were performed for HEK 293 cells and LCLs; the RNA extracted from polysomal fractions (figure 4.37 a) was analysed by Agilent microarray

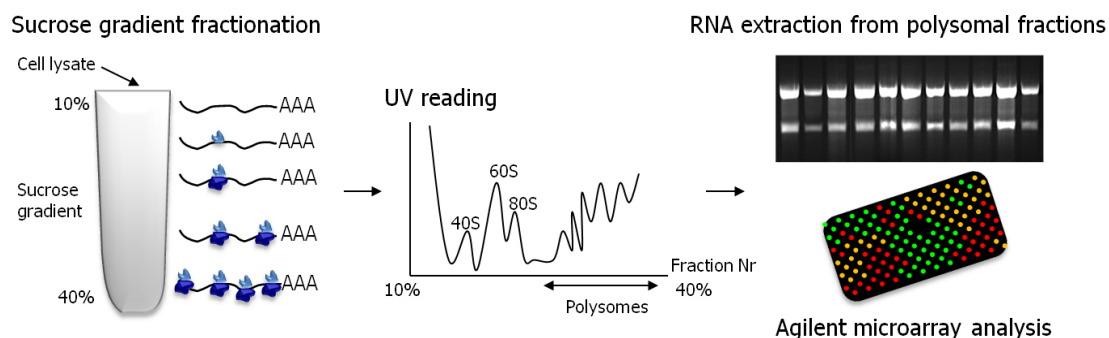


Figure 4.36: Experimental design for the analysis of effects of EBER1 on host cell translation. Cell lysates of wt, Δ EBER1 and EBER1-revertant EBV-infected cells were fractionated on sucrose density gradients. mRNA associated with polyribosomes was extracted and compared by microarray profiling.

analysis. Hybridisations were performed by Dr Oliver Dittrich-Breiholz (Medizinische Hochschule Hannover) as part of the INCA EU grant consortium. Bioinformatics analysis of the data was performed by myself, using Partek Genomics Suite software.

Unsupervised clustering of the data by covariance PCA showed clear differences in mRNA associated with polysomes in LCLs correlating with EBER1 expression (figure 4.37 b). mRNA samples of Δ EBER1 EBV-transformed LCLs separated away from wt and revertant LCLs, which demonstrates a systematic difference in the mRNA species that are bound by ribosomes in the presence of EBER1. The association of mRNAs with polysomes did not correlate with EBER1 in HEK 293 cells (not shown), which is consistent with a lack of EBER-mediated transcriptional changes in those cells (chapter 3). Association of 18 RNAs was found to change with the presence of EBER1 in LCLs if a fold-change threshold of 1.5, a p-value of <0.001 and a false discovery rate of 1% was applied to the p-value (table 4.2). EBER1 reduced the presence of the target RNA in polysomes in 56% of the targets identified, while the presence of 44% of the target RNAs was enhanced by EBER1.

The relative presence of EBER1 mRNA targets was further studied in dot plots (figure 4.38). Every dot represents the mRNA level on polysomes of a given gene in an independent RNA sample. Clear differences between wt and EBER1 deletion LCLs were observed. Moreover, the presence of mRNA of individual genes on polysomes was restored to wt levels in the revertant LCLs, confirming a specific effect of EBER1.

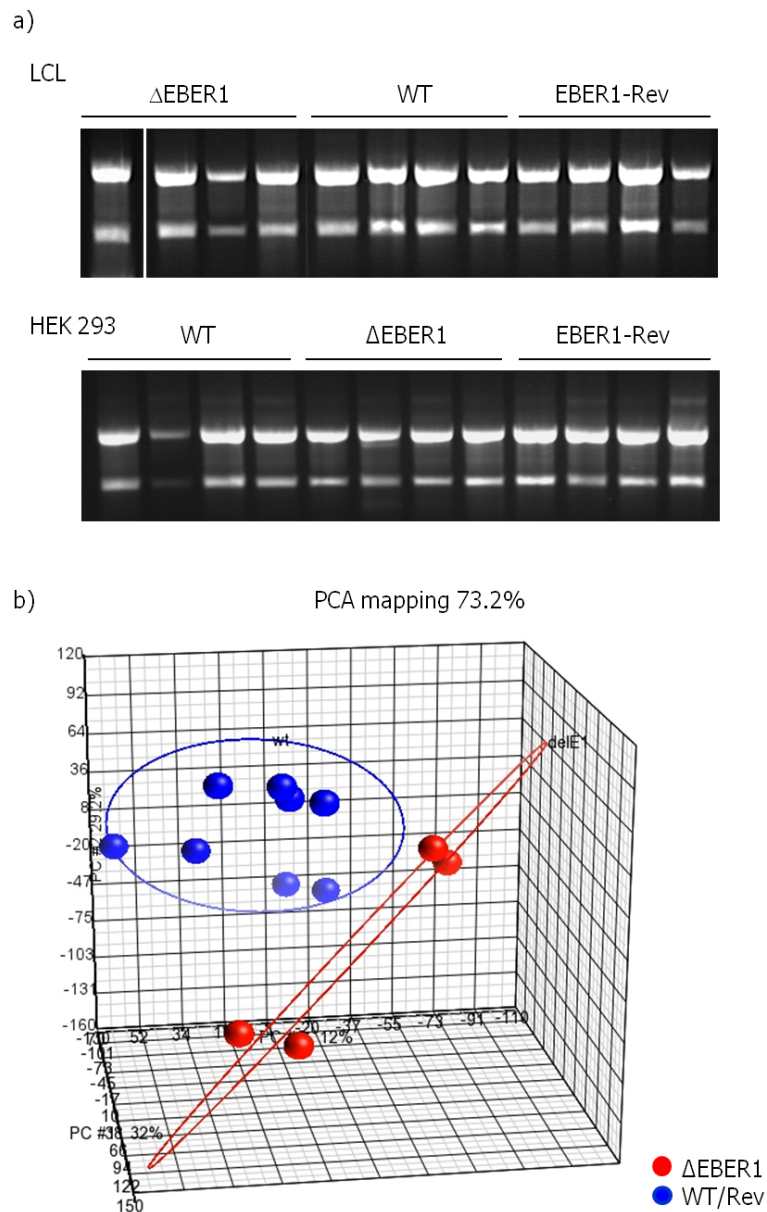


Figure 4.37: Microarray analysis of mRNA associated with polysomes.

a) Ethidium-bromide stained agarose gels showing the four independent RNA samples extracted from polysomal fractions of sucrose gradient fractionations of wt, Δ EBER1 (Δ E1) and EBER1-revertant (E1-Rev) EBV-infected LCLs (top) and HEK 293 cells (bottom). The RNA was profiled by Agilent microarray analysis. b) Principal component analysis. Unsupervised clustering of mRNA profiles disclosed differences in LCL ribosome loading correlating with EBER1 expression. Δ EBER1 samples (red) separated away from wt and revertant samples (blue).

Table 4.2: Translational targets of EBER1 with a fold-change threshold of 1.5, a p-value <0.001 and a false discovery rate of 1%.

Gene symbol	Fold-Change with EBER1	p-value	Description
ABCB1	-2.0	2.23E-05	ATP-binding cassette sub-family B member 1
ACTN1	4.2	2.36E-06	Actinin alpha 1
AK095831	11.6	7.92E-08	unknown
ARHGEF4	-20.0	5.26E-05	Rho guanine nucleotide exchange factor 4
BF803942	2.3	6.28E-06	unknown
DKFZp451G189	-2.6	1.36E-06	unknown
DTX1	-5.3	2.26E-05	Deltex homolog 1
FAM189A1	-4.4	2.36E-05	Family with sequence similarity 189, member A1
FBD8	2.1	2.50E-05	Cri-du-chat critical region mRNA
IL1R2	-4.0	8.29E-06	Interleukin 1 receptor type II
IL20Rb	1.6	3.31E-04	Interleukin 20 receptor beta
KLRC2	-22.2	2.23E-05	Killer cell lectin-like receptor subfamily C2
KLRC3	-15.3	2.66E-05	Killer cell lectin-like receptor subfamily C3
MAGI3	-3.8	3.13E-05	Membrane-associated guanylate kinase inverted 3
MED14	3.0	1.44E-04	Mediator complex subunit 14
p73	-1.7	3.73E-04	Tumour protein p73
PLOD2	9.1	1.76E-04	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
TWTF1	1.7	1.28E-05	Twinfilin, actin-binding protein, homolog 1

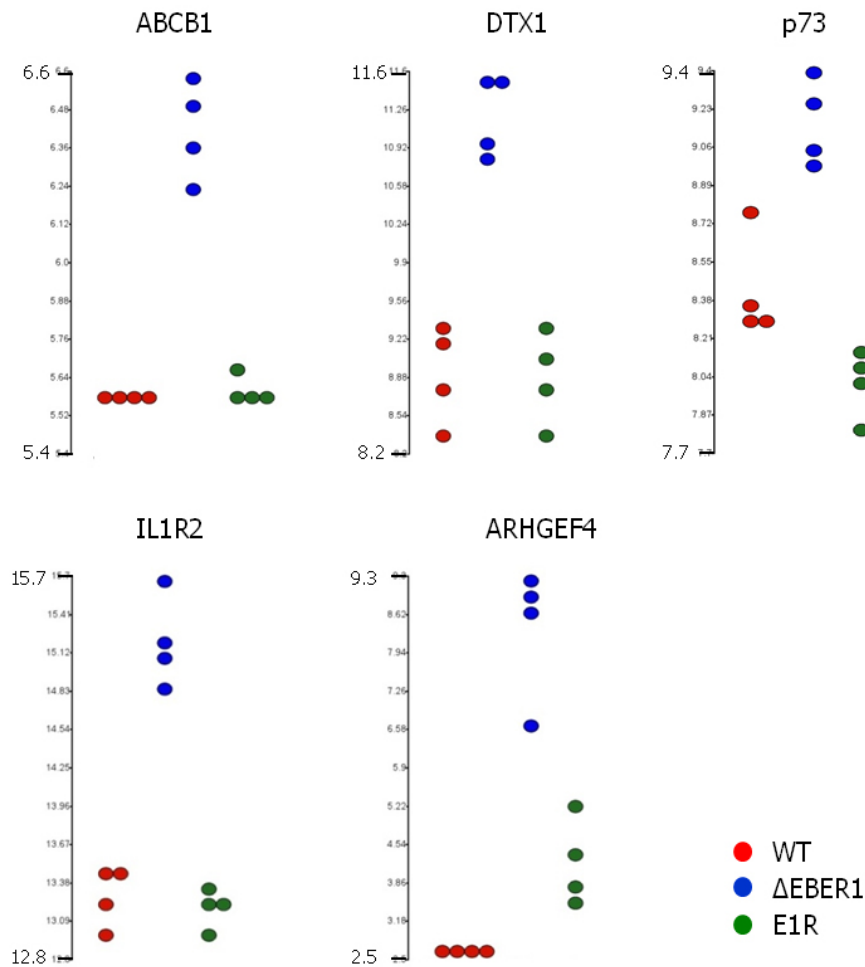


Figure 4.38: Dot plots of selected EBER1 mRNA targets.

Each dot represents the amount of RNA associated with polysomes in an individual LCL. Wt LCL RNA levels (red) differed significantly from Δ EBER1 levels (blue). RNA levels were restored to wt level in revertant LCLs (green). Given is the relative fluorescence intensity of the microarray analysis on a log 2 scale.

Western blots of the selection of EBER1 targets shown in figure 4.38 were subsequently performed to confirm changes of protein levels as a result of the observed differences in RNA association with polysomes. Interestingly, an increase in protein in the absence of EBER1 was validated for tumour protein p73 (figure 4.39 a), proposing that ribosomal fractionation and microarray analysis could be a feasible strategy to evaluate changes in the translation of specific proteins. In contrast, protein levels of Rho guanine nucleotide exchange factor 4 (ARHGEF4) were similar in wt, Δ EBER1 and EBER1-revertant LCLs (figure 4.39 b) and variation of Interleukin 1 receptor type II (IL1R2) protein levels

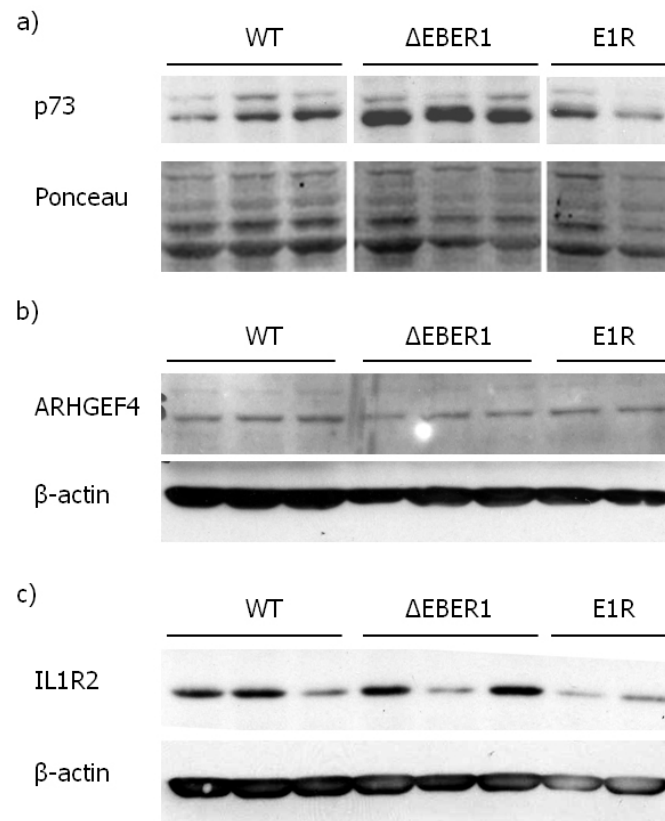


Figure 4.39: Validation of translational EBER1 targets.

Protein expression levels of a) p73, b) ARHGEF4 and c) IL1R2 were analysed in wt, Δ EBER1 (Δ E1) and EBER1-revertant (E1R) LCLs. Protein levels of p73 were decreased in the presence of EBER1, while expression of ARHGEF4 and IL1R2 was not modified by EBER1. β -actin or ponceau staining are included as loading controls.

between different LCLs did not correlate with EBER1 (figure 4.39 c). No protein band of the correct size was detected on western blots for ATP-binding cassette sub-family B member 1 (ABCB1, not shown), possibly as a consequence of very low levels of protein, as predicted by the low amounts of RNA measured on the microarray. No antibody was available for Deltex homolog 1 (DTX1).

4.4.4.3 Transformation of primary B cells and validation of the resulting LCLs

The preliminary experiment shown above used only 2-3 independent LCLs for comparison of Δ EBER1 and wt LCLs. Our lengthy experience with the main EBER microarray experiment had shown the need to analyse larger number of LCLs. To explore the function of EBER1 in the regulation of p73 more systematically, novel wt

and Δ EBER1 EBV-transformed LCLs were established by infection of 1×10^6 primary B cells with 5000, 2500 or 1250 GRU of EBV BAC recombinant virus as described in section 2.2.12 (figure 4.40 a). EBER expression in 28 created cell lines was analysed by northern blotting (figure 4.40 b) and correct expression of latent EBV proteins inspected by western blotting 7 weeks post-infection (figure 4.40 c). EBER expression patterns corresponded to the virus used for infection and no systematic differences in expression of the EBV proteins examined were observed, validating the LCLs for subsequent experiments.

4.4.4.4 Differences in p73 expression were not confirmed in LCLs established from different blood donors

Expression of p73 in 14 newly established wt and 14 newly established Δ EBER1 EBV-transformed LCLs was evaluated by western blotting using enhanced chemiluminescence (not shown) and using the Odyssey Infrared Imaging System to allow quantification of the protein bands obtained (figure 4.41). Disappointingly, a deregulation of p73 protein levels in Δ EBER1 LCLs was not confirmed in the novel LCLs. p73 was expressed at various levels independent of EBER1 status, while the amount of protein input, represented by β -actin, was nearly constant. This result shows the differences that exist between LCLs, possibly as a result of differences in the originating B cells, and emphasises the importance of large sample numbers for microarray analysis.

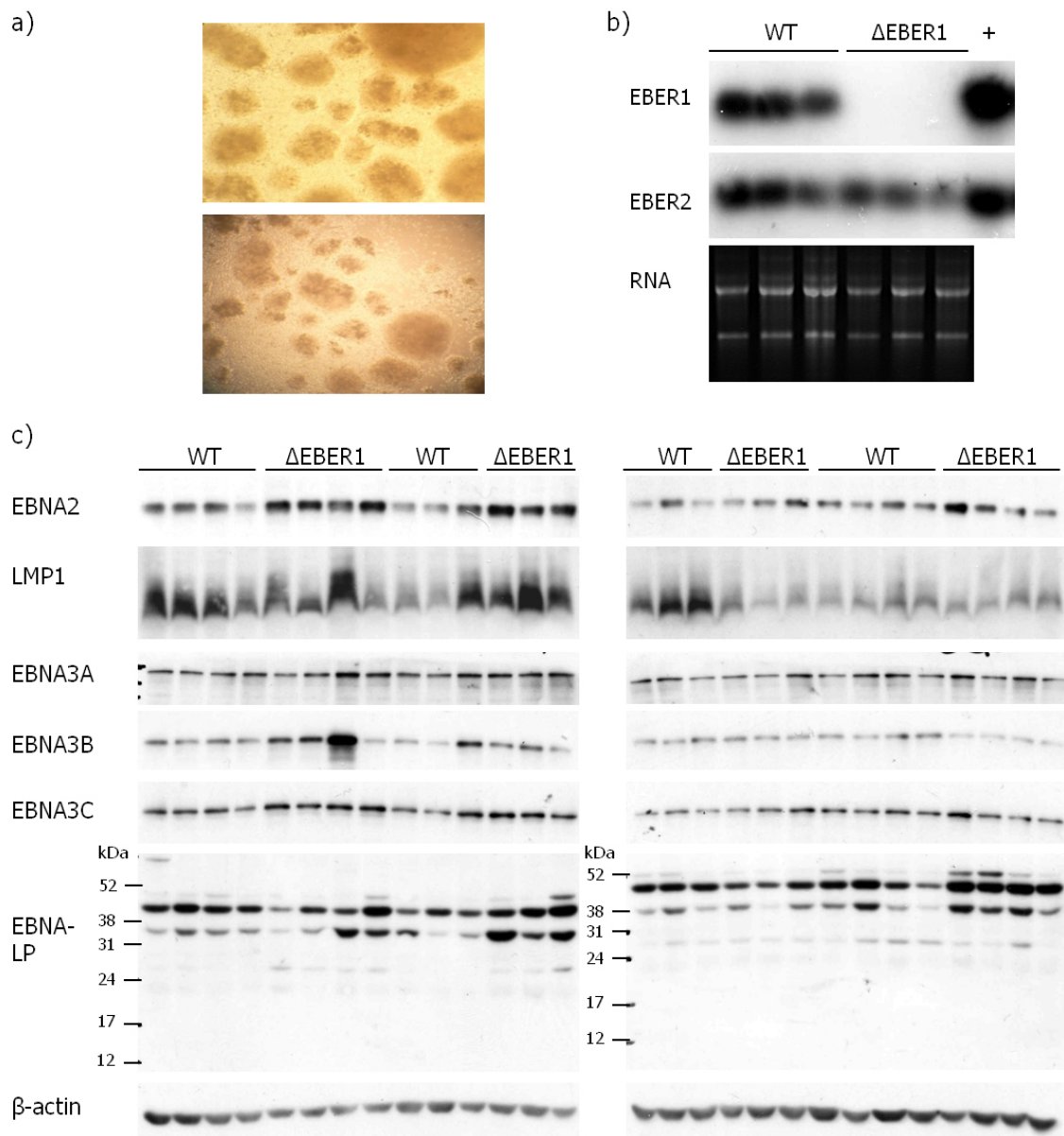


Figure 4.40: Validation of newly established LCLs.

a) Light microscopy images of LCL outgrowth (cluster formation) 9 days post-infection of primary B cells. b) Northern blot for EBER1 and EBER2 confirmed expected EBER expression patterns. +: Akata 2003 RNA. c) Expression of latent EBV proteins in wt and ΔEBER1 EBV-transformed LCLs.

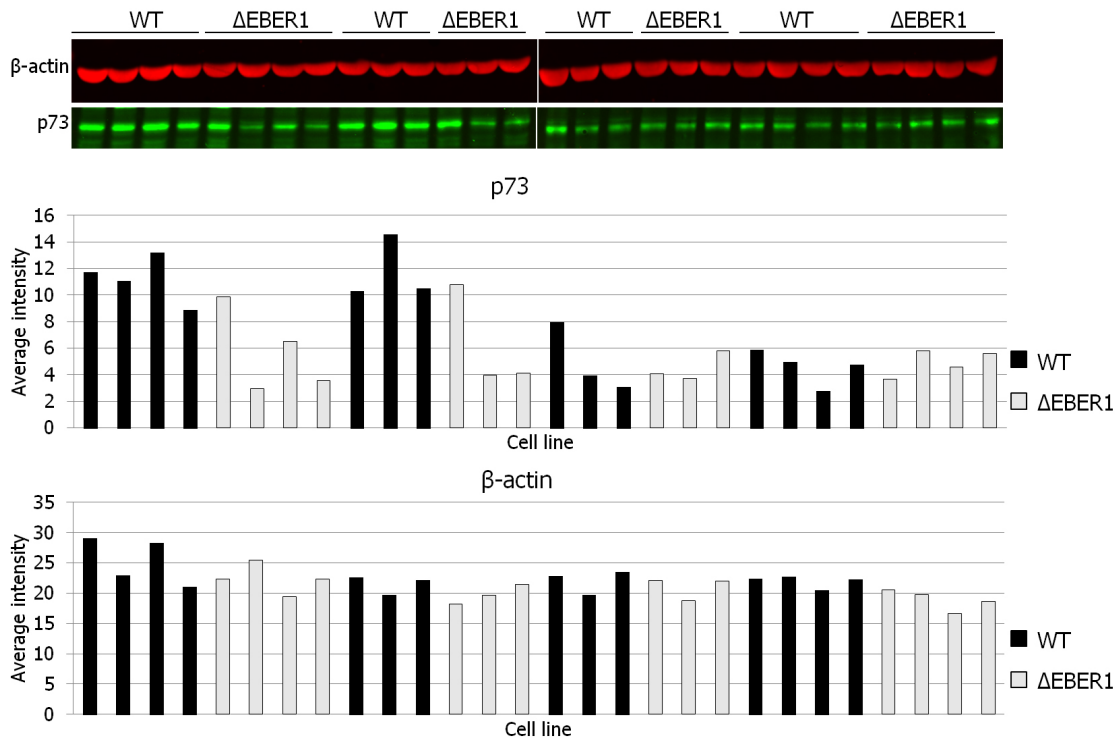


Figure 4.41: EBER1 does not alter p73 expression.

Expression of p73 in wt and Δ EBER1 LCLs as analysed using the Odyssey Infrared Imaging System (top) and the corresponding fluorescence intensity read outs (bottom). β -actin levels (red) were used as input control. p73 protein levels (green) varied between LCLs independent of EBER1 presence.

4.5 Discussion: interaction of EBER1 with RPL22

4.5.1 EBER1 delocalises RPL22 in HEK 293 cells

In the first part of this chapter, the specific interaction of RPL22 with EBER1, but not with EBER2, was demonstrated both in LCLs and HEK 293 cells (figure 4.3 and figure 4.4), which is in good agreement with previous publications [224–227]. Subsequently, the delocalisation of vector expressed FLAG-tagged RPL22 and endogenous RPL22 from the nucleoli and the cytoplasm to the nucleoplasm upon EBV infection was illustrated in HEK 293 cells (figure 4.5 and figure 4.7). The results are again consistent with experiments by Toczyski *et al* [224, 225], performed in Raji and BJAB cells.

Further, EBER1 was identified as the only viral component necessary for RPL22 delocalisation. Δ EBER1 EBV BAC was not able to induce the delocalisation of RPL22 in HEK 293 cells transfected with the mutant BAC (figure 4.8), whereas the expression

of EBER1 from Puro/oriP-pSUPER-EBER1 resulted in the complementation of EBER1 deletion in the EBV BAC and the delocalisation of RPL22 to the nucleoplasm. Moreover, expression of EBER1 from Puro/oriP-pSUPER-EBER1 led to a redistribution of RPL22 independent of any other EBV gene products in non-infected HEK 293 and BJAB cells (figure 4.9 and figure 4.10).

This original observation identified EBER1 as the only crucial viral factor for the localisation of RPL22 in EBV-infected cells. The clear results were additionally confirmed by Houmani *et al* ^[229] during the course of this study.

4.5.2 Delocalisation of RPL22 varies with the host cell type

To understand the function of EBER1 in EBV-infected cells, the delocalisation of RPL22 upon EBV infection was for the first time investigated in GC-derived AGS cells, NPC-derived HONE-1 cells and LCLs (figure 4.11 and figure 4.13). Additionally, the redistribution of RPL22 was quantified in these experiments for the first time.

In the epithelial cells, the distribution of RPL22 was clearly altered by EBV infection. Specifically, RPL22 was translocated to the nucleoplasm in 10% of AGS and 2% of HONE-1 cells, respectively (table 4.1). Impressively, RPL22 was delocalised to the nucleoplasm in 40% of HEK 293 cells stably transfected with wt EBV BAC (figure 4.7). In LCLs on the other hand, RPL22 was present in the cytoplasm of 99.5% of all cells in both wt and Δ EBER1 EBV-transformed LCLs (figure 4.13). Only 0.5% of Δ EBER1 LCLs displayed RPL22 in the nucleoli. Consistent with a delocalisation of RPL22 by EBER1, no nucleolar RPL22 was detected in wt EBV-transformed LCLs, while 0.5% of the cells displayed RPL22 in the nucleoplasm.

In contrast to other publications on the interaction of EBER1 with RPL22 ^[223–229], my study used recombinant EBV BACs in addition to EBER expression vectors to determine the importance of EBER1 for the translocation of RPL22. Moreover, the distribution of endogenous RPL22 was investigated in a set of natural EBV host cells for the first time. As a result of the detailed analysis performed and the quantification of RPL22

delocalisation, significant differences between individual cell lines were identified.

4.5.3 Explanations for the cell-specific localisation of RPL22

The striking variation in RPL22 distribution and redistribution by EBER1 between the different cell lines could be explained in several ways. Firstly, the differences might arise due to intrinsic cell-type specific differences, such as expression of cellular cofactors necessary for EBER1 action. Secondly, the differences might be cell cycle dependent. The cell cultures used in this study were not synchronised, but nucleoli were clearly observable in the phase contrast images of all cell lines. Alternatively, EBER expression levels might differ between individual cells and result in the distinct RPL22 behaviour. Most likely, the variation between the individual cell lines is the result of differences in RPL22 distribution in non-infected cells. It is assumed that EBER1 competes with 28S rRNA for binding to RPL22 in the nucleoplasm. RPL22 is subsequently co-transported into nucleoli with 28S rRNA or bound by EBER1 and retained in the nucleoplasm. Consequently, if RPL22 was not transported to the nucleoli via the nucleoplasm, but was engaged in cytoplasmic activities, RPL22 could not be retained in the nucleoplasm by EBER1 and no delocalisation could be observed. In line with this idea, RPL22 accumulated in nucleoli in only 0.5% of Δ EBER1 LCLs and was only delocalised to the nucleoplasm in 0.5% of wt EBV LCLs (figure 4.13).

The low level of RPL22 in the nucleoli of LCLs in comparison with the other cell lines tested might be the consequence of differences in ribosome formation, a process directed by the nucleoli. Ribosome biogenesis is a tightly regulated process, involving the coordination of hundreds of different proteins crucial for the production of mature rRNA, for the expression, modification and nuclear export of ribosomal proteins and for ribosome assembly ^[422]. Deregulation of many ribosomal proteins can result in p53-mediated cell cycle arrest and apoptosis ^[239, 411]. The increased accumulation of RPL22 in the nucleoli in cancer-derived cell lines in comparison with LCLs might therefore be the consequence of a deregulation of a cell-cycle control pathway involving

RPL22 and possibly p53. This signalling pathway could be altered as a result of the stronger transformation of the cancer-derived cells compared to the freshly EBV-transformed LCLs, which did not yet acquire further mutations characteristic of cancer cells.

B cell immortalisation can be divided into two steps ^[423]. Firstly, infection with EBV causes B cells to proliferate. These LCLs are not truly immortal, as their telomeres shorten, but the cells have an unusually long lifespan and LCL populations can double up to 90 times ^[423, 424]. Secondly, around 10%-15% of the transformed LCLs become immortalised by additional cellular changes. Truly immortalised LCLs generally show a downregulation of p16INK4A and pRb, mutations of p53 and an increase in telomerase activity ^[425–428]. The LCLs used in this study can therefore be regarded as pre-immortalised cells, in contrast to the cancer-derived AGS and HONE-1 cells and to HEK 293 cells, which were transformed by human adenovirus type 5 ^[321] and also have a confirmed high telomerase activity ^[429]. It would be interesting to investigate whether nucleolar accumulation of RPL22 is enhanced in long term culture of LCLs. Further, RPL22 localisation could be examined in primary cells to support the theory that RPL22 aggregation in the nucleoli is only tolerated in transformed cells.

Congruent with a transformed phenotype, NPC biopsies accumulate RPL22 in nucleoli of 35% of the tumour cells (figure 4.12). Surprisingly, however, this corresponds to a protein distribution pattern similar to EBV-negative cell lines. Unfortunately, the EBER status of the fixed NPC specimens used could not be confirmed for technical reasons, which makes the interpretation of this data difficult. Nevertheless, the vast majority of NPC is EBV-positive ^[97]. RPL22 expression and accumulation in the nucleoli could be too strong to be visibly altered by EBER1 in the tumours. An estimated 50% of RPL22 interacts with EBER1 ^[224, 229]. A higher expression of RPL22 or lower levels of EBER1 could diminish this percentage and only a small fraction of RPL22 would be delocalised by EBER1 which might fade in the high RPL22 background. Alternatively, RPL22 translocation might not be significant for the formation of NPC.

Overexpressed RPL22 was further found in the nucleoli in IB4 cells and was not

delocalised to the nucleoplasm (figure 4.19). Together with the limited translocation of RPL22 in LCLs (figure 4.13) and BJAB cells (figure 4.10), this might suggest that the delocalisation of RPL22 is not the primary function of EBER1 in a B cell background. However, more B cell-derived cell lines would need to be investigated to confirm this hypothesis. RPL22 levels could simply have been too high in FLAG-RPL22 expressing cells to be delocalised efficiently and EBER1 expression levels in the long-term cultured IB4 cells would have to be reanalysed.

4.5.4 RPL22 or EBER1 does not alter p53-dependent stress responses in MEFs and LCLs

The striking roles of ribosomal proteins in p53 signalling in combination with the distinctive distribution of RPL22 in LCLs and the difficulties of overexpressing RPL22 in these cells (section 4.3.1) highlighted RPL22 as a possible stress mediator. Further, Anderson *et al* reported an extraordinary role for RPL22 in T cell development [231]. Deletion of RPL22 in mice leads to an almost complete depletion of α/β -T cells in a p53-dependent manner, while γ/δ -T cells develop as normal. The p53-mediated cell death in absence of RPL22 is executed by PUMA and Bim [235].

Consequently, a possible role of RPL22 in cell stress responses to anti-cancer drugs was explored. No differences in cell survival were identified when analysing the response of wt MEFs and Δ RPL22 MEFs or wt EBV and Δ EBER1 EBV-transformed LCLs to cisplatin, etoposide and actinomycin D, as presented in figures 4.23 to 4.25 and figures 4.28 to 4.31. Consistently, the induction and expression of p53 and p73 was independent of RPL22 or EBER1 status of the cells.

The role of RPL22 in p53 signalling might be specifically confined to T cells due to cellular factors. The reported function of RPL22 in T cell development is highly specific and limited strictly to α/β -T cells, while no function for RPL22 was found in the development of γ/δ -T cells. It is feasible that RPL22 does not have a role in p53-mediated responses induced by chemotherapeutic agents in B cells and MEFs.

Alternatively, testing a much wider variety of cellular stresses and drugs might reveal the true role of RPL22, and possibly EBER1, in the regulation of p53 signalling in a B cell background. Alternatively, investigating the role of EBER1 interaction with RPL22 in a T cell background would be challenging, as infection of primary T cells with EBV *in vitro* is very inefficient and probably not physiological. The T cell leukemia lines normally have mutated p53, so would also be unsuitable for study.

4.5.5 RPL22 overexpression is not toxic for LCLs

Although I experienced difficulties expressing RPL22 in LCLs, a toxic effect of RPL22 overexpression and inhibition of translation or cell growth in transfected LCLs is unlikely (figure 4.15 and figure 4.16). Inducible FLAG-RPL22 was successfully expressed in more than 40% of stably transfected IB4 cells as determined by both western blot and confocal microscopy (figures 4.17 to 4.19). Even though the amount of FLAG-RPL22 in the cells was estimated to have exceeded the amount of endogenous RPL22 (figure 4.21), growth of the cells was not impaired (figure 4.20). Further, the newly constructed FLAG-RPL22-MEP4 vector was successfully expressed in LCLs in transient expression (figure 4.22).

The difficulty to overexpress RPL22 in LCLs strongly supported a function for RPL22 in p53 signalling. However, other factors could explain the poor RPL22 expression observed.

Expression of RPL22 could have been reduced significantly by promoter methylation of the plasmids ^[430], even though the same effect was seen using expression vectors that employ the CMV or the W promoter. Methylation of the vectors could be inhibited with 5-azacytidine after transfection ^[431] and protein expression analysed to test this hypothesis. Suppression of the transfected promoter in combination with the relatively low transfection efficiency of LCLs using the Neon system of max 50% (determined by Goran Gregorovic and Claudio Elgueta Karstegl) could explain the lack of detectable FLAG-RPL22 levels in some of the experiments.

4.5.6 RPL22 is expressed at normal levels and incorporated into ribosomes in EBV-infected cells

EBV infection does not alter total RPL22 expression levels in EBV-infected versus non-infected HEK 293 cells and wt versus Δ EBER1 EBV-transformed LCLs (figure 4.33).

Interestingly, a double band was observed for RPL22 in LCLs in contrast to a single band in HEK 293 cells (figure 4.33). Sucrose gradient fractionations further showed that only the slightly larger detected band was assembled into ribosomes (figure 4.35). The upper detected band might be a post-translationally modified version of RPL22, such as a phosphorylated form, and this modification might be required for successful incorporation of RPL22 into the ribosomes in LCLs.

EBV infection does not deplete RPL22 from ribosomes in HEK 293, LCLs or Akata cells (figure 4.34 and figure 4.35). Prior studies by Toczyski *et al* ^[224] confirm the normal incorporation of RPL22 into ribosomes in EBV-positive Raji cells. In contrast, Houmani *et al* ^[229] found a reduction of RPL22 in the 60S ribosomal subunit in Akata cells.

Our results suggest that RPL22 is not depleted from ribosomes and that EBER1 might therefore seek to sequester RPL22 from other functions in the nucleoli or redirect the protein to the nucleoplasm to alter its role in cellular processes to favour EBV infection. RPL22 delocalisation is mostly visible in highly transformed cell lines, but might take place to a smaller extent in more normal cells.

4.5.7 Possible functions of the interaction of EBER1 with RPL22

The majority of EBER1 is predicted to be bound by RPL22 and up to 50% of the protein is bound by EBER1 in infected cells ^[224]. This suggests a significant role of the interaction during persistent infection even if the delocalisation of RPL22 was not the primary goal of the interaction. Multiple possible functions of the association of EBER1 with RPL22 can be speculated upon, deduced from observations on cellular functions of RPL22.

4.5.7.1 Interaction of EBER1 with RPL22 could alter host cell translation

RPL22 is a component of the large ribosomal subunit, but is not essential for translation [230], which could imply a regulatory role for the protein. Similarly, other ribosomal proteins can alter translation of specific genes. Phosphorylated RPL13a is released from the ribosome in response to IFN- γ to repress translation of selected genes by binding to their 3'UTR [432, 433] and RPL26 enhances translation of p53 by binding to its 5'UTR [434].

Modifying translation of existing mRNA is a fast and efficient way to alter important cellular processes. For example, oncogenic Ras and AKT signalling pathways initially contribute to glioblastoma formation by differential recruitment of existing mRNAs to the ribosomes, while only small transcriptional effects were observed using microarray analysis of total RNA [435]. Differential regulation of translational efficiency might overshadow direct effects on transcription of selected mRNAs in oncogenesis in general [409, 436].

Therefore, a possible system to determine if EBER1 might regulate translation via its strong interaction with RPL22 or by an unknown mechanism in cooperation with other cellular and viral factors independent of RPL22 was developed in this study (section 4.4.4). To this end, mRNA associated with polysomes in a small number of Δ EBER1, wt and EBER1-revertant LCLs was isolated by sucrose gradient fractionation and identified by microarray analysis (figure 4.36). 18 RNAs were differentially associated with polyribosomes dependent on EBER1 in these LCLs (table 4.2, figure 4.38).

The genes identified to be regulated with EBER1 in microarrays using cytoplasmic RNA (chapter 3) were not found to be differentially associated with polysomes. This was surprising, as a deregulation of mRNA levels is expected to result in different levels of RNA employed in translation. Additionally, the preliminary results obtained from the arrays using polysomal RNA were only confirmed by western blotting for one out of four examples tested, which later on was not validated in novel wt and Δ EBER1 EBV-transformed LCLs (figure 4.40 and figure 4.41).

It can therefore be concluded that ribosomal fractionation and microarray analysis could be a feasible strategy to assess changes in translational efficiency in the future. However, a critical approach needs to be applied when this experiment is repeated by using LCLs from several different blood donors to control for differences in the B cell background. Moreover, larger numbers of cell lines should be tested due to the variations that might arise during the multiple experimental steps required for the preparation of the mRNA samples for the microarrays.

4.5.7.2 Interaction of EBER1 with RPL22 could alter host cell transcription

The interaction of EBER1 with RPL22 could potentially be an essential part of the mechanism by which EBER1 regulates host cell gene expression, identified in chapter 3. Alteration of the interaction of RPL22 with linker histone H1 might influence host cell transcription and RPL22 regulates more than 1000 genes in *Drosophila* [244].

Other ribosomal proteins control transcription directly or via transcriptional regulators [437]. For example, RPS3 is associated with NF- κ B p65 homodimer and p65-p50 heterodimer DNA-binding complexes and enhances DNA binding of NF- κ B to gene regulatory sites of specific target genes [438]. Another example is RPL11, which inhibits c-MYC-induced transcription and cell proliferation by impairing the recruitment of the c-MYC co-activator transformation/transcription domain-associated protein (TRRAP) and by reducing histone H4 acetylation at c-MYC target gene promoters [439]. Recent publications suggest the association with transcription sites of not only individual ribosomal proteins but whole ribosomal subunits [440].

4.5.7.3 Interaction of EBER1 with RPL22 could alter telomerase activity

RPL22 is further known to interact with telomerase and co-precipitates human telomerase RNA (hTR) and telomerase reverse transcriptase (hTERT) [242]. Enhanced telomerase activity is strongly linked to cell immortalisation and tumour formation. Interestingly, telomerase activity in EBV-positive NPC cell lines is regulated by LMP1, which strengthens the binding of NF- κ B to hTERT and translocates the complex to the

nucleus^[441]. Consistently, LMP1 enhances transcription of hTERT in B lymphoma cell lines^[442]. Regulation of telomerase is a common target of human tumour viruses as telomere shortening needs to be overcome to allow long term survival of infected cells^[443]. Cultured EBV-positive BL-cells have longer telomeres than EBV-negative cells^[444].

It is reasonable that RPL22 regulates telomerase activity by binding to hTR or hTERT. Consequently, EBER1 might regulate telomerase activity and thereby support cell immortalisation. Early LCLs are pre-immortalised transformed cells and differences in telomere length or telomerase activity correlating with EBER1 presence might only become revealed during long-term culture of wt and Δ EBER1 EBV-infected LCLs. Telomerase activity could be investigated using the telomere repeat amplification protocol^[445].

hTERT is sequestered in nucleoli by binding to nucleolin until telomeres are replicated in late S phase in healthy cells^[446]. In contrast, telomerase is not associated with nucleoli at any stage of the cell cycle in transformed cells or cells experiencing DNA-damage. Transfection of the simian virus 40 genome into a primary cell line translocates telomerase from the nucleoli to the nucleoplasm^[447]. EBER1 could potentially translocate hTERT to the nucleoplasm while delocalising RPL22 from the nucleoli. The delocalisation of hTERT by EBER1 has not previously been investigated but again offers a putative role for EBER1 during cell immortalisation.

4.5.7.4 Interaction of EBER1 with RPL22 could alter other cell processes

Alternatively, the association of RPL22 with AKT1 protein kinase, which signals downstream of phosphatidylinositol 3-kinase, implies a possible way for EBV to influence the effects of various growth factors including platelet-derived growth factor, epidermal growth factor, insulin and IGF1^[245].

On the other hand, RPL22 might primarily act as part of the antiviral immune response during EBV-infection and limit EBER1 function by inhibiting its interaction with other proteins such as PKR^[250].

RPL22 interacts with several other herpesvirus components during viral infection but the exact function of these interactions has not been clarified as yet. The interaction of RPL22 with EHV IE in the cytoplasm at late times post-infection might influence viral gene expression, as IE retained in the cytoplasm is inhibited from antagonising transactivator EICP0 [249]. RPL22 localises to HSV-1 nuclear structures, which contain the direct RPL22 interaction partners ICP4 and ICP22 plus RNA polymerase II and newly synthesised viral DNA [246, 247]. Binding of RPL22 disrupts the interaction of repressor ICP4 with its cognate promoter binding sites and might account for the derepression of late gene expression. Additionally, RPL22 might be involved in the shuttling of nascent RNA from the transcription complex to the cytoplasm or might enable an interaction of ICP4 with ICP22.

4.5.8 Interaction of EBER1 with RPL22: future directions

The strong interaction of EBER1 with RPL22 is clearly important for EBV infection and the manipulation of RPL22 function by EBER1 might enable EBV to influence a variety of host cell functions important for persistent infection and cell immortalisation. A further understanding of the roles of RPL22 in non-infected cells will be required to elucidate the exact function of EBER1 interaction with the ribosomal protein. Future work would preferably focus on the functions of RPL22 described above.

No effect of EBER1 or RPL22 on p53-mediated stress responses in LCLs or MEFs was identified so far (section 4.3). Nevertheless, testing a wider variety of cell types and stresses might reveal stress modulating functions of RPL22. Cells could be challenged with DNA damage, inhibition of transcription, depletion of nucleotide pools, oncogene expression, viral infection, heat shock, hypoxia or further toxins.

Additionally, a system to analyse a potential modification of the translation of selected host cell genes in response to RPL22 expression or EBER1 presence was already established in this study (section 4.4.4) and could be applied, using a large sample number, to evaluate a regulatory role of RPL22 in translation.

54 host cell genes are regulated with EBER1 expression in LCLs at the transcriptional level (chapter 3). siRNA knockdown of RPL22 in LCLs transformed with wt EBV or Δ EBER1 EBV BACs followed by RT-PCR for EBER1-regulated genes could show whether RPL22 expression is necessary for EBER1-mediated gene regulation.

Moreover, investigating telomerase activity in wt and Δ RPL22 MEFs by the telomere repeat amplification protocol might disclose a completely new extraribosomal function of RPL22, which could potentially be altered by EBER1.

5 Conclusions

5.1 EBER-regulated host cell genes

In this thesis, reliable and distinct sets of host cell genes regulated specifically with EBER1 or EBER2 expression in EBV-infected LCLs were identified for the first time ^[66] (chapter 3). Using microarray analysis, EBER1 and EBER2 were found to alter the expression of 54 and 115 LCL genes, respectively. This is the first report of broad range effects of the EBERs on host cell gene expression in LCLs and multiple novel genes were identified to be regulated by EBER1 or EBER2. Despite intensive investigations in the thirty years since their discovery ^[192], many questions about the functions of the EBERs have remained unanswered. Thus, analysing the complete effects of the two RNAs on host cell gene expression contributes significantly to the understanding of EBER action during infection.

Novel EBV BACs containing a deletion of EBER1 or EBER2 and revertants of the EBER mutants were constructed and used to transform primary B cells into LCLs for the microarray analysis. These LCLs allow the precise investigation of specific EBER functions in an otherwise wt EBV background in natural EBV host cells and will be a useful tool to further elucidate the mechanism of action of the EBERs. The system enables the first investigation of EBER1 or EBER2-mediated modifications of miRNA levels in infected B cells, which is currently being analysed in collaboration with Prof Bryan R Cullen (section 3.2.11). Additionally, the deletion and revertant viruses will be used by our laboratory to analyse the roles of EBERs in *in vivo* infection in a humanised mouse model in collaboration with Prof Christian Munz.

This study further strongly indicates individual roles for the two EBERs in B cell infection and underlines the crucial differences between the functions of EBER1 and EBER2. EBER2 regulates more than twice the number of genes regulated by EBER1 and only 9 host cell genes are commonly regulated by both EBER1 and EBER2 in LCLs. It is likely that EBERs associate with multiple, so far unknown and distinct cell proteins to mediate their strong and differentiated changes in gene expression. An MBP-MS2 affinity purification system was established to allow the identification of all EBER interaction partners in the laboratory in the future (section 3.2.10).

Further, the study detected EBER1 and EBER2 in the supernatant of LCLs, suggesting a potential extracellular role for both EBERs. This hypothesis is now being investigated in collaboration with Dr Michiel Pegtel.

In summary, the following goals were achieved:

- EBER-revertant bacterial artificial chromosomes were established to complement EBER deletion in the recombinant virus.
- 54 genes were identified to be regulated by EBER1 in LCLs.
- Genes regulated by EBER1 were identified to be often involved in the antiviral response and metabolic processes.
- 115 genes were identified to be regulated by EBER2 in LCLs.
- Genes regulated by EBER2 were identified to be often involved in apoptosis and membrane signalling.
- Distinct effects of EBER1 and EBER2 in the regulation of host cell gene expression were determined.
- An MBP-MS2 affinity purification system was established to identify novel EBER1 or EBER2 interaction partners.
- EBER1 and EBER2 were detected in the supernatant of LCLs.

5.2 Interaction of EBER1 with RPL22

In this part of the study, the specific interaction of EBER1 with RPL22 was confirmed but EBER2 does not bind to the protein. Moreover, EBER1 was established as the mediating factor of RPL22 delocalisation and as the only viral component necessary for the translocation of RPL22 from the nucleoli to the nucleoplasm in EBV-infected HEK 293 and BJAB cells (sections 4.2.4 and 4.2.6).

Additionally, RPL22 localisation was investigated and quantified in multiple, diverse cell lines for the first time (table 4.1). The delocalisation of RPL22 to the nucleoplasm upon EBV infection was confirmed in NPC-derived and GC-derived cell lines. Interestingly, only a minimal fraction of freshly EBV-transformed LCLs displayed delocalisation of RPL22 to the nucleoplasm. The difference in RPL22 delocalisation between the different cell lines is most likely a consequence of the RPL22 localisation in non-infected cells. The strongly transformed cancer-derived cells accumulate RPL22 in the nucleoli, whereas accumulation of RPL22 in the nucleoli of LCLs is extremely rare. This finding could indicate a possible relationship of RPL22 to the transformation process.

Based on the role of RPL22 in p53 signalling ^[231, 235] and the above described accumulation of RPL22 in the nucleoli in cancer cells which frequently acquire p53 mutations, effects of RPL22 and EBER1 on p53-induced stress responses were investigated. EBERs themselves are implicated in prevention of apoptosis ^[100, 101, 261, 272–274]. In this thesis, no differences in cell survival or expression of p53 and p73 in response to the anti-cancer drugs cisplatin, etoposide, actinomycin D and mitomycin C between wt and Δ RPL22 MEFs or wt and Δ EBER1 EBV LCLs were observed (section 4.3). In line with the tight cell subtype specificity of RPL22 function in the development of α/β -T cells, but not γ/δ -T cells ^[231], EBER1 and RPL22 might influence host cell stress responses in a strict cell type specific manner. On the other hand, testing of additional stress-inducing agents might reveal a function of EBER1 and RPL22 in B cells.

Alternatively, the interaction of EBER1 with the ribosomal protein could influence host

cell translation. Analysis of host cell translation in EBER1 deletion EBV-transformed LCLs could discover a novel function of EBER1 and help to establish whether RPL22 has a regulatory role in translation in uninfected cells in general. To enable prospective work in this direction, a ribosomal fractionation system coupled with microarray analysis was developed to analyse the association of specific mRNAs with polyribosomes in our laboratory in the future.

In summary, the following insights into the interaction of EBER1 with RPL22 were gained:

- EBER1 delocalises RPL22 from the nucleoli to the nucleoplasm in cancer-derived cell lines.
- LCLs show a predominantly cytoplasmic expression of RPL22, which is not significantly changed by EBER1.
- RPL22 is not depleted from ribosomes in EBV-infected cells.
- EBER1 does not alter induction of p53 or cell survival in response to chemotherapeutic agents in LCLs.
- RPL22 does not alter induction of p53 or cell survival in response to chemotherapeutic agents in MEFs.
- Ribosomal fractionation coupled with microarray analysis is a suitable system to study potential effects of EBER1 and RPL22 on host cell translation.

6 Appendix

Table 6.1: LCL genes regulated with EBER1. Δ E1: Δ EBER1, E1R: EBER1-revertant.

Gene Symbol	Description	Fold-Change wt/ Δ E1	p-value	Fold-Change E1R/ Δ E1	p-value
CDC42BPA	CDC42 binding protein kinase alpha (DMPK-like)	29.4	4.1E-10	27.0	7.7E-11
CSRP2	Cysteine and glycine-rich protein 2	12.6	2.9E-13	11.1	8.5E-14
SEPP1	Selenoprotein P, plasma, 1	11.7	1.2E-10	16.4	9.1E-13
CD96	CD96, T-cell surface protein tactile	11.0	1.2E-12	4.7	6.6E-10
ANK3	Ankyrin 3, node of Ranvier (ankyrin G)	9.7	7.5E-15	11.6	1.3E-16
DDAH1	Dimethylarginine dimethylaminohydrolase 1	8.7	1.9E-15	12.3	6.6E-18
CHL1	Cell adhesion molecule with homology to L1CAM	8.4	6.2E-11	8.2	8.3E-12
RHOU	Ras homolog gene family, member U	7.9	4.9E-12	11.9	1.0E-14
ADCY6	Adenylate cyclase 6	6.7	2.1E-10	8.8	1.4E-12
GAS2	Growth arrest-specific 2	5.9	2.3E-07	7.7	3.2E-09
ASS	Argininosuccinate synthetase	5.7	5.3E-11	3.5	3.1E-09
HLXB9	Homeobox HB9	5.2	1.0E-04	6.5	4.0E-06
GLIS3	GLIS family zinc finger 3	5.1	6.8E-10	8.5	3.6E-13
HOXB6	Homeobox B6	5.1	2.0E-06	8.7	2.6E-09
RNF43	Ring finger protein 43	4.8	4.8E-06	6.9	3.3E-08

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Gene Symbol	Description	Fold- Change wt/ΔE1	p-value	Fold- Change E1R/ΔE1	p-value
GPR171	G protein-coupled receptor 171	4.6	2.2E-11	3.4	1.6E-10
CRIM1	Cysteine rich transmembrane BMP regulator 1	4.5	1.4E-10	3.9	1.1E-10
KCNV1	Potassium channel, subfamily V, member 1	4.3	1.1E-10	5.8	2.6E-13
NCKAP5	Nck-associated protein 5	4.2	1.9E-12	2.9	9.7E-11
PTGS1	Prostaglandin-endoperoxide synthase 1	4.0	5.9E-07	2.9	6.6E-06
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	3.9	7.1E-11	3.1	2.8E-10
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	3.9	2.2E-06	5.9	4.4E-09
IFNA6	Interferon, alpha 6	3.7	1.4E-09	2.7	4.2E-08
AMOTL1	Angiomotin like 1	3.6	6.7E-11	3.2	5.8E-11
KL	Klotho	3.6	8.1E-10	3.2	6.8E-10
ZDHHC14	Zinc finger, DHHC-type containing 14	3.5	1.2E-06	2.9	1.9E-06
TCF2	Transcription factor 2, variant hepatic nuclear factor	3.5	1.3E-09	4.6	2.7E-12
IFNA5	Interferon, alpha 5	3.3	5.2E-07	2.5	5.6E-06
TGM2	Transglutaminase 2	3.2	1.4E-07	2.6	5.2E-07
IFNA4	Interferon, alpha 4	3.2	4.5E-07	2.4	6.2E-06
FUT4	Fucosyltransferase 4	3.1	2.0E-09	2.7	2.7E-09
SLC7A11	Solute carrier family 7, member 11	3.0	6.5E-08	2.6	9.0E-08
EFNA1	Ephrin-A1	2.6	4.0E-05	3.2	5.6E-07
ASPH	Aspartate beta-hydroxylase	2.6	4.3E-05	2.4	3.8E-05
PHGDH	Phosphoglycerate dehydrogenase	2.6	1.7E-06	2.1	1.6E-05
TGFB1I1	Transforming growth factor beta 1 induced transcript 1	2.6	3.8E-08	2.3	9.5E-08

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Gene Symbol	Description	Fold- Change wt/ΔE1	p-value	Fold- Change E1R/ΔE1	p-value
SASH1	SAM and SH3 domain containing 1	2.6	1.4E-05	2.2	2.1E-05
PLD1	Phospholipase D1, phosphatidylcholine-specific	2.5	7.5E-07	3.7	2.4E-10
IQCD	IQ motif containing D	2.4	5.3E-08	2.2	4.2E-08
EDN1	Endothelin 1	2.3	1.6E-04	2.6	8.3E-06
WWTR1	WW domain containing transcription regulator 1	2.2	2.0E-10	2.4	2.6E-12
CHST6	Carbohydrate sulfotransferase 6	-2.1	6.7E-05	-2.3	3.4E-06
PIK3R5	Phosphoinositide-3-kinase, regulatory subunit 5, p101	-2.7	3.3E-07	-2.0	1.2E-05
SETBP1	SET binding protein 1	-3.5	3.4E-06	-3.5	6.3E-07
PRUNE2	Protein prune homolog 2	-3.7	3.3E-06	-3.7	5.5E-07
LRRC32	Leucine rich repeat containing 32	-3.8	3.8E-05	-2.6	4.4E-04
NRIP1	Nuclear receptor interacting protein 1	-4.3	1.8E-14	-2.7	3.6E-12
HHEX	Homeobox, hematopoietically expressed	-4.8	1.6E-08	-3.2	4.6E-07
LEPREL1	Leprecan-like 1	-5.1	1.8E-09	-5.5	9.0E-11
TCEAL2	Transcription elongation factor A (SII)-like 2	-5.1	3.0E-09	-2.7	2.1E-06
ADARB1	Adenosine deaminase, RNA-specific, B1	-5.4	7.0E-08	-6.3	2.3E-09
CCDC74B	Coiled-coil domain containing 74B	-7.8	2.9E-08	-6.0	4.0E-08
IL12RB2	Interleukin 12 receptor, beta 2	-17.0	4.8E-11	-6.8	9.1E-09
FAM189A1	Transmembrane protein FAM189A1	-19.9	8.9E-11	-13.0	2.0E-10

Table 6.2: LCL genes regulated with EBER2. Δ E2: Δ EBER2, E2R: EBER2-revertant.

Gene Symbol	Description	Fold- Change wt/ΔE2	p-value	Fold- Change E2R/ΔE2	p-value
TBX15	T-box 15	65.4	8.1E-09	17.2	7.9E-07
MMP7	Matrix metalloproteinase 7	52.4	1.1E-06	29.3	2.2E-06
GPR125	G protein-coupled receptor 125	50.2	4.4E-14	14.3	1.3E-11
SASH1	SAM and SH3 domain containing 1	43.7	2.5E-10	13.0	3.7E-08
DACT1	Dapper, antagonist of beta-catenin, homolog 1 (Xenopus laevis)	31.5	2.2E-10	8.8	1.3E-07
LINCR	E3 ubiquitin-protein ligase neuralized-like protein 3	24.1	5.5E-09	10.8	1.2E-07
CNKSR3	CNKSR family member 3	18.9	2.1E-09	12.0	6.0E-09
CD9	CD9 antigen	15.1	8.1E-11	8.5	9.0E-10
PAG1	Phosphoprotein associated with glycosphingolipid microdomains 1	13.3	4.7E-07	7.8	2.8E-06
LRRK2	Leucine-rich repeat kinase 2	13.2	1.2E-11	6.0	1.7E-09
FXRD2	FXRD domain containing ion transport regulator 2	13.1	7.3E-07	5.4	5.2E-05
LMO3	LIM domain only 3	12.0	6.5E-11	5.4	1.3E-08
ZNF462	Zinc finger protein 462	10.0	2.3E-07	5.2	7.1E-06
GOLPH2	Golgi phosphoprotein 2	9.7	1.6E-08	5.1	6.2E-07
KCNK1	Potassium channel, subfamily K member 1	9.4	3.9E-11	4.6	6.9E-09
ACTN1	Actinin, alpha 1	9.0	4.3E-09	8.7	7.0E-10
PPIC	Peptidylprolyl isomerase C	8.7	6.4E-07	4.4	3.0E-05
IGSF4	Immunoglobulin superfamily member 4	8.1	3.8E-04	9.5	4.8E-05
MYO6	Myosin VI	8.0	7.7E-09	4.0	1.1E-06
EGLN3	Egl nine homolog 3 (C. elegans)	7.9	1.2E-06	4.3	3.4E-05

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Gene Symbol	Description	Fold- Change wt/ΔE2	p-value	Fold- Change E2R/ΔE2	p-value
MBOAT2	Membrane bound O-acyltransferase domain containing 2	7.7	2.7E-07	6.6	1.6E-07
ATP1B1	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	7.4	4.9E-06	3.9	1.6E-04
HOOK1	Hook homolog 1 (Drosophila)	7.4	3.2E-12	4.4	1.4E-10
RICH2	Rho GTPase-activating protein RICH2	7.3	8.7E-05	5.8	1.0E-04
PLA1A	Phospholipase A1 member A	6.9	1.4E-09	3.5	3.4E-07
MEX3A	RNA-binding protein MEX3A	6.8	7.5E-09	4.4	9.4E-08
ASS	Argininosuccinate synthetase	6.8	7.3E-12	3.5	3.7E-09
S100A4	S100 calcium binding protein A4	6.0	3.8E-07	5.4	1.7E-07
TMEM16K	Transmembrane protein 16K	5.9	8.0E-09	3.3	1.0E-06
CYP2J2	Cytochrome P450, family 2 subfamily J polypeptide 2	5.6	3.0E-08	4.3	7.1E-08
ACSM3	Acyl-CoA synthetase medium-chain family member 3	5.3	1.2E-10	3.0	3.5E-08
QPCT	Glutaminy-peptide cyclotransferase	5.3	1.4E-10	3.7	1.7E-09
MYBPC2	Myosin binding protein C, fast type	5.1	7.0E-06	4.8	2.7E-06
CEP70	Centrosomal protein 70kDa	5.0	9.0E-09	3.5	1.2E-07
ZNF649	Zinc finger protein 649	4.4	7.8E-11	2.8	6.9E-09
FGL2	Fibrinogen-like 2	4.3	1.9E-06	2.8	5.0E-05
NTRK2	Neurotrophic tyrosine kinase receptor type 2	4.2	9.1E-07	3.0	1.1E-05
SERPINB9	Serpin peptidase inhibitor, clade B member 9	4.2	1.4E-07	3.0	1.6E-06

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Gene Symbol	Description	Fold- Change wt/ΔE2	p-value	Fold- Change E2R/ΔE2	p-value
GDF15	Growth differentiation factor 15	4.1	4.7E-05	3.3	1.1E-04
MTAC2D1	Membrane targeting C2 domain containing 1	4.1	4.3E-06	5.1	9.1E-08
GPR56	G protein-coupled receptor 56	4.0	1.0E-04	6.2	5.6E-07
CLCF1	Cardiotrophin-like cytokine factor 1	3.9	1.8E-08	2.5	1.4E-06
KALRN	Kalirin, Huntingtin-associated protein-interacting protein	3.6	4.8E-06	2.5	9.4E-05
IGF1	Insulin-like growth factor 1	3.6	1.0E-06	2.5	2.4E-05
AKAP7	A kinase (PRKA) anchor protein 7	3.5	8.0E-08	2.8	3.8E-07
TMTC2	Transmembrane and tetratricopeptide repeat containing 2	3.5	1.5E-11	2.5	6.8E-10
EPB41L2	Erythrocyte membrane protein band 4.1-like 2	3.5	1.1E-07	2.8	3.6E-07
CPAMD8	C3 and PZP-like, alpha-2-macroglobulin domain containing 8	3.2	3.1E-06	2.8	4.1E-06
HES5	Hairy and enhancer of split 5 (Drosophila)	3.2	1.2E-05	2.7	2.4E-05
TGM2	Transglutaminase 2	3.2	1.9E-07	2.1	1.8E-05
PITPNC1	Phosphatidylinositol transfer protein, cytoplasmic 1	3.1	6.7E-06	3.5	3.3E-07
ZNF439	Zinc finger protein 439	3.1	6.2E-10	3.1	8.7E-11
NDRG1	N-myc downstream regulated gene 1	3.1	2.1E-05	4.6	4.5E-08
RNF43	Ring finger protein 43	3.1	3.0E-04	3.2	6.4E-05
HPCAL1	Hippocalcin-like 1	3.0	2.2E-07	2.2	9.6E-06

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Gene Symbol	Description	Fold- Change wt/ΔE2	p-value	Fold- Change E2R/ΔE2	p-value
CD55	CD55 molecule, decay accelerating factor for complement	3.0	1.6E-08	2.1	1.4E-06
TRBV5-4	T cell receptor beta variable 5-4	3.0	1.9E-06	2.0	1.1E-04
ZNF93	Zinc finger protein 93	2.9	9.0E-08	3.1	3.8E-09
AGRIN	Agrin	2.8	2.7E-09	2.4	4.9E-09
FAM90A1	Family with sequence similarity 90 member A1	2.8	3.0E-05	2.6	2.0E-05
ZNF682	Zinc finger protein 682	2.8	2.7E-05	2.8	5.7E-06
CLEC2D	C-type lectin domain family 2 member D	2.8	3.6E-07	2.2	3.4E-06
FBXL16	F-box and leucine-rich repeat protein 16	2.7	9.9E-08	2.2	8.8E-07
CYP2U1	Cytochrome P450, family 2 subfamily U polypeptide 1	2.7	9.6E-08	2.1	1.6E-06
TMEM23	Transmembrane protein 23	2.6	7.6E-05	2.9	4.6E-06
TNFSF10	Tumor necrosis factor superfamily member 10	2.5	4.1E-05	2.5	1.6E-05
IMPA2	Myo-inositol monophosphatase A2	2.5	8.9E-06	2.2	9.6E-06
SLC7A11	Solute carrier family 7 member 11	2.3	3.2E-06	2.4	5.0E-07
FBN1	Fibrillin 1	2.3	1.6E-08	2.4	9.5E-10
TNFRSF10C	Tumor necrosis factor receptor superfamily member 10c	2.1	4.8E-05	2.0	3.3E-05
T	T, Brachyury homolog (mouse)	-2.0	1.1E-06	-2.3	7.8E-09
TNFRSF1B	Tumor necrosis factor receptor superfamily member 1B	-2.0	2.9E-06	-2.1	3.9E-07
FOXO1A	Forkhead box O1A	-2.0	4.7E-04	-2.9	6.6E-07

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Gene Symbol	Description	Fold- Change wt/ΔE2	p-value	Fold- Change E2R/ΔE2	p-value
NMT2	N-myristoyltransferase 2	-2.1	1.8E-04	-2.2	3.2E-05
FHOD3	Formin homology 2 domain containing 3	-2.2	4.6E-10	-3.7	1.8E-15
NALP1	NACHT, leucine rich repeat and PYD containing 1	-2.2	3.2E-07	-2.1	2.6E-07
VANGL1	Vang-like 1 (Drosophila)	-2.5	1.2E-06	-2.1	5.5E-06
DNAH14	Dynein heavy chain 14, axonemal	-2.5	1.0E-04	-2.2	1.1E-04
MICAL3	Protein MICAL-3	-2.6	2.3E-09	-2.2	1.4E-08
ATF5	Activating transcription factor 5	-2.9	1.3E-06	-2.0	7.5E-05
TNFRSF19	Tumor necrosis factor receptor superfamily member 19	-3.1	4.6E-06	-2.3	4.3E-05
NOTCH2	Notch homolog 2	-3.1	4.4E-05	-3.0	1.4E-05
CHST6	Carbohydrate sulfotransferase 6	-3.2	8.7E-08	-2.3	2.9E-06
CD1A	T-cell surface glycoprotein CD1a	-3.5	1.5E-04	-2.9	2.7E-04
ARHGAP24	Rho GTPase activating protein 24	-3.8	1.3E-05	-3.7	3.4E-06
AUTS2	Autism susceptibility candidate 2	-3.9	1.5E-07	-2.6	5.6E-06
NETO1	Neuropilin and tolloid-like 1	-4.1	4.0E-04	-5.1	1.7E-05
EHD3	EH-domain containing 3	-4.1	9.5E-06	-4.8	3.9E-07
GHR	Growth hormone receptor	-4.3	4.9E-06	-2.7	1.8E-04
GPRC5D	G protein-coupled receptor family C group 5 member D	-4.4	2.4E-07	-3.9	1.5E-07
PPM1H	Protein phosphatase 1H	-4.6	1.7E-06	-6.4	1.1E-08
ADARB1	RNA-specific adenosine deaminase B1	-5.2	1.1E-07	-3.3	2.8E-06
CLEC4C	C-type lectin domain family 4 member C	-5.7	7.4E-08	-4.8	6.1E-08

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Gene Symbol	Description	Fold- Change wt/ΔE2	p-value	Fold- Change E2R/ΔE2	p-value
SGK	Serum/glucocorticoid regulated kinase	-6.1	5.3E-12	-5.1	4.6E-12
PGDS	Prostaglandin D2 synthase, hematopoietic	-6.2	1.2E-06	-5.7	4.4E-07
DNAH17	Dynein, axonemal, heavy polypeptide 17	-6.2	9.8E-07	-3.5	4.0E-05
HHEX	Hematopoietically expressed homeobox	-6.5	6.2E-10	-3.4	1.7E-07
FYB	FYN binding protein	-6.7	1.5E-06	-5.1	2.7E-06
CCDC74B	Coiled-coil domain containing 74B	-7.0	7.0E-08	-3.8	4.5E-06
HCK	Hemopoietic cell kinase	-7.4	1.2E-07	-4.0	6.0E-06
PRSS21	Serine protease 21	-7.6	6.2E-06	-6.0	7.0E-06
FCRL3	Fc receptor-like 3	-8.2	2.9E-07	-5.6	1.2E-06
SLAMF8	SLAM family member 8	-8.5	1.1E-13	-6.6	1.6E-13
HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	-8.8	1.1E-07	-4.4	7.1E-06
TLX2	T-cell leukemia homeobox 2	-9.0	1.1E-07	-12.1	1.7E-09
MSRB3	Methionine sulfoxide reductase B3	-9.2	4.4E-07	-5.3	5.5E-06
CXCL12	Chemokine (C-X-C motif) ligand 12	-9.9	1.9E-09	-4.9	1.6E-07
GIMAP5	GTPase, IMAP family member 5	-10.0	6.2E-07	-5.1	1.9E-05
CXCR3	Chemokine (C-X-C motif) receptor 3	-10.4	6.4E-09	-7.5	1.3E-08
ALPK2	Heart alpha-kinase	-10.7	1.1E-09	-6.9	6.4E-09
TIMD4	T-cell immunoglobulin and mucin domain containing 4	-12.9	6.3E-08	-5.7	4.5E-06
TMPRSS3	Transmembrane protease, serine 3	-15.7	2.2E-06	-6.1	1.3E-04

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Gene Symbol	Description	Fold- Change wt/ΔE2	p-value	Fold- Change E2R/ΔE2	p-value
GABRA4	Gamma-aminobutyric acid A receptor alpha 4	-17.1	3.3E-07	-15.2	1.1E-07
IL12RB2	Interleukin 12 receptor beta 2	-30.5	1.1E-12	-10.2	2.8E-10
TXNDC3	Thioredoxin domain containing 3	-31.6	2.1E-08	-9.6	3.2E-06

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