Deregulation of the Cell Cycle by EBV Nuclear Antigens EBNA3A and EBNA3C

by

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submitted to
Imperial College London
for the degree of Doctor of Philosophy

2012

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Abstract

Cyclin-dependent kinase inhibitor $p16^{INK4A}$ is an important tumour suppressor and inducer of cellular senescence often inactivated during the development of cancer. I investigated the mechanism by which EBV latency-associated nuclear antigens EBNA3A and EBNA3C repress $p16^{INK4A}$ expression.

Using lymphoblastoid cell lines (LCL) expressing a conditional EBNA3C, I demonstrate that EBNA3C inactivation resets the epigenetic status of $p16^{INK4A}$ to permit transcriptional activation: the polycomb-associated repressive H3K27me3 histone modification is substantially reduced, while the activation-related mark H3K4me3 is modestly increased. Activation of EBNA3C reverses the distribution of these epigenetic marks, represses $p16^{INK4A}$ transcription and allows proliferation. LCL lacking EBNA3A express relatively high levels of $p16^{INK4A}$ and have a similar pattern of histone modifications on $p16^{INK4A}$ as produced by the inactivation of EBNA3C. Since binding to the corepressor of transcription CtBP was linked to the oncogenic activity of EBNA3C and EBNA3A, LCL with viruses encoding EBNA3C- and/or EBNA3A-mutants that no longer bind CtBP were established. These novel LCL revealed that the epigenetic repression of $p16^{INK4A}$ requires the interaction of both EBNA3C and EBNA3A with CtBP. Epigenetic repression of $p16^{INK4A}$ by latent EBV may facilitate $p16^{INK4A}$ DNA methylation during lymphomagenesis.

Furthermore, by transforming the peripheral blood lymphocytes (PBL) from an individual homozygous for a deletion in CDKN2A locus with recombinant EBV viruses expressing conditional EBNA3C, we developed a system that allows inactivation of EBNA3C in LCL lacking functional $p16^{INK4A}$ protein (p16-null LCL 3CHT). EBNA3C inactivation has no impact on the proliferation rate of p16-null LCL, proving that the repression of $p16^{INK4A}$ is the main function of EBNA3C in EBV-driven LCL proliferation. The $p16^{INK4A}$ locus is epigenetically modified by EBNA3C despite the absence of functional $p16^{INK4A}$ protein. Since the selection pressure based on faster outgrowth of advantageously modified subset of cells is removed, the gradual and relatively slow kinetics of H3K27me3 restoration at $p16^{INK4A}$ following EBNA3C reactivation in p16-null LCL 3CHT seems to be genuinely related to the mechanism of EBNA3C-mediated $p16^{INK4A}$ regulation. The p16-null LCL 3CHT system further allows distinguishing genes regulated specifically by EBNA3C, rather than as a consequence of activation of $p16^{INK4A}$/Rb/E2F1 axis.

Lastly, new cellular targets of EBNA3C and/or EBNA3A from the group of microRNAs are identified in this work. Most notably, both EBNA3C and EBNA3A are shown to repress the tumour supressor miR-143/145 cluster and their precursor long non-coding RNAs in LCL.
Acknowledgments

I wish to thank the people who supported this work:

Sebastian, Valent, Dagmar, Dada, Justyna, Tina and Petra – for personal care

Prof. Martin Allday – for his guidance, deep knowledge and editorial work on the manuscript

Rob White – for providing 3CHT and CtBP-binding mutant viruses and LCL, for establishing p16-null LCL, for his help with transformation experiments and his comments on this manuscript

Jade Yee and Andrew Brown – for constant practical help in the lab

Melanie Franz and Michaela Ruhmann – for their initial work that formed a primer for this project

Gordon Peters and Alison Sinclair – for kindly providing us with p16INK4A-deleted PBL
“You will seek not a near, but a distant, objective, and you will not be satisfied with what you have done. All that you may achieve or discover you will regard as a fragment of a larger pattern, which from his separate approach every true scholar is striving to descry.”

Aims of the Harvard Society of Fellows (1938)
# Contents

Abstract 2
Acknowledgments 3
Abbreviations 6
List of figures 10
List of tables 14

1 Introduction 15
   Project background – starting point 55
   Aims of the project 57

2 Materials and Methods 58

3 Results 81
   3.1 Mechanism of $p16^{\text{INK4A}}$ epigenetic repression by EBNA3C and EBNA3A 82
      3.1.1 EBNA3C and EBNA3A co-operate with CtBP to epigenetically repress $p16^{\text{INK4A}}$ 82
      3.1.2 The association of EBNA3C and EBNA3A with components of CtBP supercomplexes 109
      3.1.3 Other potential mechanisms of $p16^{\text{INK4A}}$ de-repression 114
      3.1.4 Regulation of $p14^{\text{ARF}}$ by EBNA3C 119

   3.2 The functional significance of $p16^{\text{INK4A}}$ repression by EBNA3C 142
      3.2.1 $p16^{\text{INK4A}}$ functionally null LCL 3CHT system 142
      3.2.2 Expression of $p16^{\text{INK4A}}$ during the transformation of primary B cells by EBV 165

   3.3 EBNA3C and/or EBNA3A-regulated cellular microRNAs 184

4 Thoughts and Final Conclusions 204
   Reference list 220
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>ANRIL</td>
<td>Antisense Non-Coding RNA in the INK4 Locus</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial Artificial Chromosome</td>
</tr>
<tr>
<td>BART</td>
<td>Bamhi-A Rightward Transcript</td>
</tr>
<tr>
<td>BIM</td>
<td>Bcl-2 Interacting Mediator of cell death</td>
</tr>
<tr>
<td>BIM EL</td>
<td>Extra Long Bim isoform</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt Lymphoma</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CBF-1</td>
<td>C-Promoter Binding Factor-1</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-Dependent Kinase Inhibitor 2A</td>
</tr>
<tr>
<td>C/EBPa</td>
<td>CCAAT-Enhancer Binding Protein</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein Diacetate, Succinimidyl Ester</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<tr>
<td>CtBP</td>
<td>C-Terminal Binding Protein</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>ctrl</td>
<td>Control</td>
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<tr>
<td>CpG</td>
<td>Cytosin-Phosphate-Guanin</td>
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<tr>
<td>ddH20</td>
<td>Double Distilled Water</td>
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<td>DDR</td>
<td>DNA Damage Response</td>
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<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DNMT</td>
<td>Dna Methyltransferase</td>
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<td>EBER</td>
<td>EBV-Encoded RNA</td>
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<tr>
<td>EBNA</td>
<td>EBV-Associated Nuclear Antigen</td>
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<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<tr>
<td>E3&lt;sup&gt;CtBP&lt;/sup&gt;</td>
<td>EBV-BAC or virus genome lacking all CtBP-binding sites among the EBNA3s</td>
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<tr>
<td>E.coli</td>
<td>Escherichia Coli</td>
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<td>ECL</td>
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<td>EDTA</td>
<td>Ethylenediamine Tetra-Acetic Acid</td>
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<td>EED</td>
<td>Embryonic Ectoderm Development</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
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<td>EtOH</td>
<td>Ethanol</td>
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<td>EZH 1/2</td>
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<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
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<td>Flow Cytometry</td>
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<td>Foetal Calf Serum</td>
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<td>Gap 1</td>
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<td>GC</td>
<td>Germinal Centre</td>
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<tr>
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<td>Green Fluorescent Protein</td>
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<td>Gp</td>
<td>Glycoprotein</td>
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<td>H</td>
<td>Histone</td>
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<td>Hour</td>
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<tr>
<td>H3K27me3</td>
<td>Trimethylation of lysine 27 at histone 3</td>
</tr>
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<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
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<tr>
<td>HL</td>
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<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
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<tr>
<td>HT</td>
<td>4-Hydroxytamoxifen</td>
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<td>IM</td>
<td>Infectious Mononucleosis</td>
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<tr>
<td>IP</td>
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<td>JMJD3</td>
<td>Jumonji Domain Containing 3, Histone Lysine Demethylase</td>
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<td>JNK</td>
<td>C-Jun NH(2)-terminal protein Kinases</td>
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<td>kDa</td>
<td>Kilodaltons</td>
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<tr>
<td>KO</td>
<td>Knock-out</td>
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<tr>
<td>KPB</td>
<td>Potassium Phosphate Buffer</td>
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<tr>
<td>KSHV</td>
<td>Kaposi Sarcoma Herpesvirus</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani Broth</td>
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<tr>
<td>LCL</td>
<td>Lymphoblastoid Cell Line(s)</td>
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<tr>
<td>LCV</td>
<td>Lymphocryptovirus</td>
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<tr>
<td>lincRNA</td>
<td>Long Intergenic Non-Coding Rna</td>
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<tr>
<td>LMP</td>
<td>Latent Membrane Protein</td>
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<tr>
<td>LP</td>
<td>Leader Protein</td>
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<tr>
<td>LSD1</td>
<td>Lysine-Specific Demethylase 1</td>
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<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MEK</td>
<td>Mitogen-Activated or Extracellular Signal-regulated protein kinase Kinase</td>
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<td>min</td>
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miR MicroRNA
ncRNA Non-Coding RNA
NCR143/145 Non-Coding RNA precursor of Mir-143/145
NFκB Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NK Natural Killer
NPC Nasopharyngeal Carcinoma
NP40 Nonidet P-40
NuRD Nucleosome Remodelling and histone Deacetylation
OIS Oncogene Induced Senescence
oriP Origin Of Replication
p or pp Page(s)
PAGE Poly-Acrylamide Gel Electrophoresis
PBL Peripheral Blood Lymphocytes
PBS Phosphate Buffered Saline
PBS-T Phosphate Buffered Saline Containing 0.5% V/V Tween 20
PcG Polycomb Group proteins
PCR Polymerase Chain Reaction
PI Propidium Iodide
PMSF Phenyl Methyl Sulphonyl Fluoride
Pol II RNA Polymerase II
ppRb Hyperphosphorylated Rb
pRb Hypophosphorylated Rb
PRC Polycomb Repression Complex
PTLD Posttransplant Lymphoproliferative Disorder
Rb Retinoblastoma Protein
RBPJk/CBF1 Recombinant Binding Protein J Kappa/C-Promoter Binding Factor-1
rev Revertant
revCISP EBV-BAC or virus genome with all CtBP- binding sites among the EBNA3s reverted to WT
RIP RNA Immunoprecipitation
RNA Ribonucleic Acid
RPMI Roswell Park Memorial Institute Medium
RREB1 Ras-Responsive Element-Binding Protein
qPCR Quantitative PCR
RT Room Temperature
S Synthesis
SB Superbroth
SD Standard Deviation
SDS Sodium Dodecyl Sulphate
<table>
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<td>Sequencing</td>
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<tr>
<td>Ser 5</td>
<td>Serine 5 (of Pol II)</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short-Hairpin RNA</td>
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<tr>
<td>SILAC</td>
<td>Stable Isotope Labeling of Amino acids in Cell culture</td>
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<td>SV40</td>
<td>Simian Virus 40</td>
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<td>TAP</td>
<td>Tandem Affinity Purification</td>
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<td>TBE</td>
<td>Tris-Borate-EDTA</td>
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<tr>
<td>TE</td>
<td>Tris-EDTA</td>
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<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine</td>
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<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>TGFβRII</td>
<td>Transforming Growth Factor Beta Receptor II</td>
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<tr>
<td>TLDA</td>
<td>Taqman quantitative real-time PCR Low Density Array</td>
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<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TPA</td>
<td>Tetradecanoylphorbol Acetate</td>
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<tr>
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<td>Terminal Repeats</td>
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<td>TSG</td>
<td>Tumour Suppressor Gene</td>
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<tr>
<td>Tween 20</td>
<td>Polyoxyethylenesorbitanmonolaurate</td>
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<tr>
<td>UTX</td>
<td>Ubiquitously Transcribed TPR protein on the X chromosome</td>
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<tr>
<td>v/v</td>
<td>Volume by Volume</td>
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<td>WB</td>
<td>Western Blotting</td>
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<td>WT</td>
<td>Wild-Type</td>
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<td>w/v</td>
<td>Weight by Volume</td>
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<td>3A&lt;sub&gt;CtBP&lt;/sub&gt;</td>
<td>EBV-BAC or virus containing mutated CtBP- binding sites in EBNA3A</td>
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<td>3CHT</td>
<td>EBV-BAC or virus containing EBNA3C-HT fusion</td>
</tr>
<tr>
<td>3C&lt;sub&gt;CtBP&lt;/sub&gt;</td>
<td>EBV-BAC or virus containing a mutated CtBP- binding site in EBNA3C</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-Amino-Actinomycin D</td>
</tr>
</tbody>
</table>
## List of Figures

1.1 The origin of EBV-associated tumours
1.2 EBV episome and its latency-associated transcripts
1.3 Genomic organization of CDKN2A in relation to CDKN2B and CDKN2B-AS (ANRIL) loci
1.4 G1-S checkpoint
1.5 CtBP supercomplex
2.1 Mutations of CtBP-binding sites in EBNA3C and EBNA3A in CtBP-binding mutant EBV-BACs
2.2 Scheme of the separation of lymphocytes into high- and normal-density fractions using Ficoll-Paque reagent
2.3 Localization of the ChIP assays across the CDKN2A locus
3.1.1 EBNA3C inactivation and reactivation in LCL 3CHT
3.1.2 Validation of LCL 3CHT
3.1.3 PI and BrdU/PI profiles of LCL 3CHT with and without HT
3.1.4 Proliferation of LCL 3CHT after inactivation and reactivation of EBNA3C
3.1.5 Scheme of the CDKN2A locus and the localization of qPCR assays
3.1.6 Repression of p16\textsuperscript{INK4A} following reactivation of EBNA3C
3.1.7 Regulation of the pocket proteins in response to EBNA3C
3.1.8 Simultaneous ChIP analysis quantifying the H3K27me3 mark on p16\textsuperscript{INK4A} exon 1 and qPCR analysis of p16\textsuperscript{INK4A} transcription in response to EBNA3C
3.1.9 ChIP analysis quantifying the H3K27me3 and H3K4me3 marks at p16\textsuperscript{INK4A} locus in response to EBNA3C
3.1.10 Validation ChIPs in LCL 3CHT
3.1.11 EBNA3C-mediated regulation of p16\textsuperscript{INK4A} does not require Rb
3.1.12 EBNA3A contributes to the regulation of p16\textsuperscript{INK4A}
3.1.13 Expression of p16\textsuperscript{INK4A} in CtBP-binding mutant LCL relative to revertant and WT LCL
3.1.14 Reduction of Rb in CtBP-binding mutant LCL
3.1.15 The interaction of EBNA3A and EBNA3C with CtBP is necessary for the chromatin remodelling associated with the repression of p16\textsuperscript{INK4A}
3.1.16 EZH2 expression in LCL 3CHT
3.1.17 \( p16^{NKDA} \) promoter methylation in newly established LCL in response to EBNA3C
3.1.18 Co-immunoprecipitation with anti-CtBP and anti-LSD1 antibody in WT LCL
3.1.19 Co-immunoprecipitation with anti-Co-REST and anti-HDAC1 antibody in WT LCL
3.1.20 Co-immunoprecipitation with anti-HDAC1 and anti-LSD1 antibody in WT BL31
3.1.21 Co-immunoprecipitation with anti-LSD1 (and anti-HDAC1) antibody in WT and CtBP-binding mutant LCL
3.1.22 Phosphorylation of H2AX in LCL 3CHT and in CtBP-binding mutant LCL
3.1.23 Phosphorylation of p38MAPK in LCL in response to EBNA3C
3.1.24 Co-regulation of ANRIL ncRNA with CDKN2A (\( p16^{NKDA} + p14^{ARF} \)) mRNA in LCL 3CHT lines
3.1.25 CDKN2A mRNA and ANRIL ncRNA correlation in LCL carrying various EBV-BAC recombinant viruses
3.1.26 Comparison of the quantity of two amplicons positioned in the opposite ends of the ANRIL transcript in the same samples
3.1.27 Repression of \( p14^{ARF} \) mRNA following EBNA3C reactivation
3.1.28 \( p14^{ARF} \)-p53-p21\(^{CIP1} \) axis in LCL 3CHT after inactivation of EBNA3C
3.1.29 \( p14^{ARF} \) and p21\(^{CIP1} \) transcripts in CtBP-binding mutant LCL
3.1.30 ChIP–qPCR analysis of H3K27me3 and H3K4me3 at the \( p14^{ARF} \) promoter in LCL
3.1.31 Scheme of the CtBP contribution in the epigenetic regulation of cellular genes by EBNA3C and/or EBNA3A
3.1.32 Synchronized recruitment of PRC2 and LSD1/Co-REST to cellular gene promoters by lincRNAs
3.2.1 \( CDKN2A \) transcripts in normal cells and in individual with a 19bp deletion in the gene
3.2.2 Expression of EBV latent proteins in \( p16 \)-null and \( p16 \)-competent LCL 3CHT with and without HT
3.2.3 Characterization of the \( p16 \)-null LCL 3CHT
3.2.4 Quantification of \( p16^{NKDA} \) exon1-comprising transcripts in \( p16 \)-null and -competent LCL 3CHT with and without HT
3.2.5 Proliferation rate of \( p16 \)-null and -competent LCL 3CHT lines following EBNA3C inactivation
3.2.6 Alternative representation of the data summarized in Fig. 3.2.5. Proliferation rate of multiple \( p16 \)-null LCL 3CHT cultured with or without HT and in various time-points following EBNA3C inactivation
3.2.7 Quantity and phosphorylation of the pocket proteins in \( p16 \)-null and -competent LCL 3CHT lines with and without HT
3.2.8 Proliferation- and E2F1-dependent modulation of genes in \( p16 \)-competent LCL 3CHT
3.2.9 p14\textsuperscript{ARF}-p53-p21\textsuperscript{CIP1} pathway is not activated in p16-null LCL 3CHT following EBNA3C inactivation

3.2.10 Epigenetic regulation of p16\textsuperscript{INK4A} locus by EBNA3C in p16-null LCL 3CHT

3.2.11 ChIP-qPCR analysis quantifying the H3K4me3 across CDKN2A locus in p16-null LCL 3CHT

3.2.12 The kinetics of epigenetic modulation of p16\textsuperscript{INK4A} locus by EBNA3C

3.2.13 Histone modifications at CDKN2A locus including p14\textsuperscript{ARF} promoter in p16-null LCL 3CHT

3.2.14 Cumulative nature of H3K27me3 restoration at p16\textsuperscript{INK4A} locus following EBNA3C reactivation in p16-null LCL 3CHT

3.2.15 Characterization of p16-null EBNA3A KO and revertant LCL

3.2.16 Proliferation rate of p16-null and -competent EBNA3A KO and revertant LCL

3.2.17 Epigenetic regulation of p16\textsuperscript{INK4A} locus by EBNA3A in p16-null LCL

3.2.18 CDKN2A transcripts after EBV infection of purified primary B cells

3.2.19 Control transcripts repressed or unaltered after EBV infection of purified primary B cells

3.2.20 p16\textsuperscript{INK4A} transcripts in the normal-density cell population after EBV infection of PBL

3.2.21 Accumulation of p16\textsuperscript{INK4A} and CDKN2A transcripts in the high-density fraction of EBV-infected PBL

3.3.1 Regulation of miR-221 by EBNA3C and EBNA3A in LCL

3.3.2 Regulation of miR-138 by EBNA3A in LCL

3.3.3 Regulation of mature miR-143 and miR-145 by EBNA3C and EBNA3A

3.3.4 Regulation of the carrier miR*-143 and miR*-145 by EBNA3C and EBNA3A

3.3.5 NCR-143/145 expression in LCL 3CHT cultured with and without HT

3.3.6 The correlation of mature miR-143/145 and their long non-coding precursors in the same LCL samples

3.3.7 Regulation of NCR-143/145 by EBNA3C and EBNA3A in p16-null LCL

3.3.8 Regulation of the mature miR-143/145 by EBNA3C and EBNA3A in p16-null LCL

3.3.9 Regulation of RTKN and DNMT3A transcripts by EBNA3C and/or EBNA3A in B cell-derived lines

3.3.10 Regulation of RTKN and DNMT3A protein by EBNA3C and EBNA3A in LCL

4.1 Scheme of the roles of EBNA3C and EBNA3A in maintenance of LCL proliferation and in B cell immortalization

4.2 Direct association of EBNA3C and EBNA3A with the chromatin remodelling complex at the target cellular promoter

4.3 Direct and indirect targeting of chromatin remodelling complexes to the cellular promoters by EBNA3C and EBNA3A
4.4 Gene bookmarking by EBNA3C and EBNA3A

4.5 Transcriptional regulation of the essential component(s) of epigenetic machinery by EBNA3C and/or EBNA3A
List of Tables

1.1 EBV latency-associated expression programmes
2.1 List of most frequently used cell lines
2.2 Primer sequences for mRNA quantification using sybr green chemistry
2.3 Primer sequences for mRNA quantification using taqman chemistry
2.4 Primer sequences for mRNA quantification of housekeeping genes using sybr green chemistry
2.5 The source of the primer sequences that have been developed prior to this project
2.6 TaqMan® MicroRNA Assays
2.7 Primer sequences of the assays spaced across the CDKN2A locus
2.8 Localization of the ChIP assays across the CDKN2A locus
2.9 Primer sequences of the p14ARF and control promoter assays
Chapter 1
Introduction
**Epstein-Barr virus (EBV) epidemiology and transmission**

EBV (also known as Human Herpesvirus 4, HHV-4) is a large DNA virus belonging to the genus Lymphocryptovirus (LCV), in the subfamily Gammaherpesvirinae, family Herpesviridae. EBV co-evolved with the human species for millions of years. LCV are found only in primates, with EBV representing the only LCV that infects humans. Seroepidemiologic surveys indicate that EBV latently infects approximately 90% of the world population (Kutok and Wang, 2006).

EBV is spread from host to host mostly through saliva and the subsequent infection of both the epithelial cells and B lymphocytes in Waldeyer’s ring (Crawford, 2001). Primary infection with EBV occurs usually in early childhood and is asymptomatic; if delayed until adolescence it may manifest as a benign lymphoproliferative syndrome known as infectious mononucleosis (IM) (Crawford, 2001). Primary infection is controlled by the immune system and the distinguishing symptoms of IM, such as lymphadenopathy and splenomegaly, are largely the manifestation of this immune response (Long, Taylor and Rickinson, 2011).

Even though EBV elicits a robust immune response consisting mainly of cytotoxic T lymphocytes (CTL), EBV is still not eliminated from the body but establishes a life-long latent infection in the memory B cell compartment (Crawford, 2001; Kutok and Wang, 2006). Healthy EBV carriers display stable numbers of EBV-infected B cells in the blood (0.5–50 per million) and low level of active EBV replication in the cells of the oropharynx (Thorley-Lawson, 2001). In the immunocompetent individuals, equilibrium between virus infection and the host immune system is established and EBV latency in the majority of carriers has no serious sequel throughout life. However, the more impaired the immune system of the host, the more substantial are the consequences of EBV reactivation (Lazzarino et al., 1999; Preiksaitis and Keay, 2001).
**EBV structure and life cycle**

The EBV virion consists of a linear, double-stranded, approximately 184-kb DNA genome located within an icosahedral capsid coated with a glycoprotein-carrying lipid envelope (Johannsen *et al.*, 2004). The genome comprises short and long unique sequence domains that contain almost all the genome coding capacity, as well as internal and terminal tandem and reiterated direct repeats. EBV genome becomes circular for replication and persists in the nucleus of a latently infected cell extrachromosomally as several plasmid copies (episomes) (Hung, Kang and Kieff, 2001). The number of joined terminal repeats in the episome after circularization remains stable in all daughter cells derived from a single infected cell and can therefore distinguish clonal infection events (Katz, Raab-Traub and Miller, 1989).

An EBV major surface glycoprotein is a 350 kD antigen which binds the CD21 receptor on B cells and determines EBV’s tropism for B cells. Another envelope glycoprotein, gp42, mediates the fusion between the virus envelope and the host cell membrane. EBV can gain entry also into various types of CD21-negative cells, such as T lymphocytes, mesenchymal cells and epithelial cells, but the mechanism is less well understood (Kutok and Wang, 2006; Shannon-Lowe *et al.*, 2006; Shannon-Lowe and Rowe, 2011).

EBV infection of epithelial cells generally results in virus production and cell lysis, whereas the infection of B lymphocytes leads to activation of B cells into proliferating B blasts associated with the establishment of EBV latency. During lytic replication, about 80 viral proteins are expressed in a defined temporal order, including viral capsid antigens as the late gene products. EBV virions assemble in the nucleus and acquire the envelope by budding from the cell membrane (Kieff and Rickinson, 2007).
Apart from the lytic replication, EBV is able to execute several latency-associated gene expression programmes depending on the usage of different latency promoters (Table 1.1) (Klein, Kis and Klein, 2007). The expression of viral products is highly restricted during latency, with maximum of only 9 proteins and several RNA species. These comprise six nuclear (EBNAs) and three integral latent membrane proteins (LMPs), viral microRNA transcripts from the BamHI A region (BARTs) and non-polyadenylated EBV-encoded small RNAs (EBERs). In addition, it has been demonstrated recently that BHRF1, a viral homologue of the large BCL-2 family of proteins, usually associated with lytic EBV infection, might be a latent protein (Kelly et al., 2009). Furthermore, transient expression of several viral lytic genes that do not belong to the 'classical' latent subset is found early after infection of B lymphocytes with EBV (Kalla and Hammerschmidt, 2012).

In latency, the EBV episome is replicated by cellular DNA polymerase and distributed to daughter cells after cell division (Schaefer, Strominger and Speck, 1997; Thorley-Lawson, 2001).

<table>
<thead>
<tr>
<th>Expression programme</th>
<th>EBV genes expressed</th>
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<tbody>
<tr>
<td>Latency III (the growth programme)</td>
<td>EBERs, BARTs, EBNAs (-LP, -2, -3A, -3B, -3C, and -1),</td>
</tr>
<tr>
<td></td>
<td>LMPs</td>
</tr>
<tr>
<td>Latency II (the default programme)</td>
<td>EBERs, BARTs, EBNA1, LMP1, LMP2A</td>
</tr>
<tr>
<td>Latency I</td>
<td>EBERs, BARTs, EBNA1</td>
</tr>
<tr>
<td>Latency 0</td>
<td>EBERs, BARTs</td>
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Table 1.1. EBV latency-associated expression programmes that have been identified (Thorley-Lawson, 2001). EBERs: EBV encoded small RNAs, BARTs: Bam HI A rightward transcripts, EBNAs: EBV nuclear antigens, LMPs: latent membrane proteins
EBV persistence and B cell life cycle

In vivo, establishment of EBV persistent infection is closely connected with the maturation and differentiation of its host cell – the B lymphocyte (Thorley-Lawson, 2001). B cell maturation begins in the bone marrow and consists of the development of functional receptors and the testing for and elimination of self-reactive receptors. Mature naive B cells move to secondary lymphoid organs, such as lymph nodes and spleen, which efficiently trap antigens for exposure to T and B lymphocytes. Following encounter with the antigen, B cells are activated by CD4+ T cells in the germinal centres of the secondary lymphoid organs and differentiate into plasma cells or memory B cells (Thomas, Srivastava and Allman, 2006).

EBV resides long-term in memory B cells; however, to reach this compartment for life-long persistence, EBV infects resting naive B cells and, mimicking antigen-induced activation, drives these to proliferate as activated B blasts. EBV-infected B blasts probably migrate into germinal centres, where they differentiate to become centroblasts, centrocytes and finally long-lived resting memory B cells (Thorley-Lawson, 2001; Thorley-Lawson, Duca and Shapiro, 2008).

The differentiation of EBV-infected B cells is accompanied by the regulated shut-down of EBV gene expression by epigenetic silencing of EBV promoters and enables EBV persistence in an organism with an intact immune system. The full growth programme or latency III is expressed only transiently in the naive B cells after infection and activation with EBV. A portion of the EBV-activated B blasts undergoes germinal centre reaction. In germinal centres, the restriction of EBV protein expression continues reaching latency II. Out of centroblasts and centrocytes, some mature into memory cells that remain for the life of an individual. Within such cells EBV can persist without expressing any of its proteins (Küppers, 2003).
**EBV-associated tumours**

EBV was the first virus associated with human neoplasia when in 1964 Anthony Epstein, Bert Achong and Yvonne Barr discovered EBV particles by electron microscopy in the specimens of Burkitt lymphoma (BL) (Pagano, 1999). To this day, EBV has been linked with a wide range of tumours of lymphoid and epithelial origin, including BL, Hodgkin lymphoma (HL), post-transplant lymphoproliferative disorder (PTLD), AIDS-associated lymphomas, rare cases of T cell and NK-cell lymphoma, nasopharyngeal carcinoma (NPC) and gastric carcinoma. EBV-associated carcinogenesis is often linked to immunosuppression, but occurs also in individuals with no apparent defect in immunity (Thompson and Kurzrock, 2004).

EBV likely plays a role in the early phases of tumour development since homogeneous EBV episomes were detected with the use of the virus termini assay in several EBV-related tumours (HL, NPC and BL), suggesting that they develop from a single cell that was infected by EBV before the outgrowth (Dolcetti and Carbone, 2010). EBV is probably the most efficient transforming virus in culture, transforming >50% of resting primary B cells into continuously proliferating lymphoblastoid cell lines (LCL) that always express the full growth programme (latency III) (Young and Rickinson, 2004; Thorley-Lawson and Allday, 2008). However, only a small proportion of immunocompetent EBV carriers develop a malignancy and in the majority of the hosts latent EBV persists without serious health consequences. This is most likely a result of mutual adaptation of the virus and its host throughout their co-evolution. EBV-associated malignancies cluster in certain populations and cultures due to genetic susceptibility to viral carcinogenesis (e.g. NPC in natives of southern China, Southeast Asia, the Arctic, and the Middle East/North Africa) or in certain geographical regions where EBV co-operates in carcinogenesis with other factors such as malaria (e.g. BL in equatorial Africa) (Hoppe-Seyler and Hoppe-Seyler, 2011).
EBV-associated B cell tumours exhibit different patterns of EBV gene expression according to their origin; the tumours retain characteristics of the original B cell type and its location (Thorley-Lawson and Gross, 2004). BL originates from germinal centre or post-germinal centre B cells blocked from exiting the cell cycle in the memory compartment (latency I), HL arises from germinal centre or post-germinal centre B cells that express the centrocyte EBV latency II programme and PTLD arises from naive, memory or post-germinal centre B cells which are activated by EBV and express the latency III programme (Fig. 1.1) (Thorley-Lawson and Gross, 2004).

**Fig. 1.1.** The origin of EBV-associated tumours (reproduced from Thorley-Lawson and Gross, 2004). EBV mimics the signals provided by antigen and CD4+ T cells to enter memory B cell compartment by normal B cell differentiation pathway. EBV-related lymphomas originate from EBV-infected B cells arrested in different stages of differentiation. HL arises from a virus-infected cell that is blocked at the germinal-center stage; BL arises from a germinal-center cell that is entering the memory compartment but is driven by EBV to keep proliferating. Any B cell other than the tonsilar naive B cell (bystander B-cell) that becomes infected by EBV and expresses the growth programme cannot differentiate out of the cell cycle and, if not destroyed by CTLs, can lead to PTLD (Thorley-Lawson and Gross, 2004).
Endemic BL is an EBV-associated B cell lymphoma occurring in children in Africa which manifests mostly as facial tumours. BL exists also in a non-endemic or sporadic variant which usually presents as abdominal tumours, occurs world-wide and can be EBV-positive or negative (Ferry, 2006). The consistent causative lesion in all cases of BL was identified to be Ig/myc chromosomal translocation between the c-MYC oncogene and either the immunoglobulin heavy chain gene (80% of cases), the kappa light chain loci (15% of cases), or the lambda light chain loci (5% of cases) (Kutok and Wang, 2006; Thorley-Lawson and Allday, 2008). Similar to BL, EBV also contributes to the development of only a proportion (30-50%) of HL. In HL, malignant Hodgkin-Reed Sternberg cells typically comprise less than 1–2% of the total tumour mass, while the remainder is formed by non-neoplastic cell populations in lymph nodes (Flavell and Murray, 2000; Gallagher et al., 2003). The transformation process of Hodgkin-Reed Sternberg cells is not yet fully understood, and is thought to involve a combination of EBV-mediated constitutive NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation, and EBV-unrelated acquisition of apoptosis-resistant phenotype and increased genetic instability (Thomas et al., 2002).

PTLD consists of LCL-like cells that express the full growth programme since the CTL response to latent proteins is not adequate. PTLD is a frequent complication of iatrogenic immunosuppression; it arises in up to 10% of solid organ transplant recipients and its incidence relates directly to the degree and length of immunosuppression. Early lesions are polyclonal; the tumours arise multifocally in extranodal sites, usually affecting the transplanted organ or gastrointestinal tract and exhibiting aggressive behaviour associated with high mortality (Taylor, Marcus and Bradley, 2005). The treatment options are either the reduction or withdrawal of immunosuppression, often associated with graft loss, or transfer of autologous (or even allogeneic) EBV-specific CTL (Taylor, Marcus and Bradley, 2005). AIDS-associated lymphomas form a more heterogeneous group of tumour types and their
association with EBV is more variable and dependent on the type of tumour (Kutok and Wang, 2006).

**EBV latent antigens**

As opposed to majority of other human tumour viruses, EBV episomes persist in latently infected cells and EBV-associated tumour cells without DNA integration and the consequences of insertional mutagenesis (Groger, Morrow and Tykocinski, 1989). EBV uses its viral proteins, which in part mimic several cellular growth factors, transcription factors, and antiapoptotic factors, to create a favourable environment for persistence of the virus within the host cell.

EBV genome encodes at least 86 genes but only a fraction is necessary for the transformation and immortalization of primary B cells into LCL. Specifically EBNA1, EBNA2, LMP1, and EBNA3C are absolutely required (Young and Murray, 2003). Although the primary role of EBNA1 is in episome maintenance, it might also promote malignant transformation by conferring higher resistance to apoptosis in the context of c-MYC translocation and by promoting telomere dysfunction via induction of oxidative stress (Kamranvar and Masucci, 2011). EBNA2 and LMP1 are often considered to be the EBV principal oncoproteins. EBNA2 is partially interchangeable with an activated Notch receptor (Notch-IC), exploits the Notch signalling pathway and through the interaction of DNA-bound RBPJk/CFB1-complex transactivates a subset of Notch-regulated cellular promoters, including the c-MYC promoter (Gordadze et al., 2001; Kohlhof et al., 2009). In addition to transactivation of cellular genes, EBNA2 together with cellular transcription factors creates the LMP-1 promoter activating complex (Wang et al., 1990; Wang, Grossman and Kieff, 2000). LMP1, in a similar manner to CD40, acts as constitutively active TNF-receptor, leading to induction of NFkB and c-Jun NH(2)-terminal protein Kinases (JNK) pathways (McFarland, Izumi and Mosialos, 1999; Wan et al., 2004).
EBV nuclear antigens EBNA3C and EBNA3A

EBNA3s are thought to have arisen from the tandem duplication events of a single EBNA3 gene during evolution of EBV. The proteins they encode – EBNA3A, B and C originate from a large transcript under the control of W or C promoter (Wp, Cp), that is spliced and translated to produce all six EBNAs (Fig. 1.2) (Young and Murray, 2003; Kelly et al., 2006; White et al., 2010). EBNA3s are expressed as a part of the latency programme III in vitro. In vivo, cells expressing latency III programme including EBNA3C and EBNA3A have been found in blood during acute IM, but not in the blood of healthy or immunosuppressed individuals during persistent infection (Tierney et al., 1994; Miyashita et al., 1997; Babcock et al., 1999).

EBNA3B is not essential for EBV-mediated B cell immortalization in vitro (Chen et al., 2005) and was not investigated in this project. EBNA3A was traditionally considered essential for immortalization (Tomkinson, Robertson and Kieff, 1993; Farrell, 1995); however, it has been shown recently that EBV-BAC with the deletion of the entire EBNA3A open reading frame can still immortalize primary B cells, although with lesser efficiency and slower outgrowth of the newly established LCL in comparison to LCL established with wild-type (WT) EBV-BAC viruses (Hertle et al., 2009; Skalska et al., 2010; Rob White, personal communication). However, EBNA3C appears to be absolutely essential for immortalization (Maruo et al., 2009; Rob White, personal communication).

Because of their large size (>900 aa), and the probability that large regions are unfolded, full-length EBNA3C and EBNA3A proteins have thus far proven refractory to structural analysis and determination of crystal structure. However, it has been established that EBNA3C contains a proline-rich and glutamine-proline-rich domains and a leucine zipper, bZIP domain. A leucine zipper is usually part of the DNA-binding domain in various
transcription factors; however the bZIP domain of EBNA3C is an atypical leucine zipper which does not appear to mediate DNA binding. The EBNA3C bZIP domain has been implicated in EBNA3C self-association and oligomerization, as well as in the interaction with transcription factor RBPJk/CBF1 (West et al., 2004).

The concentration of EBNA3A and EBNA3C transcripts in a cell at any given time is estimated to be only one or few copies, however the protein half-life of both molecules is relatively long (>24h) (Touitou et al., 2005).

Fig. 1.2. EBV episome and its latency-associated transcripts (reproduced from Young and Murray, 2003). The figure depicts the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The solid blocks represent coding exons for each of the latent proteins and the arrows indicate the direction in which they are transcribed. EBNA3s (together with the nuclear antigens EBNAs 1, 2 and –LP) are produced from the long transcript common for all six EBNAs and initiated from adjacent promoters Cp and Wp. EBNA-LP transcripts contain variable numbers of repetitive exons. During latency I and II, EBNA1 transcripts are transcribed from Qp (as opposed to Cp or Wp). LMPs 1 and 2 are transcribed from independent EBNA2-activated promoters. LMP2A and LMP2B transcripts consist of multiple exons located on either side of the terminal repeats (TR) region. EBV small RNAs EBER1 and EBER2 originate from the part of the genome adjacent to the EBV origin of replication (oriP), while BamA region of the EBV genome gives rise to multiple EBV microRNAs (Young and Murray, 2003).
EBNA3C and EBNA3A function as transcriptional regulators modulating the expression of both viral and cellular genes (Waltzer et al., 1996; Young et al., 2008; Zhao et al., 2011a). EBNA3C and EBNA3A are thought to contribute to the switch from latency III to latency II by epigenetic silencing of the Cp and Wp (Thorley-Lawson and Allday, 2008). EBNA3C and EBNA3A both serve as a negative regulator of EBNA2 expression and/or function. EBNA2 extensively associates with the Notch-regulated transcription factor (TF) RBPJk/CBF1 (Johannsen et al., 1996; Waltzer et al., 1996). In the absence of EBNA2, RBPJk/CBF1 acts as a repressor through recruitment of repressive chromatin remodelling complexes. EBNA2 binding to RBPJk/CBF1, together with other co-activators, leads to gene activation (Young and Rickinson, 2004). EBNA3 proteins have the capacity to compete with EBNA2 for RBPJk/CBF1 binding and repress EBNA2 activation of Cp, limiting the “feed forward” upregulation of EBNA2 expression (Johannsen et al., 1996). The association of EBNA3C and EBNA3A with RBPJk/CBF1 is essential for the growth of LCL (Maruo et al., 2005 and 2009).

EBNA3C and EBNA3A exhibit robust repressor activity when targeted directly to DNA by fusion with the DNA-binding domain of Gal4. EBNA3C binds HDAC1 (Radkov et al., 1999; Knight et al., 2003), while both EBNA3C and EBNA3A bind the co-repressor of transcription CtBP (Touitou et al., 2001; Hickabottom et al., 2002).

In primary rodent fibroblasts, EBNA3C and EBNA3A have both been shown to be oncogenic in cooperation with activated Ras, similar to oncoproteins of small DNA viruses such as HPV E7 or adenoviral E1A (Touitou et al., 2001; Hickabottom et al., 2002). EBNA3C has been reported to bind the retinoblastoma protein (Rb) and via an SCF ubiquitin ligase may direct the proteasome-mediated degradation of Rb. It can interact with cyclin A (and perhaps other cyclins) and may modulate the activity of p27^KIP1 (Knight et al., 2004; Knight, Sharma and Robertson, 2005). EBNA3C is thought to directly bind to the tumour suppressor protein p53 and block its transcriptional activity. EBNA3C might also suppress p53 function by additional mechanisms including interaction with p53-regulatory
proteins, the inhibitor of growth family proteins ING4 and ING5, and stabilization of MDM2, a negative regulator of p53 (Saha et al., 2009 and 2011).

EBNA3C overexpression was shown to disrupt the mitotic spindle checkpoint and induce nuclear division separated from cytokinesis, leading to multinucleated cells (Parker, Toutou and Allday, 2000). EBNA3C might facilitate the spread of damaged DNA through inactivation of the mitotic spindle checkpoint and transcriptional repression of BubR1 (Gruhne, Sompallae and Masucci, 2009). However, EBNA3C seems to be important for attenuation of the EBV-induced ATM/CHK2-mediated DNA damage response in the early phases of primary B cell transformation (Nikitin et al., 2010).

The microarray studies performed in our lab revealed that EBNA3C and EBNA3A extensively cooperate in the regulation of a surprisingly large number of cellular genes, including the gene encoding the pro-apoptotic tumour suppressor BIM (Anderton et al., 2008; White et al., 2010). Both EBNA3C and EBNA3A have been recently shown to repress expression of the cyclin-dependent kinase (CDK) inhibitor p16\textsuperscript{INK4A} and thus maintain proliferation of LCL (Maruo et al., 2006; Hertle et al., 2009).

**CDKN2A locus in tumour suppression and senescence**

The CDKN2A locus in the human genome encodes two tumour suppressor proteins – p16\textsuperscript{INK4A} and p14\textsuperscript{ARF}. The transcripts of p16\textsuperscript{INK4A} and p14\textsuperscript{ARF} consist of a unique first exon and shared second and third exons. However, the proteins are structurally unrelated since p14\textsuperscript{ARF} utilizes an alternative reading frame to that used for p16\textsuperscript{INK4A}. In different contexts, these tumour suppressors can be activated and repressed by various agents independently, or sometime co-regulated (Gil and Peters, 2006). CDKN2B locus located just upstream of CDKN2A encodes p15\textsuperscript{INK4B}, another tumour suppressor with functions
similar to p16\textsuperscript{INK4A} (see below). In addition, a non-coding RNA termed ANRIL is transcribed antisense to the \textit{INK4-ARF} locus. The \textit{ANRIL} gene (\textit{CDKN2B-AS}) contains 20 exons, spans a region of 126.3 kb, and gives rise to approximately 4kb-long transcripts in the antisense orientation of the \textit{p15\textsuperscript{INK4B} - p16\textsuperscript{INK4A} - p14\textsuperscript{ARF}} gene cluster (Pasmant et al., 2007).

![Genomic organization of CDKN2A in relation to CDKN2B and CDKN2B-AS (ANRIL) loci](image)

Fig. 1.3. Genomic organization of \textit{CDKN2A} in relation to \textit{CDKN2B} and \textit{CDKN2B-AS (ANRIL)} loci (reproduced from Pasmant et al., 2007). Boxes represent exons. p16\textsuperscript{INK4A} protein is encoded by exons 1\textsubscript{α}, 2, and 3 of p16/CDKN2A, whereas p14\textsuperscript{ARF} protein is encoded by exon 1\textsubscript{β}, spliced to exons 2 and 3 of p16/CDKN2A in a different reading frame and transcribed using a different promoter. The \textit{ANRIL} gene overlaps the two exons of \textit{p15/CDKN2B} and is transcribed in the orientation opposite to the \textit{p15\textsuperscript{INK4B} - p16\textsuperscript{INK4A} - p14\textsuperscript{ARF}} gene cluster (Pasmant et al., 2007).

p16\textsuperscript{INK4A} is induced by DNA damage, reactive oxygen species and various other forms of intracellular stress. Aberrant oncogenic signals such as activated Ras also lead to p16\textsuperscript{INK4A} induction (Macleod, 2008). p16\textsuperscript{INK4A} protein is relatively stable and p16\textsuperscript{INK4A} is primarily regulated at the level of transcription; ETS1 is the main positive transcriptional regulator of p16\textsuperscript{INK4A}; whereas ID1 and BMI1 function as negative transcriptional regulators of p16\textsuperscript{INK4A} (the latter also regulates p14\textsuperscript{ARF}) (Ohtani, et al., 2001; Satyanarayana and Rudolph, 2004; Kotake, Zeng and Xiong, 2009). p16\textsuperscript{INK4A} is a cyclin-dependent kinase inhibitor that activates the cell cycle inhibitory activity of Rb tumour suppressor by inhibiting its phosphorylation either directly or indirectly. Firstly, p16\textsuperscript{INK4A} binds cyclin D-dependent kinases CDK4 and CDK6, preventing cyclin D binding and subsequent kinase function. In
addition, by releasing additional CDK inhibitors p21\textsuperscript{CIP1} and p27\textsuperscript{KIP1}, which are sequestered in cyclin D/CDK4 and CDK6 complexes, p16\textsuperscript{INK4A} also suppresses CDK2 activity (Knudsen and Knudsen, 2008).

p14\textsuperscript{ARF} is generally induced by oncogenic signals or release of activating E2F factors (E2F1-3). It binds an E3-ubiquitin ligase MDM2 and inhibits its activity. MDM2 inhibition increases the stability of p53, leading either to transcriptional activation of p53-target genes followed by apoptosis, or more frequently to cell cycle arrest in G1 and G2 phase. Apart from MDM2-p53-p21\textsuperscript{CIP1}‐mediated arrest, p14\textsuperscript{ARF} is also able to arrest cells independently of p53, by inhibiting the expression of a number of other transcription factors (Eymin et al., 2003; Sherr et al., 2005; Gil and Peters, 2006; Amente et al., 2007).

Expression of p16\textsuperscript{INK4A} and p14\textsuperscript{ARF} in tissues \textit{in vivo} is usually low (Gil and Peters, 2006); however, they have been shown to increase with age in most tissues in mice and in several organ systems in human (including skin, kidney tissues and lymphoid progenitors) (Signer et al., 2008; Romagosa et al., 2011). p16\textsuperscript{INK4A} has been proposed as a reliable marker of biological aging (i.e. aging at a cellular level) as opposed to the chronological age (i.e. age in years). However, p16\textsuperscript{INK4A} is not merely a marker of aged tissues, but it directly promotes aging by limiting tissue regeneration and repair. Repression of p16\textsuperscript{INK4A} and p14\textsuperscript{ARF} is crucial for stem cell self-renewal as well as for reprogramming of differentiated cells into pluripotent cells (Li et al., 2009). Both p16\textsuperscript{INK4A} and p14\textsuperscript{ARF} are mediators of senescence, a state of permanent proliferative arrest and a mechanism of tumour suppression (Gil and Peters, 2006).

Senescence is activated in primary untransformed cells due to telomere shortening after a certain number of cell divisions or prematurely as a tumour-suppressive mechanism in response to oxidative stress, DNA damage or aberrant mitotic (‘oncogenic’) signalling. In recent years, it has been shown that premature oncogene-induced senescence (OIS), alongside death cell programmes, prevents cells from undergoing oncogenic
transformation not only under the conditions of cell culture, but also in vivo. Senescent cells were found in human benign melanocytic nevi, precursors for melanoma, as well as in murine lung adenomas, T cell lymphomas and prostate tumours (Prieur and Peeper, 2008). Generally, OIS is found in pre-malignant or early stages of tumours and is absent in advanced tumours, suggesting that the tumour cells have developed mechanisms to bypass senescence (Larsson, 2011). Unlike apoptosis, senescence does not lead to tissue loss which can trigger compensatory proliferation in surrounding cells in an attempt to maintain tissue homeostasis (Fan and Bergmann, 2008). Senescent cells exit the cell cycle but stay in situ and metabolically active, influencing their neighbouring cells with active secretory programme. p16\textsuperscript{INK4A} is thought to play a major role in tumour suppression and senescence in humans, and some cells depend entirely on p16\textsuperscript{INK4A} for OIS. p19\textsuperscript{ARF} (the murine homologue of p14\textsuperscript{ARF}) mediates the OIS in mice; however, p14\textsuperscript{ARF} seems to be less important in humans (Ben-Porath and Weinberg, 2005; Gil and Peters, 2006).

\textit{p16\textsuperscript{INK4A}} is inactivated in nearly 50\% of all human cancers, including pancreatic carcinoma, carcinoma of head and neck, oesophagus, biliary tract, liver, lung and bladder, in colon and breast carcinomas; leukemia, lymphomas and glioblastomas (Romagosa et al., 2011). \textit{p16\textsuperscript{INK4A}} is inactivated predominantly by somatic homozygous deletion or \textit{p16\textsuperscript{INK4A}} promoter DNA methylation; \textit{p16\textsuperscript{INK4A}} inactivation by mutation is rare (Merlo et al., 1995; Li, Poi and Tsai, 2011). Germline mutations and deletions in \textit{p16\textsuperscript{INK4A}} are found in very rare familial cancer syndromes, mainly familial melanoma (Hussussian et al., 1994).

\textit{p16\textsuperscript{INK4A}} is found overexpressed in benign or pre-malignant lesions with functional \textit{p16\textsuperscript{INK4A}}-Rb pathway, where it demonstrates the protective effect of OIS. However, \textit{p16\textsuperscript{INK4A}} is also overexpressed in several fully malignant cancers and the detection of \textit{p16\textsuperscript{INK4A}} is used as a diagnostic biomarker in cervical, head and neck and perianal cancers (Romagosa et al., 2011). In human papilloma-virus (HPV)-related neoplasms, \textit{p16\textsuperscript{INK4A}} overexpression is the
consequence of Rb inactivation by HPV oncoprotein E7. In HPV-unrelated cancers, an inverse correlation between Rb loss and p16\(^{INK4A}\) expression levels is also common, and most likely in such cases p16\(^{INK4A}\) overexpression might also be attributed to the loss of negative Rb-p16\(^{INK4A}\) feedback loop (Lu et al., 2003; Little and Stewart, 2010).

In addition to negatively regulating the cell cycle, p16\(^{INK4A}\) was shown to block invasion, angiogenesis and promote apoptosis in several cancer models. Apart from its usual localization in the nucleus, p16\(^{INK4A}\) was also detected in the cytoplasm where it could engage in some of these additional functions (Evangelou et al., 2004; Romagosa et al., 2011).

Recently, the antisense non-coding RNA associated with the INK4A-ARF locus has been identified. The first exon of ANRIL is located in the promoter region of the p14\(^{ARF}\) gene and overlaps two exons of the p15\(^{INK4B}\) gene (Pasmant et al., 2007). At least 8 splice variants of ANRIL with tissue-specific expression were identified. In addition, distinct single nucleotide polymorphisms in the ANRIL locus seem to modulate its expression (Cunnington et al., 2010). Several common disease genome-wide association studies showed the co-regulation of p15\(^{INK4B}\), p16\(^{INK4A}\), p14\(^{ARF}\), and ANRIL gene expression in human tissues (Pasmant et al., 2011). Furthermore, deletion in a mouse region, homologous to the portion of human 9p21 that includes seven exons of ANRIL, resulted in a significantly reduced expression of both p16\(^{INK4A}\) and p15\(^{INK4B}\) in several organs and tissues through a cis-regulatory element (Visel et al., 2010). In contrast, in a prostate cancer model, an inverse correlation has been found between the ANRIL and p16\(^{INK4A}\) transcript abundance and ANRIL transcripts have been implicated in epigenetic repression of both p16\(^{INK4A}\) and p15\(^{INK4B}\) in cancers (Yap et al., 2010; Kotake et al., 2011).
G1-S checkpoint of the cell cycle

Recently, Coudreuse and Nurse (2010) demonstrated in fission yeast that the eukaryotic core cell cycle can be built on a circuit of two CDK activity thresholds; sequence and timing of which are imposed by a single CDK oscillator. In eukaryotic cells, CDKs are inactive without binding the cyclin partner and need to be phosphorylated at the key residue. Cyclins are the oscillators, synthesized and destroyed in temporal order throughout the cell cycle. However, the long-accepted model of specific subsets of CDKs and cyclins playing distinct roles in each phase of the cell cycle in mammalian cells has been increasingly challenged recently, e.g. when a functional cell cycle was observed in mice embryos that lack cdk2, cdk4 and cdk6 (Hochegger, Takeda and Hunt, 2008).

Cell cycle checkpoints are the control mechanisms that ensure the fidelity of cell division and warrant that alterations in the DNA molecule or incorrectly segregated chromosomes are not transmitted onto the daughter cell. Several switch-like checkpoints control the cell cycle: G1-S checkpoint is activated by either DNA damage or the DNA replication fork arrest, G2 checkpoint is activated if damaged DNA is detected and finally the mitosis checkpoint is activated by improperly connected kinetochores and spindle microtubules (de Bruin and Wittengerg, 2009; Malumbres and Barbacid, 2009).

G1-S checkpoint regulates the transition through the G1 phase and the entry into the S phase of the cell cycle. The G1-specific CDK-cyclin complexes are negatively regulated by the members of the INK4 or KIP/CIP families of cyclin dependent kinase inhibitors. Following antiproliferative or aberrant pro-proliferative ('oncogenic') signals, the formation of CDK4/6-cyclin D complexes is inhibited by the members of the INK4 family such as p16^{INK4A} or p15^{INK4B}, while the CDK2 complexes with cyclins E and A are disrupted by p21^{CIP1} or p27^{KIP1} (Fig. 1.4) (Knudsen and Knudsen, 2008).
Following mitogenic stimuli, the complexes of cyclin D with CDK4/CDK6 phosphorylate Rb. Rb phosphorylation is further augmented by the activity of CDK2 complexes with cyclins E and A. Rb phosphorylation disrupts its association with E2F transcription factors and enables E2F-mediated gene expression and S-phase progression. Anti-proliferative signals (or the inappropriate activation of certain oncogenes) induce p16\(^{INK4A}\) which disrupts the formation of cyclin D-CDK4/CDK6 complexes, prevents Rb phosphorylation and arrests the cells in the G1 phase of the cell cycle. Similarly, p21\(^{CIP1}\) and p27\(^{KIP1}\) inhibit the formation of cyclin E/A complexes with CDK2 (Knudsen and Knudsen, 2008).

**Pocket protein family, Rb-E2F1 pathway**

The pocket protein family consists of three members - Rb, p130 and p107 which control the progression from G1 to S phase and prevent unscheduled entry into the cell cycle. They all have a pocket domain containing the motif that recognises the LxCxEx amino acid sequence and mediates protein-protein interactions (Dahiya *et al.*, 2000). Pocket proteins exhibit some functional overlap but not complete redundancy; Rb is abundant in cells most of the time, whereas p107 is expressed generally in proliferating cells, and p130 in G0.
arrested cells. Rb is a master tumour supressor mutated in 30% of all human cancers, while mutations of p130 and p107 are rare (Classon and Dyson, 2001).

Rb and p107 play major roles in proliferating cells, while p130 is primarily active in G0 arrested cells. All three proteins are regulated during the progression of cell cycle by cyclic phosphorylation and dephosphorylation; however, specific members of pocket proteins preferentially interact with distinct groups of E2F transcription factors and specific pocket protein/E2F complexes may regulate different subsets of target genes (Du and Pogoriler, 2006).

Pocket proteins are hypophosphorylated in non-cycling cells or at early G1 when they bind members of E2F family proteins, mask the transactivation domain of E2Fs and inhibit the transcriptional activation of E2F-regulated genes. Pocket protein/E2F complexes actively repress transcription on promoters containing E2F-binding sites through the recruitment of SWI/SNF complexes, histone deacetylases and other chromatin modifying enzymes (Ferreira et al., 2001; Zhang and Dean, 2001; Macaluso, Montanari and Giordano, 2006). After the phosphorylation of pocket proteins by G1-specific cyclin-CDK complexes, pocket protein-E2F binding is disrupted, E2F transcription factors are released and initiate expression of genes essential for S phase entry, DNA replication and mitosis. In the period from anaphase to G1, pocket proteins are re-activated by protein phosphatase 1 (Cobrinik, 2005). They can also be activated during the active cell cycle by CDK inhibitor induction, down-regulation of cyclins or via protein phosphatase 2A (Cobrinik, 2005; Parisi, Bronson and Lees, 2009).

At least one component of the Rb pathway (that includes D-type cyclins, CDK4 and CDK6, Rb itself or CDK inhibitors of INK4 family such as p16\textsuperscript{INK4A} or p15\textsuperscript{INK4B} is functionally inactivated in the majority of cancers (Knudsen and Knudsen, 2008).
Genetic and epigenetic causes of cancer

Cancer is the disease of cell proliferation and differentiation. It is now thought to develop from a population of tumour-initiating cells that have accumulated multiple alterations in genes regulating normal cellular turn-over, growth and differentiation. Key regulatory genes become either aberrantly overactive (oncogenes) or underactive (tumour suppressor genes, TSG). Tumour-initiating cells gain stem-cell like properties, the ability to re-grow the tumour and resistance to conventional cancer therapies (Zhou et al., 2009). As the tumour develops in time, the cell's genetic make-up, growth properties and resistance diverge and the tumour becomes increasingly heterogeneous. In addition, the complexity of the tumour is increased with the mutual interaction between the transformed cells and their microenvironment or stroma consisting of epithelial cells, activated stromal cells and extracellular matrix (Tlsty and Coussens, 2005).

Somatic genetic changes in cancer comprise deletion or mutation of TSG or amplification and activating mutations of oncogenes, including chromosomal translocations. Loss of heterozygosity in the case of tumour suppressors occurs in a somatic cell where the first allele of a gene has been inherited inactivated by a germ-line deletion/mutation and the remaining functional allele becomes inactivated during the lifetime of an individual (Bishop, 1987).

Genetic causes have been in the centre of cancer research in the past. However, research findings of the recent decades have shown that activation of oncogenes or inactivation of TSG are caused by both genetic and epigenetic alterations (Lund and van Lohuizen, 2004; Esteller, 2007; Sharma, Kelly and Jones, 2010). 'Epigenetic' is a term used to describe modifications other than changes of primary DNA sequence that regulate gene expression, are heritable during mitosis and some might be in meiosis and do not require the continuous presence of the initiating signal. It is the inherited genome activity that does not depend on the naked DNA sequence (Esteller, 2011). Epigenetic regulation links the
genetic background and the environment (Brower, 2011) and provides dynamic variability above the static genome. Epigenetic processes play an essential role during development and tissue differentiation, and are frequently deregulated in non-Mendelian and complex diseases, including cancer. Epigenetic information is stored in multiple dimensions, comprising DNA, RNA and protein and is mediated by DNA methylation, post-translational histone modifications, nucleosome remodelling and various non-coding RNA species (Chahwan, 2011).

The term DNA methylation is used for the process of methylation of cytosines that precede guanines in CpG dinucleotides of the genome to yield 5-methylcytosine. CpGs in the genome are asymmetrically distributed; areas with a high frequency of CpG sites, termed CpG islands, are characteristic for gene promoters. In normal cells, methylation of CpG dinucleotides mediates the X-chromosome inactivation in females as well as silencing of repetitive genomic regions and parasitic elements such as endogenous retroviruses. Most CpG islands are generally unmethylated in normal cells (Robertson, 2005). The transmission of DNA methylation in mitosis is well understood. It proceeds as a templated copy event; the methylation pattern of the original chromatid is precisely copied by DNA methyltransferase DNMT1 into the newly synthesised DNA strand (Hervouet, Vallette and Cartron, 2010). However, the process of active demethylation is still controversial (Klug et al., 2010). Additional chemical modifications of DNA, such as 5-hydroxymethylcytosine and 5-carboxylycytosine, were found in the past years and their interplay with methylcytosine and function in health and disease is currently being investigated (Ficz et al., 2011; He et al., 2011).

Epigenetic modifications include, in addition to the chemical modifications of the DNA itself, the modification of the DNA-associated proteins - histones. In the nucleus, DNA must be heavily condensed and packaged into a tight structure. The basic packaging unit is formed by a nucleosome in which 147 base pairs of DNA are wrapped around an octamer of core histone proteins, H3, H4, H2A and H2B. About 75-90% of DNA is wrapped in nucleosomes
and the remaining unwrapped DNA serves as a linker DNA and is associated with the H1 linker histone (Segal and Widom, 2009). When visualized by electron microscopy, this assembly appears as beads on string - ‘beads’ being the individual nucleosomes and the ‘string’ being the linker DNA (Füllgrabe, Kavanagh and Joseph, 2011).

N-terminal histone tails are decorated by a variety of posttranslational covalent modifications including acetylation, methylation, phosphorylation, ubiquitylation, glycosylation, ADP-ribosylation, carbonylation and SUMOylation (Strahl and Allis, 2000). The functional outcome of different modifications depends on the modified residue (e.g. K27, K4) and the degree of modification (mono-, di-, etc.). Histone modifications work in concert rather than in isolation (Zhang and Reinberg, 2001; Perissi et al., 2010). The histone code hypothesis suggests that transcriptional outcome depends on the combination of various histone modifications and that distinct combinations of histone modifications determine specific chromatin-related functions and processes (Jenuwein and Allis, 2001).

Research from recent years has clearly shown that histone modifications do not form a static landscape, but on the contrary, are regulated in a dynamic, reversible fashion. Gene transcription, DNA replication and repair all involve disruption and reassembly of the chromatin structure (Ho and Crabtree, 2010). The dynamic nature of chromatin is ensured by the families of antagonistic enzymes that add and remove histone modifications, so that the ‘writers’ are opposed by ‘erasers’ (e.g. methyltransferases/demethylases, acetyltransferases/deacetylases). Histone modifications impact on the electrostatic charge of the histone resulting in changes to the chromatin structure or histone binding to DNA. Histone acetylation and methylation play a major role in determining the degree of chromatin condensation and the access of transcriptional machinery, and perhaps other proteins, to DNA (Suganuma and Workman, 2011). Furthermore, modified histones provide a selective binding platform for regulatory proteins. Many regulatory proteins evolved conserved domains, such as bromodomains or chromodomains, which bind
specific histone modifications and thus 'read' the histone code and mediate the transcriptional repression or activation (Füllgrabe, Kavanagh and Joseph, 2011). Apart from modification of the core histones, another level of epigenetic regulation is provided by histone variants with altered primary sequence and unique functions, e.g. H2AZ or H2AX (Khare et al., 2012).

Non-coding RNAs involved in epigenetic regulation include a variety of species, such as microRNA (that will be discussed in detail later), short ncRNA (<200bp) and long ncRNAs (>200bp). Long mammalian ncRNAs, such as Xist, HOTAIR and Kcnq1ot1, modify chromatin structure by several mechanisms; they serve as a scaffold for chromatin remodelling enzymes or target the chromatin remodelers to the gene promoters or regulatory regions by carrying a sequence-specific information (Gupta et al., 2010; Kaikkonen, Lam and Glass; 2011). Short ncRNA also emerged as mediators of gene repression and heterochromatization. A group of 50-200nt short RNAs transcribed from the 5' end of polycomb target genes interact with polycomb repression complex (PRC) 2 through a stem-loop structure and cause gene repression in cis. These short RNAs need to be depleted from polycomb target genes to allow activation during cell differentiation (Kanhere et al., 2010).

Nucleosome remodelling refers to the ability of nucleosomes to alter their position at promoters. Depending on the exact localization on DNA, nucleosomes act as both general activators and repressors of gene expression (Segal and Widom, 2009). Nucleosomes are further arranged by long-range interactions into a higher order three-dimensional chromatin structure, the organization and functions of which are not yet well defined. In addition, entire chromosomes are arranged non-randomly in the nucleus and there seems to be a relationship between the organization of the nuclear space and gene transcription (Cremer and Cremer, 2010; Wang et al., 2011a).
The combinations of various modifications of specific residues at the histone tails and DNA methylation followed by altered nucleosome positioning govern the resulting transcriptional status of a gene (Kia et al., 2008; Cedar and Bergman, 2009). Initially, the term ‘epigenetic’ was frequently interpreted as permanent. Recently, it has been appreciated that epigenetic configuration once established (at any level mentioned above) is not being fixed but can respond to both extrinsic and intrinsic signals.

Epigenetic deregulation contributes to the onset and progression of cancer and seems to be as significant as genetic mutations. All the layers of epigenetic regulation are distorted in cancers. Epigenetic regulators are often inactivated by somatic genetic mutations resulting in alteration of the expression of multiple genes simultaneously (Tuma, 2010).

Malignant tissues exhibit global DNA hypomethylation leading to an increase in genomic instability. However, the CpG islands within promoter regions of specific sets of genes, in normal tissues constitutively unmodified, are frequently hypermethylated in cancer (Esteller, 2011). Tumour suppressor genes such as hMLH1, BRCA1, VHL, Rb, p16INK4A, p14ARF, and p15INK4B are more frequently inactivated by such anomalous promoter methylation than by mutation. Increased methylation in cancer also affects the regions with lower CpG density up to 2000 base pairs distant from CpG islands, termed CpG island shores (Irizarry et al., 2009). DNA methyltransferases DNMT3A and DNMT3B, mediating ‘de novo’ DNA methylation, are frequently differentially expressed or mutated in cancers.

The aberrations of histone modifications in cancer occur either locally at promoters by improper targeting of histone-modifying activities or globally at the level of the whole nuclei. Global deacetylation and methylation of specific residues at histones H3 and H4 is a hallmark of cancer and includes global loss of acetylation of histone H4 at lysine 16 (H4K16ac), hypoacetylation of histone H4 lysine 12 (H4K12ac), loss of H4K20me3, and H3K18ac (Ellis, Atadja and Johnstone, 2009; Niller, Wolf and Minarovits, 2009; Füllgrabe, Kavanagh and Joseph, 2011). Global changes of histone modifications are a result of
deregulated epigenetic master regulators, such as amplification or mutation in deacetylases or EZH2 (Esteller, 2011). The consequences of altered histone modifications include genomic instability and aberrant gene expression, i.e. overexpression of oncogenes and loss of TSG expression, which result in the incompetence of the cell cycle checkpoints and DNA repair (Füllgrabe, Kavanagh and Joseph, 2011).

A strong link has been identified between the characteristics of the embryonic stem cells and the profile of epigenetic deregulation arising in cancer. Genes encoding tumour suppressor proteins and microRNAs that become aberrantly methylated and repressed in cancers seem to be pre-marked by the bivalent domains (see below) in embryonic stem cells. Cancer cells, or the subpopulation of tumour-initiating cells, by mimicking the embryonic gene expression programme, retain inappropriate self-renewal capacity and multi-lineage potential (Iliou et al., 2011).

**Epigenetic regulation of p16\(^{INK4A}\) locus**

The p16\(^{INK4A}\) promoter region is epigenetically regulated by an interplay of repressive modification - trimethylation of lysine 27 at histone H3 (H3K27me3) and activating trimethylation of lysine 4 at histone H3 (H3K4me3) in human stem cells (Li et al., 2009), primary fibroblasts (Barradas, et al., 2009) and cancer cells (Kia et al., 2008) alike. H3K27me3 and H3K4me3 modifications co-localize at the p16\(^{INK4A}\) promoter and form a bivalent chromatin domain. Bivalent domains carry the potential of the alternate transcriptional status. Such domains are thought to silence certain genes while keeping them poised for rapid activation (Bernstein et al., 2006; Krivtsov and Armstrong, 2007). In differentiated cells, activated genes lose H3K27me3; while gene sets which encode functions that are irrelevant for a particular cell identity retain H3K27me3 and remain repressed.
Polycomb group proteins (PcG), that establish the H3K27me3 mark, control the expression of hundreds of genes with roles in differentiation, development and cell proliferation. In vertebrates, PcG assemble into two discrete complexes. The minimal subunit of the polycomb repression complex 2 (PRC2) consists of EED, SUZ12, RbAp46/48 and histone methyltransferase EZH2 (or EZH1 in certain cell types) which methylates H3K27 (Simon and Kingston, 2009). H3K27me3 is a very stable and abundant histone modification that results from monomethylation of H3K27me2. With exception of a single viral protein (*Paramecium bursaria Chlorella Virus 1* methyltransferase), PRC2 containing either EZH2 or EZH1 is the only complex found so far that both di- and tri-methylates H3K27. It is likely that mono-methylation of H3K27 is catalyzed by a complex different from PcG (Margueron and Reinberg, 2011).

Although the core components EZH1/2, SUZ12, EED and RbAp46/48 are conserved across species, several other components of PRC2 in mammals have been identified recently, including JARID2, AEBP2 and PCL (Nekrasov et al., 2007; Kim, Kang and Kim, 2009; Li et al., 2010). These additional components do not seem to be strictly required for PRC2 enzymatic activity *in vitro* but are necessary for optimum PRC2 activity *in vivo*. Another polycomb repressive complex PRC1 includes BMI1, RING1A/B, and CBX family proteins (CBX2, CBX4, CBX6, CBX7, and CBX8) (Vincenz and Kerppola, 2008; Schuettengruber et al., 2009). It has been generally accepted that PRC1 is recruited by methylated H3K27 and acts downstream of PRC2 as a direct executor of silencing. The chromodomains of CBX family proteins can specifically recognize H3 trimethylated on K27, while the PRC1 ubiquitin ligases, RING1A and RING1B, monoubiquitylate Lys 119 of histone H2A (H2AK119ub). H2AK119ub restraints poised RNA Polymerase II (Pol II) at bivalent promoters and inhibits transcriptional elongation (Sugamuna and Workman, 2008; Tiwari et al., 2008; Simon and Kingston, 2009).

However, recently, the redundancy of PRC1 and PRC2 in gene repression was described and it was suggested that both complexes might act in parallel (Leeb et al., 2010). Both,
genes targeted by PRC2 that lack H2AK119ub, and genes targeted by PRC1 in the absence of PRC2, were found (Schoeftner et al., 2006; Ku et al., 2008; Margueron and Reinberg, 2011). PRC2 with the H3K27me3 mark and PRC1 complexes separately have the ability to compact the chromatin structure and silence the target genes (Eskeland et al., 2010).

Targeting of PRC complexes to the mammalian gene promoters is not yet fully understood. In Drosophila, PRC dock at the composite DNA elements termed polycomb response elements; however, similar DNA motifs in mammals involved in the global targeting of polycomb were not identified. Recent studies point to the role of sequence-specific TFs and/or non-coding RNA, acting either in trans or in cis (Bracken and Helin, 2009). The transcript antisense to INK4/ARF - ANRIL - has been implicated in direct epigenetic repression of $p16^{\text{INK4A}}$. ANRIL was shown to bind to PRC1 component CBX7 and mediate the recruitment of the polycomb proteins to the $CDKN2A$ locus (Yap et al., 2010).

The repression of $p16^{\text{INK4A}}$ is mediated by PcG in both humans and mice. The $p19^{\text{ARF}}$ (the mouse homologue of $p14^{\text{ARF}}$) is repressed by PcG in mice; however it is unclear whether PcG play equally important roles in $p14^{\text{ARF}}$ repression in human cells (Kotake et al., 2007; Barradas et al., 2009).

Trithorax group proteins were identified in Drosophila as antagonists of the PcG silencers. Trithorax group proteins function in transcriptional activation and form complexes similar to PcG but less well defined. H3K4 methylation is established by the SET1 and MLL family of histone methyltransferases (Ruthenburg et al., 2007). MLL1, MLL2, MLL3, MLL4, MLL5, Set1A and Set1B each contains a SET domain mediating H3K4-specific methyltransferase activity; however, their mechanism of action, target genes and the distinct functions of different MLLs are still unclear (Ansari and Mandal, 2010). MLL complexes catalyze mono-, di- and trimethylation of H3K4 and each of these modifications has distinct functions (Cosgrove and Patel, 2010).
During the induction of $p16^{\text{INK4A}}$, whether oncogene-induced or senescence-associated, PRC1 and PRC2 complexes are removed from the $p16^{\text{INK4A}}$ locus and the H3K27me3 mark at the $p16^{\text{INK4A}}$ promoter decreases. This is in some cases accompanied by the recruitment of trithorax MLL1 activators and the concomitant increase in the H3K4me3 mark (Agherbi et al., 2009; Kotake, Zeng and Xiong, 2009). For example, in the rhabdomyosarcoma cell line lacking functional SWI/SNF chromatin remodelling complexes, the reconstitution of SWI/SNF function leads to eviction of PcG silencers and loss of H3K27 trimethylation, simultaneously with MLL1 recruitment and H3K4 trimethylation, resulting in $p16^{\text{INK4A}}$ induction (Kia et al., 2008). The H3K27me3 demethylase JMJD3/KDM6B contributes to plasticity of the $p16^{\text{INK4A}}$ locus, and functions in a switch from the repressive chromatin in response to oncogenic stress (Agger et al., 2009; Barradas et al., 2009). Functional Rb, which negatively regulates $p16^{\text{INK4A}}$ expression, may collaborate with PcG on $p16^{\text{INK4A}}$ repression via H3K27me3 (Kotake et al., 2007).

Additionally, it has been demonstrated that overexpression of an essential regulator of DNA replication, CDC6, represses the entire CDKN2A locus (together with the neighbouring CDK inhibitor $p15^{\text{INK4B}}$) through recruitment of histone deacetylases and H3K9 methylation (Gil and Peters, 2006; Borlado and Mendez, 2008).

In a variety of cancers, including lung, colon and prostate carcinomas as well as lymphomas, $p16^{\text{INK4A}}$ promoter undergoes DNA methylation; $p16^{\text{INK4A}}$ is one of the most frequently aberrantly methylated genes in cancer (Das and Singal, 2004).
C-terminal binding protein (CtBP) family

The CtBP family in vertebrates consists of two proteins CtBP1 and CtBP2 which are largely homologous (henceforth referred to as CtBP). CtBP major splice variants function predominantly as transcriptional co-repressors in the nucleus, while the minor splice isoforms perform various cytosolic functions. A range of DNA binding TFs recruit CtBP through a PLDLS motif to which CtBP binds; CtBP forms dimers to bridge the proteins containing PLDLS motifs (Chinnadurai, 2009).

CtBP is found in the supercomplexes consisting of enzymes mediating co-ordinated epigenetic modifications that are targeted by CtBP-binding sequence-specific TFs to the promoters of target genes (Fig. 1.5) (Shi et al., 2003; Kuppuswamy et al., 2008). CtBP recruits chromatin remodelers including histone deacetylases (HDAC1/2), the lysine-specific demethylase (LSD1) and histone methyl transferases (G9a and GLP). In addition, CtBP nuclear complexes contain two SUMO E3 ligases, HPC2 and PIAS1, and also certain other co-repressors such as Co-REST (co-repressor for element-1-silencing transcription factor) that might link the enzymatic constituents to CtBP (Kuppuswamy et al., 2008). Apart from the gene-specific repression, CtBP have been indicated in global repression by antagonizing the action of transcriptional co-activator p300 and the associated histone acetyltransferases (Chinnadurai, 2007). CtBP has also been implicated in the PcG-mediated transcriptional repression. Although the requirement for CtBP in repression of PcG target genes is currently well-defined in Drosophila rather than vertebrates, CtBP might also recruit human PcG through PRC2 subunit HPC2 (Shi et al., 2003). Furthermore, binding of transcription factor YY1 to DNA and the subsequent PcG-mediated silencing of some genes in mammals is dependent on CtBP (Sewalt et al., 1999; Atchison et al., 2003; Basu and Atchison, 2010).
Fig. 1.5. CtBP supercomplex (reproduced from Kuppuswamy et al., 2008). The CtBP dimer can interact with chromatin-anchoring transcriptional repressors through a PLDLS-dependent interaction. CtBP can recruit a variety of chromatin-remodelling enzymes such as HDAC1/2, CoREST/LSD1 complex, G9a/GLP complex, Ubc9 and E3 ligases (HPC2 and PIAS1).

LSD1/KDM1

LSD1 (also known as KDM1) is an enzyme which, by an FAD-dependent oxidative reaction, demethylates mono- and di-methylated lysines, specifically histone 3 lysines 4 and 9 (H3K4 and H3K9). It is found in the repressor complexes containing Co-REST and NuRD (nucleosome remodelling and histone deacetylation) (Forneris et al., 2007; Wang et al., 2009b). LSD1 function in the demethylation of H3K4me1/me2 mediates gene repression, while demethylation of H3K9me1/me2 results in gene activation (Metzger et al., 2005; Wang et al., 2007a). LSD1 mediates the epithelial-to-mesenchymal transition
characterized by reprogramming of specific promoters across the genome (McDonald et al., 2011). It regulates the critical balance between H3K4 and H3K27 methylation and through the regulation of bivalent domains maintains pluripotency (Adamo et al., 2011). LSD1/Co-REST and PRC2 members are co-recruited to promoter regions of HOX genes by long intergenic non-coding (linc) RNA HOTAIR and such synchronized targeting of these chromatin remodelers by lincRNAs might serve as a general mechanism in the maintenance of bivalency (Tsai et al., 2010).

LSD1 also demethylates non-histone templates such as DNMT1 and E2F1 (Wang et al., 2009a; Kontaki and Talianidis, 2010). The LSD1/Co-REST complex serves as an HIV Tat K51-specific demethylase and is required for the activation of HIV transcription in latently infected T cells (Sakane et al., 2011).

**Cellular microRNAs in cancer**

In humans, 721 miRs have been identified so far (Philippidou et al., 2010). MiRs regulate the expression of hundreds of genes through sequence-specific binding to mRNA and subsequent inhibition of translation and/or degradation of target mRNAs. Mature miRs are between 19 and 22 nucleotides in length and their production is regulating by transcription, posttranscriptional processing and export. Nucleotides 2–7 of the mature miR sequence create the 'seed region' which specifies the specific mRNA to which the miR will bind. The binding between the miR’s seed region and the target mRNA is mostly in perfect base pair complementarity, this does not always apply for the miR's flanking regions. A gene can harbour binding sites for several miRs and a single miR can regulate as many as 200 genes. The targets of one miR can have diverse functions. The miRs usually cause quantitatively modest changes in the target protein expression. They generally fine-tune the
expression of a group of genes rather than robustly modulating a single target (Nelson et al., 2003; Didiano and Hobert, 2006; Croce, 2009; Janga and Vallabhaneni, 2011).

MiRs were discovered initially as developmental regulators; therefore it is not surprising that they were recognised to play a major role in tumour development. In cancers, miRs are globally downregulated and the miR processing machinery (e.g. DICER) is frequently mutated (Esteller, 2011). Simultaneously, specific subsets of microRNAs with either oncogene or tumour suppressor functions are deregulated in cancers (Lu et al., 2005; van Kouwenhove, Kedde and Agami, 2011).

**MicroRNA 143 and 145 (miR-143/145)**

Genomic loci of both miR-143/145 are co-located within 1.3 kb at chromosome 5q32. MiR-143/145 originate from non-coding transcripts approximately 11, 7.5, and 5.5 kb-long termed NCR143/145 (Non-coding RNA encoding miR-143/145). The expression of NCR143/145 is coordinated with that of resident miRs in normal and cancer tissues (Iio et al., 2010). Apart from both miRs being produced as a bicistronic unit from the long transcripts, miR-145 can be also generated independently from a shorter 1.9-kb transcript (Iio et al., 2010). MiR-143/145 co-operatively promote differentiation and repress proliferation of smooth muscle cells; miR-145 alone can induce differentiation of multipotent neural crest stem cells into vascular smooth muscle (Cordes et al., 2009). MiR-143/145 are both up-regulated approximately 3.5-fold during senescence in human foreskin fibroblasts and transfected synthetic mimic of miR-143 inhibits the proliferation of fibroblasts in a dose-dependent manner (Bonifacio and Jarstfer, 2010).

MiR-143/145 and their precursors NCR143/145 are down-regulated in a variety of cancers, including B cell malignancies (Iio et al., 2010). The target genes of miR-143 include DNMT3A and KRAS in colorectal cancer (Ng et al., 2009; Chen et al., 2009) and ERK5 in
prostate cancer (Clape et al., 2009). MiR-145 targets consist of EGFR and NUDT1 in lung adenocarcinoma (Cho, Chow and Au, 2011), YES and STAT1 in colon cancer (Gregersen et al., 2010) and BNIP3 in prostate cancer (Chen et al., 2010a).

Not much is known about the regulation of miR-143/145 locus. Activation of Notch receptors by Jag-1 increases promoter miR-143/145 activity, and this is dependent on intact RBPJk/CFB1 consensus sites within the promoter in vascular smooth muscle cells (Boucher et al., 2011). In pancreatic cancers, miR-143/145 is repressed by oncogenic Ras and this requires the Ras-responsive element-binding protein (RREB1) (Kent et al., 2010).

**MiR-221 and miR-138**

MiR-221 (together with miR-222) are encoded on chromosome X and induce cell growth and cell cycle progression via direct targeting of p27^KIP1 and p57^KIP2 in various human malignancies (Gillies and Lorimer, 2007; Galardi et al., 2007; Chun-zhi et al., 2010; Pineau et al., 2010). MiR-221/222 inhibit cell apoptosis by targeting the product of the pro-apoptotic gene PUMA and enhance cell migration by down-regulating protein tyrosine phosphatase μ in human glioma cells (Zhang et al., 2010; Quintavalle et al., 2011). MiR-221/222 decrease expression of epithelial-specific genes and increase expression of mesenchymal-specific genes, promoting the epithelial-to-mesenchymal transition in breast cancer (Stinson et al., 2011). MiR-221 is strongly down-regulated upon differentiation and maturation of skeletal muscle cells (Cardinali et al., 2009). NFkB and c-Jun induce miR-221 (and miR-222) expression in glioblastoma and prostate cancer (Galardi et al., 2011) and the inhibition of EGFR (epidermal growth factor receptor) or MEK (mitogen-activated or extracellular signal–regulated protein kinase kinase) downregulates miR-221 expression in breast cancer (Stinson et al., 2011).
As opposed to the pro-proliferative miR-221, miR-138 originating from chromosome 3, seems to possess tumour-suppressive capabilities. Down-regulation of miR-138 has been observed in various cancers, including head and neck squamous cell carcinomas. Expression of miR-138 suppresses cell invasion and leads to cell cycle arrest and apoptosis (Liu et al., 2009). MiR-138 targets the histone H2AX 3’-UTR, reducing histone H2AX expression and inducing chromosomal instability following DNA damage (Wang et al., 2011b).

**Tumour viruses and epigenetic regulation**

Viruses annually account for over 1.3 million cancer cases worldwide (Hoppe-Seyler and Hoppe-Seyler, 2011). Human hepatitis B and C viruses are associated with 80% of hepatocellular carcinomas, HPV is present in >95% of cervical carcinomas, EBV is associated with 30% of HL, 95% of endemic BL, 15% of sporadic BL, >95% of NPC and 60%–80% PTLD, including 100% of early-onset PTLD patients (Chan et al., 1995; Butel, 2000; Liao, 2006; Carbone, Gloghini and Dotti, 2008; Cohen et al., 2011). These viruses contribute to the cancer development by insertional mutagenesis and/or by oncoproteins that disrupt the cell cycle and apoptosis regulating pathways. However, generally virus alone is not sufficient for carcinogenesis and additional co-factors are required (Liao, 2006; Fernandez and Esteller, 2010).

Tumour viruses often infect cells that are fully differentiated and have left the cell cycle. Non-cycling, resting cells need to be induced to proliferate to support viral replication and further host to host spread (Ferrari, Berk and Kordistanti, 2009). The development of cancer is often the by-product of such virally-induced host cell cycle deregulation. Oncogenic viruses typically target the same pathways that are altered in many cancers of non-infectious aetiology. The common feature is inactivation of p53 and Rb pathway (Cann,
2011). However, oncogenesis is not only the by-product of S phase initiation in non-dividing cells, but also of the viral long-time persistence strategy (e.g. modifying the genes involved in apoptosis and senescence) and frequently develops only after decades of viral persistence (Butel, 2000; Liao, 2006; Fernandez and Esteller, 2010).

During viral latency, shut-down of the majority of viral protein expression, for the reasons of long-term immune evasion, typically occurs and is regulated by epigenetic machinery usually involving both viral and host factors (Günther and Grundhoff, 2010; Tempera, et al., 2010). Moreover, viruses can modify the epigenome of the host cell on all levels including DNA methylation, histone modification and microRNA expression.

Paramecium bursaria Chlorella virus 1, which infects algae, encodes its own viral histone methyltransferase, which methylates histone H3K27 and represses certain host genes (Mujtaba et al., 2008). Although this is the only viral histone lysine methyltransferase identified to date, multiple viruses have been shown to usurp or regulate parts of the cellular epigenetic machinery.

EBV, KSHV, HBV and HPVs all express viral oncoproteins which target cellular key epigenetic regulators, such as DNMTs and histone-modifying enzymes (Hoppe-Seyler and Hoppe-Seyler, 2011). The most reported virally-induced epigenetic modification is DNA methylation of cellular TSG, such as E-cadherin, p16INK4A and p73, through induction of DNMTs (Paschos and Allday, 2010).

Tumour viruses modify histones both globally and at specific promoters. Global reduction (~70%) and redistribution of H3K18ac occurs during transformation of primary human fibroblasts with adenoviral small E1A (Horwitz et al., 2008; Ferrari et al., 2008). EBV LMP1 induces increased JMJD3/KDM6B expression in germinal-centre B cells and can in consequence modulate H3K27me3 pattern genome-wide (Anderton et al., 2011). Similarly, human papillomavirus type 16 E6/E7 cause alterations in EZH2, BMI1 and H3K27–specific
demethylase UTX/KDM6A (Hyland et al., 2011). In an independent study, human papillomavirus E7 was found to epigenetically reprogramme cells through induction of both UTX/KDM6A and JMJD3/KDM6B histone demethylase expression (McLaughlin-Drubin, Crumb and Münger, 2011). Published examples of targeted silencing of specific tumour suppressors by repressive chromatin include repression of the BIM promoter by EBV (Paschos et al., 2009) or TGFβRII promoter by KSHV (Di Bartolo et al., 2008).

Lastly, tumour viruses not only use their virally-encoded microRNA in tumorigenesis; they also modulate the expression of cellular microRNAs (Pfeffer and Voinet, 2006; Seto et al., 2010; Dreher et al., 2011; Gottwein et al., 2011).

Viral proteins and senescence

Genome-wide analysis of genes upregulated in cancer showed that nearly 50% of them were downregulated upon senescence (Rovillain et al., 2011). This suggests that overcoming senescence is a crucial step in malignant transformation and nearly half of the genes upregulated in cancer are related to it (Rovillain et al., 2011). Overcoming the mechanisms of senescence induction is a common strategy for tumour viruses.

Hepatitis B virus X protein overcomes stress-induced premature senescence by repressing p16INK4A expression via promoter DNA methylation (Oishi et al., 2007; Kim et al., 2010). When ectopically expressed in rat embryonic fibroblasts and human epithelial-like osteosarcoma cell line, EBV LMP1 is capable of suppressing p16INK4A promoter in a promoter reporter assay. Additionally, LMP1 prevents primary mouse embryonic fibroblasts from entering into replicative senescence in vitro and prevents Ras-induced premature senescence in rat embryonic and human diploid fibroblasts (Yang et al., 2000). In human epithelial cells, LMP1 promotes nuclear export of ETS2, thereby reducing the level of
p16\(^{\text{INK4A}}\) gene expression and simultaneously promotes nuclear export of E2F4 and E2F5, the essential downstream mediators for a p16\(^{\text{INK4A}}\)-induced cell cycle arrest (Ohtani \textit{et al.}, 2003). HTLV-1 Tax can directly bind and inhibit p16\(^{\text{INK4A}}\) (Low \textit{et al.}, 1997). Furthermore, p16\(^{\text{INK4A}}\) is lost in KSHV-associated primary effusion lymphoma (Platt, Carbone and Mittnacht, 2002) and KSHV-cyclin activates CDK6, alters its substrate specificity, and renders CDK6 insensitive to inhibition by p16\(^{\text{INK4A}}\) (Swanton \textit{et al.}, 1997; Kaldis \textit{et al.}, 2001).

In addition to blocking the p16\(^{\text{INK4A}}\) function, viruses overcome the induction of senescence directly by inhibiting p21\(^{\text{CIP1}}\) (Park \textit{et al.}, 2011) or indirectly by the modulation of Rb and p53-pathways by a variety of mechanisms (Ferrari, Berk and Kurdistani, 2009).

**Systems for the study of EBV pathogenesis**

EBV-infected B cells are rare in healthy virus carriers (1–50 per million B cells) and are very difficult to analyse directly (Kuppers, 2003; Thorley-Lawson and Gross, 2004). B cell-derived EBV-carrying cell lines are the most common model used to study EBV. Burkitt lymphoma (BL) cells and LCL differ phenotypically. BL are small and round, similar to non-activated resting B cells while LCL are large and irregular and resemble antigen-activated lymphoblasts (Nilsson and Ponten, 1975; Klein, Kis and Klein, 2007). LCL always express latency III programme. Although BL lines that maintain the type I expression have been established, in culture BL typically drift to latency III programme (Gregory, Rowe and Rickinson, 1990).

One of the greatest obstacles in the study of EBV-related pathogenesis is the lack of animal models. γHV68 virus has been used as a surrogate for EBV and KSHV in the infection of mice; it leads to a robust acute infection in the lung and a long-term latent infection in the spleen (Dong \textit{et al.}, 2010). Furthermore, EBV-initiated tumour lines can be
introduced into severe combined immunodeficiency (SCID) mice allowing the study of in vivo functions of EBV (Kaul et al., 2007). More recently, immunodeficient mice have been reconstituted with components of the human immune system to produce humanized mice (Strowig et al., 2011) and these can be infected with recombinant EBV in order to analyse viral genes function (White et al., under revision).

Rhesus macaque LCV contains a repertoire of genes similar to that of EBV, and experimental infection of naive rhesus macaques with rhesus macaque LCV accurately reproduces some aspects of lytic and latent EBV infection. Such non-human primate models could be instrumental in gaining insight into the mechanisms of immune evasion by LCV and by extension EBV (Moghaddam et al., 1997). However the macaque immune system is poorly characterized and there are financial and ethical constrains on developing such models.

EBV viruses cannot be easily produced due to the lack of permissive cell lines. Therefore in order to establish genotype-phenotype connection and study viral ORFs, a herpesviral genome was cloned into the bacterial artificial chromosome (BAC). This allows manipulation of the large virus genome as a plasmid in E. coli. Genomes can be shuttled between E.coli and eukaryotic cells and the viruses produced after transfection of EBV-BACs into 293 epithelial producer cell lines (Delecluse et al., 1998). EBV-BAC is an essential tool to genetically modify gammaherpesvirus genomes and to create the deletion mutants. Revertant viruses are established alongside the deletion mutants to prevent the bias caused by any effects arising from second site mutations. Revertants also allow distinguishing whether the observed biology results from the interruption of a protein sequence or whether it is caused by an alteration to a cis-element. A more physiological study of viral proteins and RNA is carried out in the context of the entire virus rather than expression of isolated EBV components (WT or mutated), since frequently viral proteins and RNA species work in concert and are inter-dependent. Lastly, using EBV-BACs, rather than over-expression systems, leads to physiological expression levels of all viral
components in the infected BL or LCL (Kanda et al., 2004; Delecluse et al., 2008; Feederle, Bartlett and Delecluse, 2010).

The 4-hydroxytamoxifen-inducible fusion protein system

In contrast to the systems based on transcriptional induction, fusions with hormone-binding domains of steroid receptors allow posttranslational functional control of a chimerical protein depending on the presence of a small molecule ligand (Guo et al., 2008).

Posttranslationally inducible fusion proteins were traditionally produced by fusion with the hormone-binding domain of the oestrogen receptor. The caveats associated with this system were the need for the removal of phenol red and steroid hormones from culture medium, unsuitability for in vivo usage due to high levels of circulating steroid hormones and the remaining inherent transactivation activity of the hormone-binding domain (Littlewood et al., 1995). The system was advanced by the introduction of a point mutation into the murine oestrogen receptor that renders it unable to bind oestrogen. This modified receptor is responsive to the synthetic oestrogen antagonist 4-hydroxytamoxifen, and the hormone-binding domain is completely transcriptionally inactive.

As documented on numerous fusion proteins such as c-myc ER\textsuperscript{TAM}, in the presence of the ligand, the fusion protein is transcriptionally active in the nucleus. After ligand removal, the fusion protein translocates from the nucleus to the cytoplasm where it is captured in inhibitory complexes with cytoplasmic proteins, especially heat shock proteins, and inactivated. In some cases, inactive complexes might be targeted for proteasomal degradation. So in addition to inactivation, the quantity of fusion protein in cell might decrease in the absence of the ligand (Sipo et al., 2006; Chu et al., 2008; Stevanato et al., 2009).
Project background – starting point

More than a decade ago EBNA3C was shown to act in a similar manner to E7 of papillomaviruses by enabling G1 to S progression through inactivation of the Rb axis; however, the mechanism was not determined (Parker et al., 1996). Recently, in our lab a conditional EBNA3C was constructed by fusing the C-terminus of EBNA3C with 4-hydroxytamoxifen (HT)-dependent mutant murine oestrogen receptor. This gene was then engineered into EBV strain B95.8 using a BAC system and the resulting virus was used to produce LCL (LCL 3CHT) from primary human B cells (Melanie Franz and Michaela Ruhmann, personal communication). Previously, Maruo et al. (2003) showed using a similar system based on the Akata strain of virus that after withdrawal of HT from the medium, EBNA3C gradually disappears, LCL arrest in the G1 phase of the cell cycle and exhibit elevated levels of apoptosis. The G1 arrest is accompanied by the accumulation of p16\textsuperscript{\text{INK4A}} and decrease in the hyperphosphorylated form of Rb.

In analogous conditional EBNA3A system, EBNA3A inactivation leads to LCL growth arrest and cell death (Maruo et al., 2006). Consistent with this finding, a recent study using recombinant EBV viruses lacking EBNA3A and microarray technology showed that although EBNA3A knock-out (EBNA3A KO) LCL can be established, the absence of EBNA3A (similarly to EBNA3C) leads to reduced proliferation and increased apoptosis. EBNA3A KO LCL fail to suppress p16\textsuperscript{\text{INK4A}} and display decreased levels of Rb mRNA (Hertle et al., 2009).

Microarray data produced in our lab have revealed that EBNA3A and EBNA3C co-operate in the regulation of subset of cellular genes (White et al., 2010). Furthermore, the physical association of EBNA3A and EBNA3C proteins was firmly established. The EBNA3s can be co-precipitated with each other from lysates of EBV-infected B cells. The presence of all three EBNA3s is not necessary for successful co-immunoprecipitations, neither are other EBV proteins. The interaction of EBNA3C with EBNA3A was further convincingly confirmed
using the LCL produced with recombinant EBV-BAC viruses expressing strep-FLAG (TAP)-tagged EBNA3C (Gill Parker, Kostas Paschos and Oak Watanatanasup, personal communication).

Taking the BIM locus as a model for regulation by EBV, it has been demonstrated for the first time that EBNA3A and EBNA3C can mediate heritable repression of a cellular gene via epigenetic mechanisms including H3K27me3 and DNA methylation (Anderton et al., 2007; Paschos et al., 2009; Paschos et al., under revision). Following genome-wide studies in our lab, which integrated the profile of cellular transcripts with the profile of epigenetic marks at cellular promoters, have strongly suggested that EBNA3C and EBNA3A manipulate cellular gene expression through epigenetic mechanisms, most notably the polycomb-imposed repressive histone mark H3K27me3 (Kostas Paschos and Ian Groves, personal communication; White et al., 2010).

CtBP has been shown to mediate the repression of p16\textsuperscript{INK4A} (but not p14\textsuperscript{ARF}) in primary human fibroblasts and keratinocytes via H3K27me3 (Mroz et al., 2008). Our group has demonstrated that LCL immortalized by recombinant EBV viruses with engineered mutations that abolish CtBP-binding sites present in EBNA3C and EBNA3A (henceforth referred to as CtBP-binding mutant LCL) grow out poorly following B cell transformation and express elevated levels of p16\textsuperscript{INK4A} in comparison to LCL transformed with WT EBV-BAC viruses (WT LCL) (Rob White, personal communication).

Lastly, all known tumour viruses interact with the cellular miR network (Hoppe-Seyler and Hoppe-Seyler, 2011). Latent EBV is known to regulate cellular microRNA expression (Yin et al., 2008; Imig et al., 2011), and the role of certain EBV latent antigens such as LMP1 in the regulation of cellular miRs has been well documented (Cameron et al., 2008; Anastasiadou et al., 2010). However, the participation of EBNA3C and/or EBNA3A on posttranscriptional gene regulation of cellular genes via microRNAs has not been investigated so far.
Aims of the project

For decades, EBNA3C and EBNA3A have been implicated in transcriptional regulation of both viral and cellular genes, but little is known about the precise molecular mechanisms involved or about the consequences of the EBNA3C and/or EBNA3A-mediated gene regulation in lymphomagenesis. In this project, I aimed to address:

1) the details of \( p16^{INK4A} \) repression by EBNA3C and EBNA3A in LCL and by extension perhaps the mechanism of EBNA3C and EBNA3A-mediated epigenetic regulation of other cell gene(s)

2) the functional significance of EBNA3C and EBNA3A-mediated \( p16^{INK4A} \) repression in LCL proliferation and B cell transformation and immortalization

3) a putative new role of EBNA3C and/or EBNA3A in posttranscriptional gene regulation
Chapter 2
Materials and Methods
All chemical grade reagents were supplied by BDH chemicals, UK and were of AnalR grade purity unless otherwise stated. All autoradiography film (Hyperfilm™), Amplify™ and Enhanced Chemi-Luminescence (ECL™) reagents were supplied by GE Healthcare, UK.

**Solutions and Buffers**

Unless otherwise stated, all solutions were prepared in ddH₂O.

**6x Agarose gel loading buffer**

20% (w/v) Sucrose

0.1% (w/v) Bromophenol blue

10% (v/v) 10xTBE

**Alkaline SDS**

1% SDS

0.2 M NaOH

**Blocking Solution for Western Blots**

5% (w/v) skim milk powder (Sigma, UK) was reconstituted in 1xPBS/0.05% Tween (Sigma, UK)

**4-hydroxytamoxifen (HT)**

4mM (10 000x) stock was prepared by resuspending 5mg of HT (Sigma) in 3.22 ml of 95-100% ethanol. The aliquots of the stock were stored at -20°C in dark.

**Immunoprecipitation (IP) lysis buffer**

50 mM Tris-HCl, pH 8.0

150 mM NaCl

10% Glycerol

0.5% Triton X-100

2 mM Phenylmethylsulfonyl fluoride (PMSF)

2 mM mixture of proteinase inhibitors (Roche Molecular Biochemicals)
Phosphate Buffered Saline (PBS) [Dulbecco’s PBS without CaCl$_2$ and MgCl$_2$ (Dulbecco and Vogt, 1954)], pH 7.2

12.5mM NaCl
2mM Na$_2$HPO$_4$
1mM NaH$_2$PO$_4$

100mM PMSF (phenylmethylsulfonyl fluoride)
Dissolved in isopropanol and stored at –20°C.

Protease Inhibitor Cocktail Solution

1 tablet of Complete™ Protease Inhibitors Cocktail (Roche) was dissolved in 2ml ddH$_2$O. Stored at -20°C for up to 2 months.

Resolving gel for Western Blotting

<table>
<thead>
<tr>
<th></th>
<th>7.5%</th>
<th>10%</th>
<th>12.5%</th>
<th>15%</th>
</tr>
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<tbody>
<tr>
<td>30% Acrylamide</td>
<td>1.98ml</td>
<td>10 ml</td>
<td>3.30ml</td>
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</tr>
<tr>
<td>1 M Tris-HCl (pH 8.8)</td>
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<td>2.96ml</td>
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<tr>
<td>SDS (10% w/v)</td>
<td>79.2μl</td>
<td>79.2μl</td>
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<tr>
<td>ddH$_2$O</td>
<td>2.96 ml</td>
<td>8.7 ml</td>
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<tr>
<td>APS (10% w/v)</td>
<td>26.4μl</td>
<td>26.4μl</td>
<td>26.4μl</td>
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<tr>
<td>TEMED</td>
<td>5.3μl</td>
<td>5.3μl</td>
<td>5.3μl</td>
<td>5.3μl</td>
</tr>
</tbody>
</table>

5x Radioimmunoprecipitation (RIPA) Lysis Buffer

0.75M NaCl
5% (v/v) NP40
2.5% (w/v) Deoxycholate
0.5% (w/v) SDS
0.25M Tris-HCl pH 8.0
10x SDS Running Buffer for SDS-PAGE
250mM Tris
1.92M Glycine
1% (w/v) SDS

2x SDS Sample Buffer
100mM Tris-HCl (pH 6.8)
20% (v/v) Glycerol
1% (v/v) β-mercaptoethanol
1% (w/v) SDS
0.025% (w/v) Bromophenol blue

Stacking gel (5%) for Western Blotting

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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</thead>
<tbody>
<tr>
<td>30% Acrylamide</td>
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<tr>
<td>1 M Tris-HCl (pH 6.8)</td>
<td>620µl</td>
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<tr>
<td>SDS (10% w/v)</td>
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<tr>
<td>ddH2O</td>
<td>3.47ml</td>
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<tr>
<td>APS (10% w/v)</td>
<td>24.8µl</td>
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<tr>
<td>TEMED</td>
<td>5µl</td>
</tr>
</tbody>
</table>

10x TBE (Tris-Borate-EDTA) Buffer
89mM Tris
89mM Boric acid
10mM EDTA

TE
10mM Tris-HCl pH 7.5
1mM EDTA pH 8.0

Transfer Buffer for Western Blots
300ml 10xSDS Running Buffer
700ml EtOH
2l ddH2O
## Western Blotting (WB), Immunoprecipitation (IP) and Flow Cytometry (FC) - Primary Antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Species</th>
<th>Supplier</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU-FITC</td>
<td>B44</td>
<td>Mouse</td>
<td>BD Biosciences</td>
<td>FC 20ul per sample</td>
</tr>
<tr>
<td>Co-REST</td>
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<td>Mouse</td>
<td>BD Biosciences</td>
<td>IP 1:50</td>
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<tr>
<td>CtBP1</td>
<td>D2D6</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
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<td>CtBP</td>
<td>---</td>
<td>Rabbit</td>
<td>Prof. Martin Allday</td>
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<tr>
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<td>Rabbit</td>
<td>Abcam</td>
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<td>aa 466-482</td>
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<td>Abcam</td>
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<td>E2F1</td>
<td>cKH20 and KH95</td>
<td>Mouse</td>
<td>Millipore</td>
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<td>---</td>
<td>Human serum</td>
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<td>---</td>
<td>Sheep</td>
<td>Exalpha</td>
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<td>EBNA3B</td>
<td>---</td>
<td>Sheep</td>
<td>Exalpha</td>
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<tr>
<td>EBNA3C</td>
<td>A10</td>
<td>Mouse¹</td>
<td>Dr. Martin Rowe</td>
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<td>EBNA-LP</td>
<td>JF186</td>
<td>Mouse¹</td>
<td>Dr. Lindsey Spender</td>
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<td>ERK5</td>
<td>---</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
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<tr>
<td>Fascin</td>
<td>IM20, C-terminus</td>
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<td>Active Motif</td>
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<td>DAKO</td>
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<td>Rat</td>
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<tr>
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<td>C-terminus</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>IP 1:200</td>
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<tr>
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<td>N-terminus</td>
<td>Rabbit</td>
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<td>p14ARF</td>
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<td>Rabbit</td>
<td>Cell Signaling</td>
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<tr>
<td>p16INK4A</td>
<td>JC8</td>
<td>Mouse¹</td>
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<td>p53</td>
<td>DO-1</td>
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<td>phospho-p38</td>
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<td>γ-H2AX</td>
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<tr>
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<td>Sigma</td>
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¹ Tissue culture supernatant
## Western blotting - Secondary Antibodies

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Species-Conjugate</th>
<th>Supplier</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Ig</td>
<td>Rabbit-peroxidase conjugated</td>
<td>DAKO</td>
<td>WB 1:2000</td>
</tr>
<tr>
<td>Mouse Ig</td>
<td>Sheep-peroxidase conjugated</td>
<td>GE Healthcare</td>
<td>WB 1:2000</td>
</tr>
<tr>
<td>Rabbit Ig</td>
<td>Goat-peroxidase conjugated</td>
<td>DAKO</td>
<td>WB 1:2000</td>
</tr>
<tr>
<td>Rat Ig</td>
<td>Rabbit-peroxidase conjugated</td>
<td>DAKO</td>
<td>WB 1:2000</td>
</tr>
<tr>
<td>Sheep Ig</td>
<td>Rabbit-peroxidase conjugated</td>
<td>DAKO</td>
<td>WB 1:2000</td>
</tr>
</tbody>
</table>

All secondary antibodies were diluted in 5% Milk/0.05% Tween-20/PBS.
Cell culture

Established LCL were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin. BL31 cell lines were cultured in the same medium as LCL with the addition of 1 mM sodium pyruvate (Sigma) and 50 µM α-thioglycerol (Sigma). For cultures of BL31 containing recombinant hygromycin-resistant EBV-BACs, 100 µg/ml hygromycin B (Roche) was added. LCL 3CHT were cultured with 400nM 4-hydroxytamoxifen (HT, Sigma).

Cells recovered from liquid nitrogen were cultured for about 10 days before the start of any experiment. At the end of an experiment (a maximum of 35 days), the cells were discarded. Twenty-four hours before any experimental treatment, cells were seeded at a density of 2.5x10⁵ cells/ml.

Generation of recombinant EBV-BACs

All recombinant EBV-BACs used in this work were already available in the lab. EBNA3C-HT fusion EBV-BAC was produced by M. Franz and R. White (Skalska et al., 2010), EBNA3A KO and revertants by E. Anderton (Anderton et al., 2007) and CtBP-binding mutants and revertants by R. White (Skalska et al., 2010).

An EBNA3C-HT fusion (3CHT) in the B95-8 background was constructed with the same linking sequence and 4-hydroxytamoxifen-sensitive murine oestrogen receptor that has previously been described in the Akata background (Maruo et al., 2006). The connection between the 3C and HT is a single proline residue between the last amino acid of EBNA3C and amino acid 281 of the murine oestrogen receptor alpha (modified by the replacement of glycine at position 525 by arginine to make it 4-hydroxytamoxifen-specific). This fusion
was recombined into the B95-8 BAC (Delecluse et al., 1998) using previously described methods (White et al., 2003; Anderton et al., 2007) to produce two independent BACs containing 3CHT (A and C).

A set of CtBP-binding-mutant viruses were generated in which the EBNA3A and/or EBNA3C binding site(s) for CtBP were replaced with previously characterized mutations (Touitou et al., 2001; Hickabottom et al., 2002) that lack the ability to bind CtBP (Fig. 2.1). This was achieved by a sequential set of recombinations, initially mutating the pair of CtBP binding sites in EBNA3A (to generate the 3A<sup>CtBP</sup> mutant). The EBNA3C binding site for CtBP in this was then mutated to create a virus genome lacking all CtBP binding sites among the EBNA3s (E3<sup>CtBP</sup>). Then the EBNA3A mutant sequence was replaced with WT sequence, leaving only the EBNA3C sequence as mutant (3C<sup>CtBP</sup>) and finally the EBNA3C sequence was reverted to WT sequence, generating the CtBP revertant (rev<sup>CtBP</sup>).

![Fig. 2.1](image-url)

**Fig. 2.1.** Mutations of CtBP-binding sites in EBNA3C and EBNA3A in CtBP-binding mutant EBV-BACs. EBNA3C contains a single consensus CtBP binding site (PLDLS) which has been mutated into ALDAS. EBNA3A contains two non-consensus CtBP binding sites which synergize to produce efficient binding to CtBP (Touitou et al., 2001; Hickabottom et al., 2002). ALDLS site has been mutated into ALDAA and VLDLS binding site into VLDAA.
Infection of peripheral blood lymphocytes (PBL) with recombinant EBV

Recombinant BACs were transfected into HEK293 cells [(ATCC, CRL-1573), a kind gift of Claire Shannon-Lowe, University of Birmingham]; clonal hygromycin B-resistant cell lines were then selected and screened for integrity of the EBV genome by episome rescue and pulsed-field gel analysis of BAC restriction digests. Infectious virus was produced by the transfection of EBV-BAC-containing 293 cells with BZLF1 and BALF4 expression constructs (Neuhierl et al., 2002), and after 4 days, supernatant was filtered through 0.45 μm filters. Virus titre was assessed by infection of Raji cells and counting green cells on an inverted fluorescent microscope after enhancement of GFP expression by overnight treatment with 5 nM tetradecanoylphorbol acetate (TPA) and 1.25 mM sodium butyrate. Virus titres were typically in the range of 50 to 250 Raji green units per microlitre of tissue culture supernatant.

PBL for generation of LCL 3CHT-A and -C were isolated from buffy coat residues (UK blood transfusion services) by centrifugation over Ficoll-Paque. EBNA3A KO LCL, CtBP-binding mutant LCL and LCL 3CHT-B, D and E (described herein) were generated by infection of PBL isolated from donated EBV-seronegative blood from five donors (annotated 1-5) (a kind gift of Ingo Johannessen, University of Edinburgh). LCL 3CHT-A were produced by the infection of PBL from mixed donors by EBV-3CHT-A virus and LCL 3CHT-C by EBV-3CHT-C virus. LCL 3CHT-B, D and E were made by infection of PBL in blood from a single donor 1, 3 and 2 respectively with EBV-3CHT-A virus. EBNA3A KO and revertant LCL, as well as CtBP-binding mutant and revertant LCL were established using the viruses and PBL from various single donors listed in the table 2.1. Essentially, between 50 μl and 1 ml of virus was added to 10^6 PBL (typically 2-8% of which are B-cells by FACS for CD20; not shown) in a well of a 24 well plate, and cultured in RPMI supplemented for the first 2-3 weeks with 15% FCS and Cyclosporine A (500 ng/ml). When LCL grew out to the volume of about 60ml at a density of 3x10^5 cells/ml or greater, multiple aliquots were
frozen in liquid nitrogen. This took 4-8 weeks for WT LCL, revertant LCL and LCL 3CHT (cultured with HT) and 6-12 weeks for the EBNA3A and CtBP-binding mutant LCL.

Infection of p16-functionally null PBL with recombinant EBV

We have acquired PBL containing a germline homozygous 19bp-deletion in the second exon of CDKN2A locus, known as p16-Leiden (Gruis et al., 1995; de Vos tot Nederveen Cappel et al., 2003), as a kind gift from Gordon Peters (Institute for Cancer Research, UK) and Alison Sinclair (University of Surrey). The details of transcripts and proteins produced from the CDKN2A locus are described in Chapter 3.2.; this homozygous deletion in the CDKN2A locus renders the cells null for p16ink4a function (Brookes et al., 2002; Hayes et al., 2004).

PBL containing the p16-Leiden deletion were isolated from the blood of the male born in 1972, with the clinical history of multiple atypical naevi developed in childhood and puberty and seven superficial spreading melanomas developed and removed by the age of 25 (Brookes et al., 2002). PBL were used to produce p16-functionally null LCL 3CHT lines by infection with two independent E3CHT BACs [two lines with EBV-3CHT-A virus (A1, A2) and two using EBV-3CHT-C virus (C1, C2)] Due to the limited number of these rare PBL, only a single pair of p16-functionally null EBNA3A KO and revertant LCL was produced. The p16-functionally null LCL were established using the exactly same procedure described above for production of the p16-competent lines. The p16-competent and -null cell lines most frequently used during the course of this study are listed in the table 2.1.
<table>
<thead>
<tr>
<th>Annotation</th>
<th>Virus</th>
<th>Donor</th>
<th>LCL clone</th>
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</tr>
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<td>C19</td>
<td>Mixed donor PBL</td>
</tr>
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<td>Single donor 3 PBL</td>
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<td>Single donor 2 PBL</td>
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<td>3A rev</td>
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</tr>
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<td>Single donor 4 PBL</td>
</tr>
<tr>
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<td>3A CtBP</td>
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</tr>
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<td>3C CtBP LCL</td>
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<tr>
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<tr>
<td>WT LCL</td>
<td>WT BAC</td>
<td>i6</td>
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<td>WT BAC</td>
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<td>A13</td>
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<td>p16-null LCL 3CHT A2</td>
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<td>C19</td>
<td>Single donor p16-null PBL 1</td>
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<td>p16-null LCL 3CHT C2</td>
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<td>C19</td>
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<tr>
<td>p16-null EBNA3A rev LCL</td>
<td>3Ar rev</td>
<td>M4</td>
<td>Single donor p16-null PBL</td>
</tr>
</tbody>
</table>

**Table 2.1. List of the most frequently used cell lines.** p16-null LCL are highlighted in blue. Additional p16-competent LCL 3CHT produced with 3CHT-C(19) virus by infection of PBL from donor 1, 2 and 3 and CtBP-binding mutant and revertant LCL produced with viruses listed in the table by infection of PBL from donor 1 and 5 were used in replicate experiments.
**Analysis of cell gene expression during B cell transformation by EBV**

Fresh EBV B95-8 virus was produced as follows. B95-8 cell line, made functionally conditional for BZLF1/Zta activation of lytic replication by an in-frame fusion with a mutant oestrogen receptor (Johannsen et al., 2004), was a kind gift from Eric Johannsen (Harvard University). This line was induced to produce virus by adding HT at 200nM final concentration for 4 days. The supernatant containing EBV B95-8 virus was harvested by centrifugation at 1300 rpm for 4 min at 4°C. To concentrate the viral yield by approximately 100-fold, the supernatant was ultracentrifuged at 25000 rpm for 1h at 4°C, then resuspended in RPMI, aliquoted, snap-frozen and stored in liquid nitrogen.

Infectivity of the supernatant was assayed by infection of the Raji cells. Ten-fold dilutions of viral supernatant harvested from 293 cells and concentrated by ultracentrifugation (1 ml, 0.1 ml and 0.01 ml made up to 1 ml volume using RPMI/10% FCS) were used to infect $10^6$ Raji cells in 1ml RPMI/10% FCS. Raji cells were harvested after 40h for western blotting for EBNA-LP expression. The amount of virus was judged sufficient for infection of $10^6$ Raji cells in the cases when EBNA-LP was detected as a well-expressed ladder of proteins as opposed to a single band detectable in the Raji cells prior infection with EBV B95-8 virus. 1-5 µl of concentrated B95-8 virus was sufficient to infect $10^6$ Raji cells.

Excess (10 µl of the virus per $10^6$ primary B cells) was used in the B cell transformation experiment in an attempt to infect every primary B cell. Two experimental designs were employed (with duplicate experiments for each design): either PBL or B cells, purified by anti-CD19 coated MACS micro-beads (using a MACS separator from Miltenyi Biotec according to the manufacturer’s instructions), were used in EBV infection. In the case of PBL infection, Cyclosporine A (500ng/ml) was added to the culture medium of PBL after the infection and the first harvesting time-point was set 7 days post-infection, when the majority of PBL other than B cells should be eliminated from the culture (i.e. T cells and NK cells would be eliminated; however, macrophages/monocytes would still survive).
In order to avoid harvesting heterogeneous cell populations, the EBV-infected cells were separated using Ficoll-Paque reagent into the high-density, most likely apoptotic cells (Belloni et al., 2008 and 2010), and normal-density cells. The equal volume (7ml) of culture medium with cells was poured on the top of 7ml of Ficoll-Paque reagent in 15 ml tube and centrifuged for 30 min at 1380 rpm at RT. The lymphocytes of normal density separated at the interphase between the culture medium and the Ficoll-Paque reagent, while the high-density cells were pelleted at the bottom of the tube (Fig. 2.2). The normal density fraction was transferred to the new tube, mixed and pelleted by centrifugation for 5 min at 1300 rpm. The same end points were analyzed in both, the high-density and the low-density fraction. Transcript quantities were assessed by qPCR, and multiple reference genes (GNB2L1, ALAS1 and RPLP0) were used to accurately normalize the expression data.

Fig. 2.2. Scheme of the separation of lymphocytes into high- and normal-density fractions using Ficoll-Paque reagent. High-density fraction is pelleted at the bottom of the tube, while the lymphocytes of the normal density separate in the interphase between the Ficoll-Paque reagent and the culture medium.
Flow cytometry

To assess the cell cycle distribution, cells were harvested at the times indicated and fixed in 80% ethanol. Fixed cells were resuspended in PI solution [(PBS containing 18 µg/ml propidium iodide (PI) and 8 µg/ml RNase A (both Sigma Aldrich)] and incubated at 4°C for 1h. Flow cytometry was performed using a Beckton-Dickinson FACSort flow cytometer and analysed with CellQuest software.

To analyse the proportion of cells entering and passing through S phase, 2x10^6 cells were incubated with 5-bromo-2'-deoxyuridine (BrdU, Sigma) at a concentration of 10 µM for 1h, after which cells were immediately harvested, centrifuged at 1300 rpm for 5 min, washed twice in 2 ml of 1% bovine serum albumin (BSA) in PBS, resuspended in 500 µl of ice-cold 70% ethanol and incubated on ice for 30 min before storage at -20°C or direct analysis. Cells were then washed in PBS before thorough resuspension in 750 µl of 2N HCl containing 0.5% (vol/vol) Triton X-100 for 30 min at room temperature (RT) to denature the labelled, double-stranded DNA. Acid was neutralized by resuspending cells in 750 µl of 0.1M sodium tetraborate (pH 8.5) and incubation at RT for 5 min. Cells were centrifuged and resuspended in 20 µl of fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Becton Dickinson, United Kingdom), which was then further diluted with 380 µl of 1% BSA-0.5% Tween 20-PBS. After incubation in the dark at RT for 60 min, cells were washed twice in 0.5% Tween 20-PBS and resuspended in 500 µl of PI solution. Flow cytometry was performed using a DAKO CyanADP flow cytometer and analysed with Summit software.
Western immunoblotting

Cells were washed twice in PBS and lysed in RIPA buffer, equivalent to the volume of the cell pellet, for 10 min on ice. After centrifugation at 4°C for 10 min, the supernatant was removed and protein concentration was estimated colorimetrically using the Bio-Rad detergent-compatible assay. 20-40 µg of protein was added to an equal volume of 2xSDS protein sample buffer and loaded onto SDS-polyacrylamide gel of appropriate percentage. Alternatively, in some experiments the whole cell lysates were loaded, after lysing cell pellets directly in 2xSDS protein sample buffer (50µl of buffer per 10^6 cells) and sonicating to fragment DNA using Bioruptor sonicator (UCD-200; Diagenode) on a high setting for a total of 15 min (30 sec ‘on’/30 sec ‘off’ intermittent sonication). Gels were transferred at constant voltage 100W for 1h onto a Protran nitrocellulose membrane (Schleicher and Schuell Bioscience). Membranes were blocked with 5% milk powder in PBS/0.05% Tween 20 for 1h at RT. After incubation with primary antibody overnight at 4°C, membranes were washed in changes of PBS-0.05% Tween 20 for a total of 1h, incubated with secondary antibody conjugated to horseradish peroxidase (1:2000 in PBS-M) for 1h at RT, then washed as previously, and visualized by enhanced chemiluminescence (Amersham) as recommended by the supplier.

RNA and Quantitative real time PCR (qPCR)

For qPCR, RNA was extracted from approximately 5x10^6 cells for each cell line using the RNeasy mini kit from Qiagen and following the manufacturer’s instructions. One microgram of each RNA sample was reverse-transcribed using SuperScript III First-Strand Synthesis Supermix for qPCR (Invitrogen). Between 0.5-1% of cDNA product (equivalent to 5-10 ng RNA) was used per qPCR reaction. In the case of low-abundance transcripts (e.g. ANRIL
and p14ARF), up to 20% of cDNA product was used in a qPCR reaction performed in an upscaled volume to avoid PCR inhibition.

The majority of qPCR measurements were performed using sybr green chemistry. Platinum Sybr Green qPCR SuperMix UDG kit (Invitrogen) was used with Applied Biosystems (ABI) 7900HT real-time PCR machine. The cycling conditions were generally 95°C for 2 min, followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C on a fast block. Dissociation curve analysis was performed during each run to confirm absence of non-specific products. Sequences of the assays used with sybr green chemistry are listed in table 2.2. Primer assays were either developed using Primer Express software (ABI), or published previously (Table 2.5).

qPCR measurements using the taqman chemistry were performed with TaqMan® Gene Expression Assays (ABI) listed in table 2.3 and Taqman Fast Universal PCR Mastermix (ABI). For low-abundance non-coding RNA (ncRNA) detection, the TaqMan® Gene Expression Mastermix was used with the standard mode of cycling (initial 10min hold at 95°C, followed by 40 cycles of 15 sec at 95°C and 1min at 60°C).

Standard curves, used to standardise amplification efficiency, were produced by six 5-fold serial dilutions of a mixture containing all cDNA samples used. Results were analyzed with qbasePLUS software (Biogazelle). Precise normalization was achieved using internal average control calculated from the controls (housekeeping genes - table 2.4) with the highest stability rating (usually ALAS1 and GNB2L1). The error bars in the graphs represent the standard errors from three replicate qPCR reactions for each mRNA in a single experiment. To obtain statistical significance data were analysed by unpaired student t-test. P value p<0.005 was considered as highly significant.
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<tr>
<th>Assay</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
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<td>ANRIL (15/16)</td>
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<tr>
<td>BIM EL</td>
<td>GCTGTCTCGATCCTCCAGTG</td>
<td>GTTAACCTCGTCCTCAATAACG</td>
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<td>CDKN2A</td>
<td>CATAGATGCCGCGGAGGCT</td>
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<td>NOTCH2</td>
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<td>p16NK4A</td>
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<td>TGFβRII</td>
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Table 2.2. Primer sequences for mRNA quantification using sybr green chemistry.

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<td>CDKN2A</td>
<td>Hs 00923894_m1</td>
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<tr>
<td>MALAT-1</td>
<td>Hs 00273907_s1</td>
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Table 2.3. Primer sequences for mRNA quantification using taqman chemistry.

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<td>RPLP0</td>
<td>ACTTGCGATTCTCGTCTCTCTTG</td>
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Table 2.4. Primer sequences for mRNA quantification of housekeeping genes using sybr green chemistry.
### Table 2.5. The source of the primer sequences that have been developed prior to this project.

<table>
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<td>GNB2L1</td>
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<td>Loc 3 and loc 10</td>
<td>Iio <em>et al.</em>, 2010</td>
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<td>p16&lt;sup&gt;INK4A&lt;/sup&gt;</td>
<td>Kia <em>et al.</em>, 2008</td>
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<tr>
<td>RPLP0</td>
<td>Christin Down, unpublished data</td>
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**MicroRNA profiling by TaqMan quantitative real-time PCR Low Density Array (TLDA)**

RNA was isolated using *mir*Vana™ miRNA Isolation Kit (Ambion) including the optional step for enrichment of small RNA (<200 bp) which enables more sensitive detection of low-level small RNAs. TaqMan® MicroRNA RT Kit and Megaplex Primer Pool A (ABI) were used to reverse transcribe up to 381 microRNAs in a single reaction according to the manufacturer’s instructions. 300ng of RNA was reverse transcribed per reaction and the cDNA product was used in qPCR without pre-amplification.

377 human microRNAs were profiled by real-time qPCR using the TaqMan® Array MicroRNA A Card v 2.0 (ABI). cDNA was diluted, mixed with TaqMan® Universal PCR Master Mix II (ABI) and loaded into the pre-configured micro fluidic card. Real-time reaction was run on a 7900HT Real-Time PCR System (ABI) and data analyzed using the SDS RQ manager software (ABI). Two biological replicates (two LCL 3CHT lines cultured with or without HT and two EBNA3A KO LCL and respective revertants) were analyzed. C<sub>t</sub> values were normalized to endogenous U6 snRNA and comparative 2<sup>−ΔΔCt</sup> method was used to evaluate the fold change of miRNAs in LCL with or without functional EBNA3C or EBNA3A.
MicroRNA qPCR detection

Both, the mirVana™ miRNA Isolation Kit (Ambion) with the step enriching for small RNA and the miRCURY RNA Isolation Kit (Exiqon) that isolates total RNA (ranging from large messenger and ribosomal RNAs down to microRNAs and other small RNAs) were used. 10ng RNA was reverse transcribed using microRNA specific RT primers (supplied with TaqMan® MicroRNA Assays) and TaqMan® MicroRNA RT Kit (ABI). RNA isolated by miRCURY RNA Isolation Kit allowed for reverse transcription and quantification of both microRNAs and their longer RNA precursors or target mRNA in the same RNA preparation.

MicroRNAs were quantified by qPCR using the TaqMan® MicroRNA Assays listed in the table 2.6, the TaqMan® Universal PCR Master Mix II and ABI 7900HT real-time PCR machine with emulation mode (initial 10 min hold at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C).

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<td>RNU6B</td>
<td>001093</td>
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Table 2.6. TaqMan® MicroRNA Assays and U6snRNA (RNU6B) endogenous control
Quantification of promoter DNA methylation

Genomic DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s protocol. 200–500 ng of genomic DNA was converted by bisulphite using EZ DNA Methylation Kit (Zymo Research). Bisulfite treatment lead to the complete conversion of cytosine to uracil in unmethylated DNA, leaving 5-methylcytosine residues unaffected. The uracil was amplified as thymine in subsequent PCR reaction. The degree of $p16^{INK4A}$ promoter methylation was assessed quantitatively by duplex real-time qPCR using EpiTect MethyLight Hs_CDKN2A Assay (Qiagen). Two CpGs in $p16^{INK4A}$ promoter (21964737-21964742 of NT_008413.17) were interrogated. The FAM labelled TaqMan probe detected the methylated site, while the VIC® labelled TaqMan probe detected the unmethylated site in the same reaction. Starting amount of 10 ng bisulfite converted DNA was used in each reaction with EpiTect MethyLight PCR kit (Qiagen). EpiTect Control DNA set (Qiagen) supplied control methylated and unmethylated bisulfite converted DNA to assess the specificity of primers, as well as methylated and unmethylated (unconverted) genomic DNA to monitor the efficiency of bisulphide conversion.

Chromatin immunoprecipitations (ChIP)

All ChIP were carried out using the Chromatin Immunoprecipitation Assay Kit or EZ ChIP Kit from Upstate (Millipore), according to the manufacturer’s protocol. To obtain sheared chromatin with DNA of 200 bp – 1000 bp in length, extracted chromatin from 2x10^6 cells per ChIP was sonicated in 200 µl lysis buffer for five 20 sec sonication rounds, using a Heat Systems Sonicator Ultrasonic Processor XL at 10% intensity or Bioruptor sonicator (UCD-200; Diagenode) on a high setting for a total of 12 min (30 sec ‘on’/30 sec ‘off’ intermittent sonication).
ChIP for histone modifications was performed using anti-H3K27me3 antibody, anti-H3K4me3 antibody and rabbit IgG serum as a negative control (all from Upstate). Precipitated DNA was cleaned using QIAquick Gel Extraction Kit (Qiagen) and was assayed by qPCR. Input DNA Ct was adjusted from the 5% used in the qPCR to 100% equivalent by subtracting 4.32 (Log2 of 20) cycles. ‘Percent input’ precipitated was then calculated by 100 x 2^ (Ct adjusted input – Ct IP). Non-specific background was estimated by precipitation with IgG (data generally not shown since all values were below 0.03% of input). The error bars represent standard deviations from triplicate qPCR reactions for both input and IP. Sequences of the primers mapping the modifications across the CDKN2A locus are listed in table 2.7. The position of these assays is specified in the figure 2.3 and table 2.8. The sequences of the p14ARF and control promoter assays are listed in the table 2.9.

<table>
<thead>
<tr>
<th>CDKN2A ChIP assay</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>GGAGCGATGTGATCCGTTATC</td>
<td>TGAAATCCCAATCGTCTTCCAC</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>CTCAAAGCGGATAATTCAGAGC</td>
<td>AAGCCTTAAGAACAGTGCCACAC</td>
</tr>
<tr>
<td><strong>C (i)</strong></td>
<td>CCCCTTGCCTGGAAAGATAC</td>
<td>AGCCCCTCCTCTTTCTTCCT</td>
</tr>
<tr>
<td><strong>C (ii)</strong></td>
<td>AGAGGGTCTGCGAGCGG</td>
<td>TCGAAGCGCTACCTGATTC</td>
</tr>
<tr>
<td><strong>C1</strong></td>
<td>GCCAAGGAAAGGAATGAGGAG</td>
<td>CCTCAGATCTTCTCAGCATTGC</td>
</tr>
<tr>
<td><strong>D</strong></td>
<td>TAGGAGGCCCCATTAAAGCATA</td>
<td>TGATGTGCGGAGGATTGAGG</td>
</tr>
</tbody>
</table>

Table 2.7. Primer sequences of the assays spaced across the CDKN2A locus.

All assays were developed by Bracken et al., 2007 with the exception of C(i) which is from Kia et al., 2008.
Fig. 2.3. Localization of the ChIP assays across the CDKN2A locus. Schematic of the human CDKN2A locus showing the location of coding exons (boxes) and transcription start sites (horizontal arrows) – not drawn to scale. The vertical (A-D) arrows refer to the approximate locations of primer pairs used for qPCR analysis of precipitated chromatin.

<table>
<thead>
<tr>
<th>CDKN2A ChIP assay</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.5kb downstream of p14&lt;sup&gt;ARF&lt;/sup&gt; TSS</td>
</tr>
<tr>
<td>B</td>
<td>1kb upstream p16&lt;sup&gt;INK4A&lt;/sup&gt; promoter</td>
</tr>
<tr>
<td>C (i)</td>
<td>85bp in 16&lt;sup&gt;INK4A&lt;/sup&gt; exon 1 (starts at nt 21964992 of NT_008413.17)</td>
</tr>
<tr>
<td>C (ii)</td>
<td>3’ end of 16&lt;sup&gt;INK4A&lt;/sup&gt; exon 1 (starts at nt 21964638 of NT_008413.17)</td>
</tr>
<tr>
<td>C1</td>
<td>200bp downstream of 16&lt;sup&gt;INK4A&lt;/sup&gt; exon 1</td>
</tr>
<tr>
<td>D</td>
<td>800bp downstream 16&lt;sup&gt;INK4A&lt;/sup&gt; exon 3</td>
</tr>
</tbody>
</table>

Table 2.8. Localization of the ChIP assays across the CDKN2A locus
Two assays designed in a very similar location were used for the GC-rich region of 16<sup>INK4A</sup> exon 1 (C i and ii) to ensure precision.

<table>
<thead>
<tr>
<th>Promoter assay</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TACTAGCGGTGTTTACGGGCCG</td>
<td>TCGAACAGGAGGAGCAAGAGCCGA</td>
</tr>
<tr>
<td>γ-globin</td>
<td>GCCCTGACCAATAGCCTTGACA</td>
<td>GAAATGACCCATGGCGTCTG</td>
</tr>
<tr>
<td>p14&lt;sup&gt;ARF&lt;/sup&gt; (i)</td>
<td>GTGGGTCCCAGTCTGTCAGTTA</td>
<td>CTTTTGGCACCAGAGGTGAG</td>
</tr>
<tr>
<td>p14&lt;sup&gt;ARF&lt;/sup&gt; (ii)</td>
<td>CGCCGTGTCCAGATGTCG</td>
<td>TGCTCTATCCGCAATCAG</td>
</tr>
</tbody>
</table>

Table 2.9. Primer sequences of the p14<sup>ARF</sup> and control promoter assays
GAPDH promoter assay was developed by Millipore
γ-globin – Bottardi <i>et al.</i>, 2011
p14<sup>ARF</sup> (i) - Bracken <i>et al.</i>, 2007
p14<sup>ARF</sup> (ii) - Kia <i>et al.</i>, 2008
**Immunoprecipitation**

$10^7$ cells were harvested and washed once in ice-cold PBS. The cells were collected by centrifugating at 1300 rpm for 4 min and the pellet re-suspended in 600 μl of IP lysis buffer. The cell lysates were incubated at 4°C for 20 min and the debris was pelleted by centrifugating at 13 000 rpm at 4°C for 15 min. The supernatant was split into 200 μl aliquots per immunoprecipitation reaction. 20 μl of protein G-Sepharose beads was added to each aliquot and mixed at 4°C for 1 h to pre-clear the lysate. The beads were next pelleted by centrifugation at 1000 rpm at 4°C for 1 min and the supernatant was transferred to a fresh tube. Complexes were then precipitated with an antibody specific for the protein of interest and the mixture was incubated at 4°C on an orbital rotor for 2 h. After this, 30 μl of protein G-Sepharose beads were added to the mixture and it was left to rotate at 4°C for 1 h. The beads were next washed 4 times in IP lysis buffer. 30 μl of SDS sample buffer was added to the pelleted beads, boiled, centrifuged, and the supernatant was loaded on a SDS-PAGE gel for Western blot analysis.
Chapter 3
Results
3.1 Mechanism of $p16^{INK4A}$ epigenetic repression by EBNA3C and EBNA3A

3.1.1 EBNA3C and EBNA3A co-operate with CtBP to epigenetically repress $p16^{INK4A}$

Objectives

Maruo et al. (2006) reported that conditional EBNA3C inactivation in LCL leads to robust growth inhibition and accumulation of $p16^{INK4A}$. This important report raised a series of questions regarding the nature of the cell cycle arrest following EBNA3C inactivation and the mechanism of $p16^{INK4A}$ repression by EBNA3C. In addition, since EBNA3A inactivation in LCL was shown to result in similar growth inhibition (Maruo et al., 2003), it became of interest whether EBNA3A also contributes to $p16^{INK4A}$ repression in LCL. It has been well established in the past that EBNA3C and EBNA3A are transcriptional regulators of both viral and cellular genes (Waltzer et al., 1996; Bourillot et al., 1998; Radkov et al., 1999; Lin et al., 2002); however, the mechanism by which they modulate gene expression has remained elusive. The regulation of $p16^{INK4A}$ expression by conditionally active EBNA3C in LCL therefore offered an attractive model to study in detail the mechanism of EBNA3C-mediated repression of this crucial cell-cycle regulator.
Results

Inactivation of EBNA3C leads to proliferative arrest of LCL 3CHT that can be reversed by re-adding HT

The construction of the EBV-BAC containing EBNA3C-HT fusion and subsequent production of LCL 3CHT cell lines has been described previously (see Materials and Methods pp. 64-67). It has been predicted from the use of other oestrogen receptor fusion systems, such as EBNA2 (Kempkes et al., 1995), that after removal of HT from the medium, the protein fused to the HT domain would be rapidly inactivated; in addition, some fusions are also targeted for proteasomal degradation. In LCL 3CHT, three to seven days after removal of HT from the medium, EBNA3C protein had almost entirely disappeared from the whole cell lysates. After re-adding HT into the medium, EBNA3C protein levels were mostly reconstituted within 24 hours (Fig. 3.1.1). There were no consistent changes in the expression of other late EBV proteins that correlated with EBNA3C inactivation for up to 26 days (Fig. 3.1.2).

![Fig. 3.1.1. EBNA3C inactivation and reactivation in LCL 3CHT.](image)

**Fig. 3.1.1. EBNA3C inactivation and reactivation in LCL 3CHT.** Cells that had been grown in culture medium containing HT were washed and re-suspended in medium with HT omitted. After culturing for the number of days indicated, protein extracts were western blotted and probed with an anti-EBNA3C MAb to reveal EBNA3C-HT. After 17 days some cells were transferred to medium containing HT and after 1, 5 or 8 days samples were again taken for western blotting. The WB was re-probed with anti-γ-tubulin (γ-tub) to ensure equal loading of the proteins. EBNA3C-HT protein is considerably decreased by day 7 following HT removal but is reconstituted within first day after HT re-adding. WB shown is a representative of at least three replicate experiments.
Fig. 3.1.2. Validation of LCL 3CHT. Western blot of latency-associated EBV proteins in representative LCL 3CHT-A, -B and -E cultured for 26 days with (+) or without (-) HT. No consistent changes in the expression of EBV latent proteins following EBNA3C inactivation were detected. Data shown are representative of two replicate experiments.

Maruo et al. (2006) demonstrated that EBNA3C inactivation leads to cessation of LCL proliferation. Consistent with this report, PI staining followed by flow cytometric analysis confirmed that there were reduced numbers of cells in the S phase and to a lesser extent in G2/M phase in LCL 3CHT cultured for 24 days without HT in comparison to LCL 3CHT cultured with HT (Fig. 3.1.3A). In order to test whether EBNA3C inactivation prevents S phase entry, BrdU incorporation was used to assess the amount of newly synthesised DNA. LCL 3CHT cultured for 33 days with or without HT were pulsed for 1h with BrdU, harvested immediately after the pulse, fixed, co-stained with anti-BrdU-FITC conjugate and PI and analysed by flow cytometry. The BrdU/PI profile of cells cultured for a prolonged time without active EBNA3C showed a dramatic decrease in cells in S phase of the cell cycle (i.e. cells which have highly incorporated BrdU and their DNA content was between
2N and 4N) and accumulation of the cells in the G1 phase of the cell cycle (i.e. cells which did not incorporate BrdU and their DNA content was 2N) (Fig. 3.1.3B).

**Fig. 3.1.3.** PI and BrdU/PI profiles of LCL 3CHT with or without HT. (A) LCL 3CHT cells cultured for 24 days with or without HT, fixed, stained with PI and analysed by flow cytometry. M1 gate represents sub G1 phase, M2 - G1 phase, M3 - S phase and M4 - G2/M phase of the cell cycle. PI profile of LCL 3CHT cultured for 24 days without HT shows reduction of cell population in the S phase and G2/M phase of the cell cycle compared to LCL 3CHT cultured with HT. A representative image of two separate experiments each including two LCL 3CHT lines is shown alongside the mean and SD of percentage of cells in the S phase. (B) LCL 3CHT cells cultured for 33 days with or without HT, pulsed for 1 hour with BrdU, fixed, stained with anti-BrdU-FITC/PI and analysed by flow cytometry. The gated population, which comprises the cells that have undergone DNA replication while incubated with BrdU, is considerably reduced in the absence of HT. The image shown is representative of two measurements each including two LCL 3CHT lines.
In the next step, the kinetics of the onset and progression of LCL proliferative arrest following EBNA3C inactivation were assessed. The apparent reduction in LCL proliferation was seen within 14 days of culturing LCL 3CHT without HT, by which time BrdU incorporation was reduced by about 60% in comparison to proliferating LCL 3CHT cultured with HT (controls). When the LCL 3CHT were cultured without HT for an extended period of time (33 days), the reduction of cells entering S phase progressed and reached 70 to 90% (Fig. 3.1.4A).

In order to test whether the growth arrest could be reversed after EBNA3C reactivation and whether the period LCL 3CHT were cultured without HT would have any effect on the restoration of proliferation, HT was re-added after 14 and 33 days without HT. Surprisingly, EBNA3C had little immediate effect on cell proliferation, but rather cells increased their proliferation very gradually over a number of days. The full restoration of proliferation after reactivation of EBNA3C took in the region of weeks in both instances and took longer where cells had been cultured for a greater time without functional EBNA3C: if the HT was re-added following 14 days without HT, the full proliferation rate was reached in 12 days (Fig. 3.1.4B), while if the HT was re-added at the later time-point (after 33 days without HT), the period needed to re-establish the full proliferation rate was increased to 16 days (Fig. 3.1.4C). Although the times needed for the restoration of the original proliferation rate differed only by days, this suggests that at the level of the cell population -if a total population of cells is analyzed rather than a single cell- the proliferative arrest following EBNA3C inactivation is not a static condition and the speed of its reversal after EBNA3C reactivation is dependent on the degree of its progression.
Fig. 3.1.4. Proliferation of LCL 3CHT after inactivation and reactivation of EBNA3C. (A) Two LCL 3CHT (-A and -C) generated from independent 3CHT-BACs using PBL from mixed donors analysed by BrdU/PI (as in Fig. 3.1.3B) after 14 and 33 days without HT. Histograms show BrdU incorporation relative to the control, cycling population (ctrl) grown with HT. (B) LCL 3CHT-A and -C cultured for 14 days in the absence of HT (0) and after 4, 7 and 12 days after re-addition HT. (C) A similar experiment performed on cells cultured for 33 days without HT. Proliferation of LCL 3CHT is progressively reduced during weeks following EBNA3C inactivation and can be fully restored after EBNA3C is reactivated; however, the restoration occurs gradually over number of days and appears to depend on the intensity of the previous proliferative arrest. The error bars represent the standard deviation from the means of two technical replicates for each sample.
Proliferation of LCL 3CHT correlates with the regulation of p16\(^{\text{INK4A}}\) expression

Maruo et al. (2006) described how p16\(^{\text{INK4A}}\) accumulates in the absence of functional EBNA3C. Here, I tested whether the changes in p16\(^{\text{INK4A}}\) levels in response to EBNA3C inactivation and reactivation correlate with (and could account for) the changes in proliferation of LCL 3CHT.

The DNA sequence of the unique first exon of p16\(^{\text{INK4A}}\) is GC rich and contains substantial secondary structure. A qPCR assay specific for p16\(^{\text{INK4A}}\) only transcripts, which detects amplicon located within the first exon of p16\(^{\text{INK4A}}\) (p16\(^{\text{INK4A}}\) assay), is quantitative only in the presence of higher quantities of the template. Therefore, an assay quantifying an amplicon located within the second and third exon shared by p16\(^{\text{INK4A}}\) and p14\(^{\text{ARF}}\) was designed (CDKN2A assay), which is quantitative over 5 log units of template (Fig. 3.1.5).

![Diagram](image_url)

**Fig. 3.1.5.** Scheme of the CDKN2A locus and the localization of qPCR assays (adapted from Walker and Hayward, 2002a and 2002b). p16\(^{\text{INK4A}}\) and p14\(^{\text{ARF}}\) transcripts share the second and third exons but not the first. The p16\(^{\text{INK4A}}\) transcript uses exon 1\(\alpha\), whereas the p14\(^{\text{ARF}}\) transcript uses exon 1\(\beta\). The unshaded portions of each exon correspond to the untranslated regions of the transcripts. p16\(^{\text{INK4A}}\) qPCR assay detects amplicon in exon 1\(\alpha\), p14\(^{\text{ARF}}\) qPCR assay detects an amplicon in exon 1\(\beta\), while the CDKN2A assay detects an amplicon across the exon2/exon3 boundary. The details of the primers are listed in the Material and Methods, table 2.2 and table 2.3. The localization of sybr green CDKN2A assay and taqman CDKN2A assay (ABI) is similar.
Using the comparative Ct method and comparing in each sample the Ct values of CDKN2A assay and the assay that detect only p16\textsuperscript{INK4A} (p16\textsuperscript{INK4A} assay) or only p14\textsuperscript{ARF} (p14\textsuperscript{ARF} assay), it was estimated that the majority of the transcripts measured by CDKN2A assay in LCL 3CHT cultured with or without HT were p16\textsuperscript{INK4A} transcripts (data not shown).

After 14 days in culture in the absence of HT, there was a 2-2.5 fold increase in CDKN2A transcripts. After re-adding HT into the medium, CDKN2A transcripts gradually decreased over 12 days (Fig. 3.1.6A). When the period of culture in the absence of HT was extended to 33 days, there was a 3-5 fold increase in the level of CDKN2A transcripts. After re-adding HT into the medium, CDKN2A transcripts gradually decreased, but it seemed to require a longer period of at least 16 days (Fig. 3.1.6C). This was consistent with fold changes measured by the assay specific for p16\textsuperscript{INK4A} only transcripts (Fig. 3.1.6B and 6D). Both, the accumulation of CDKN2A transcripts in LCL 3CHT cultured 33 days without HT relative to the cycling controls cultured with HT and the decrease of CDKN2A transcripts after HT was re-added for 16 days relative to day 0, were found statistically significant (Fig. 3.1.6C).

Correspondingly, after reactivation of EBNA3C in LCL 3CHT, p16\textsuperscript{INK4A} protein expression gradually diminished (Fig. 3.1.6E). The levels of p16\textsuperscript{INK4A} protein correlated with p16\textsuperscript{INK4A} mRNA. The regulation of p16\textsuperscript{INK4A} in LCL 3CHT seems to be exclusively or at least predominantly at the level of the transcript as has been previously shown in various types of cell, including LCL (Gil and Peters, 2006; Maruo et al., 2006).

In the context of the total LCL 3CHT cell population, with extended time of culture without functional EBNA3C, p16\textsuperscript{INK4A} progressively accumulated and the population arrest became more profound. Depending on the degree of p16\textsuperscript{INK4A} accumulation and the intensity of proliferative arrest, different time periods were needed to fully repress p16\textsuperscript{INK4A} and restore the original rate of proliferation after reactivation of EBNA3C. Taken together, p16\textsuperscript{INK4A}
levels in LCL 3CHT cultured with or without HT seemed to be in inverse relation to the proportion of cells entering S phase.

**Fig. 3.1.6.** Repression of p16<sup>INK4A</sup> following reactivation of EBNA3C. (A) After 14 days without HT (0), total RNA was extracted from aliquots from two LCL 3CHT populations (-A and -C). HT was re-added to the remaining cells and further RNA samples were taken at the times indicated.
CDKN2A transcripts were quantified by qPCR. The histogram corresponds to CDKN2A mRNA relative to that in control cycling populations of each LCL 3CHT (ctrl). (B) As in (A) but using a p16<sup>INK4A</sup>-specific qPCR. (C) & (D) Similar assays to those described in (A) and (B) after 33 days without HT. ** p<0.005; *** p<0.001 (E) Western blot – probed with a p16<sup>INK4A</sup>-specific MAb – of cell lysates from LCL 3CHT-C cells to which HT was re-added after 14 or 33 days in its absence (0<sub>14</sub> and 0<sub>33</sub> respectively). The control (ctrl) is LCL 3CHT-C cells continuously cultured with HT. CDKN2A/p16<sup>INK4A</sup> mRNA and p16<sup>INK4A</sup> protein gradually decrease following EBNA3C reactivation. The results are representative of two similar experiments each including two LCL 3CHT lines.

Inactivation of EBNA3C leads to dephosphorylation of the retinoblastoma protein (Rb), reduced expression of p107 and an increase in p130; activation of EBNA3C reverses these processes

In the next step I tested the consequences of p16<sup>INK4A</sup> accumulation following EBNA3C inactivation on the downstream components of the p16<sup>INK4A</sup>-Rb axis. LCL 3CHT were cultured with HT (controls) or without HT for 14 and 33 days and subsequently, HT was re-added at day 14 and at day 33. In arrested cells, Rb was initially hypophosphorylated; as the arrest intensified with extended time of culture without HT, hypophosphorylation seemed to be accompanied by Rb downregulation (Fig. 3.1.7A). Simultaneously, with the length of time in culture in the absence of functional EBNA3C, p130 protein accumulated and p107 was diminished (Fig. 3.1.7B, samples annotated 0<sub>14</sub> and 0<sub>33</sub>). Consistently with previous data monitoring LCL 3CHT proliferation and p16<sup>INK4A</sup> repression in response to re-addition of HT, after reconstitution of functional EBNA3C, Rb was gradually phosphorylated and upregulated (Fig. 3.1.7B). Expression of p130, although still detectable, decreased as cells entered the proliferation cycle and conversely, p107 was upregulated (Fig. 3.1.7B).
Fig. 3.1.7. Regulation of the quantity and phosphorylation of pocket proteins in response to EBNA3C (A) Western blot analysis of three LCL 3CHT lines (-A, -B and -D) performed on whole cell lysates from cells cultured for 26 days with (+) or without HT (-). As the amount of EBNA3C protein decreases and p16INK4A accumulates, so hyperphosphorylated Rb (ppRb) protein disappears and only hypophosphorylated Rb (pRb) is detected. The levels of E2F1 and γ-tubulin (γ-tub) remain unchanged irrespective of the culture conditions. (B) Western blot analysis of extracts from LCL 3CHT-C after re-addition of HT to cultures starved of HT for either 14 or 33 days (0₁₄ and 0₃₃ respectively). A pan-specific anti-Rb MAb and phospho-specific MAb (Ser 807-811) both show an increase in hyperphosphorylated Rb (ppRb) after HT was added. After 12-16 days the degree of phosphorylation is equivalent to that in the proliferating control LCL 3CHT population (ctrl). As Rb becomes phosphorylated, expression of p107 increases and expression of p130 decreases. The level of γ-tubulin (γ-tub) does not alter throughout the experiment. Data shown are representative of at least three separate experiments in (A) and two biological replicates (LCL 3CHT lines) in (B).
Regulation of H3K27me3 and H3K4me3 across the p16<sup>INK4A</sup> locus in LCL 3CHT requires EBNA3C

The repression of p16<sup>INK4A</sup> by H3K27me3 mark and PcG silencers has been previously well characterized in primary fibroblasts. In pre-senescent proliferating fibroblasts H3K27me3 mark forms a broad peak centred on the first exon of p16<sup>INK4A</sup>. Induction of senescence is associated with eviction of PcG silencers and a profound reduction of H3K27me3 on the chromatin associated with p16<sup>INK4A</sup> exon 1 (Barradas et al., 2009). The p16<sup>INK4A</sup> exon 1 serves as a promoter-like transcription-defining region in p16<sup>INK4A</sup> locus.

We hypothesised that the transcriptional up-regulation of p16<sup>INK4A</sup> in the absence of functional EBNA3C might result from the loss of PcG-mediated repression. Therefore, ChIP combined with a set of qPCR assays detecting amplicons spaced across the entire CDKN2A locus was performed to assess the level of H3K27me3 at p16<sup>INK4A</sup> in LCL 3CHT cultured with or without HT (Fig. 3.1.8A). After EBNA3C inactivation, H3K27me3 at the p16<sup>INK4A</sup> exon 1 gradually decreased over the period of several weeks (Fig. 3.1.8B). This process could be reversed by reconstitution of functional EBNA3C after re-addition of HT with similar delay (Fig. 3.1.8B sample annotated +). The progressive reduction of H3K27me3 at p16<sup>INK4A</sup> with the length of time in culture without functional EBNA3C inversely correlated with the gradual p16<sup>INK4A</sup> transcriptional induction (Fig. 3.1.8C and D).
Fig. 3.1.8. Simultaneous ChIP analysis quantifying the H3K27me3 mark on p16$^{INK4A}$ exon 1 and qPCR analysis of p16$^{INK4A}$ transcription in response to EBNA3C. (A) Schematic of the human CDKN2A locus showing the location of coding exons (boxes) and transcription start sites (horizontal arrows) – not drawn to scale. The vertical (A-D) arrows refer to the approximate locations of primer pairs used for qPCR analysis of precipitated chromatin (as described in Materials and Methods, Table 2.7 and Table 2.8). (B) ChIP analysis of H3K27me3 distribution on p16$^{INK4A}$ exon1 (site C- two assays in similar location, see Table 2.8). Histogram shows the decline in H3K27me3 in LCL 3CHT-A cultured for up to 35 days without HT and H3K27me3 restoration after HT was re-added for 16 days (annotated as +). (C) Similar histogram as in (B) shows a decline in H3K27me3 relative to a cycling LCL 3CHT (day 0) in LCL 3CHT-C. (D) Corresponding increase in p16$^{INK4A}$ mRNA in LCL 3CHT-C quantified by qPCR.
Relatively high quantities of an activation-associated H3K4me3 were detected at $p^{16}{^\text{INK4A}}$ exon 1 compared to the surrounding regions even when EBNA3C was active and $p^{16}{^\text{INK4A}}$ was repressed (i.e. in LCL 3CHT with HT and with HT re-added) (Fig. 3.1.9B). This suggests that $p^{16}{^\text{INK4A}}$ exon 1 in LCL contains a bivalent chromatin domain which can facilitate prompt $p^{16}{^\text{INK4A}}$ induction following anti-proliferative or aberrant pro-proliferative signals. ChIP analyses were performed to assess the quantities of both modifications at the $p^{16}{^\text{INK4A}}$ locus in LCL 3CHT in response to EBNA3C. EBNA3C inactivation affected epigenetic marks at the $p^{16}{^\text{INK4A}}$ locus in a way consistent with transcriptional activation; the repressive H3K27me3 mark was reduced while the activation-related H3K4me3 modestly increased. After EBNA3C reactivation, the epigenetic modifications at $p^{16}{^\text{INK4A}}$ locus were reversed (Fig. 3.1.9A and B).

**Fig. 3.1.9.** ChIP analysis quantifying the H3K27me3 and H3K4me3 marks at the $p^{16}{^\text{INK4A}}$ locus **in response to EBNA3C.** (A) ChIP analysis of H3K27me3 distribution across the $CDKN2A$ locus in LCL 3CHT-A proliferating in the presence of HT, after 30 days without HT and 20 days after re-adding HT. (B) Similar ChIP analysis of H3K4me3. Repressive chromatin is detected at $p^{16}{^\text{INK4A}}$ exon 1 when EBNA3C is active and the chromatin is remodelled to support transcription following EBNA3C inactivation.
To confirm specificity of $p16^{\text{INK4A}}$ regulation, H3K27me3 and H3K4me3 quantities at $p16^{\text{INK4A}}$ locus were compared to the quantities at the surrounding upstream region located 4.5kb downstream of $p14^{\text{ARF}}$ transcription start site (assay A in Fig. 3.1.9A). As described previously (Barradas et al., 2009), in human primary fibroblasts H3K27me3 mark is broadly distributed at CDKN2A locus and spans into the chosen control region A. Similarly, the control region in LCL 3CHT was still associated with some although low H3K27me3 binding. However, H3K4me3 was present in insignificant amounts in the control region compared to $p16^{\text{INK4A}}$ exon 1. The peak binding of H3K27me3 (in the presence of functional EBNA3C) and H3K4me3 detected at $p16^{\text{INK4A}}$ exon 1 strengthens the evidence that the regulation of $p16^{\text{INK4A}}$ locus is specific. In addition, the presence of two inversely regulated modifications excludes the possibility that the reduction in histone methylation is due to nucleosome re-positioning and reduction of the total histone H3 at $p16^{\text{INK4A}}$ exon 1.

Parallel ChIP experiments were performed with anti-IgG antibody of the same species as the anti-H3K27me3/H3K4me3 antibodies to assess the level of background. The background binding was found to be insignificant in all lines and conditions. In addition, the anti-H3K27me3 and anti-H3K4me3 ChIPs were validated using qPCR assays located in control promoters either repressed ($\gamma$-globin) or active (GAPDH) in LCL 3CHT regardless of EBNA3C activity (Fig. 3.1.10A and B, qPCR of GAPDH and $\gamma$-globin in LCL 3CHT with or without HT – data not shown). $\gamma$-globin genes are developmentally regulated and usually expressed only in the fetal liver, spleen and bone marrow; otherwise they are repressed by H3K27me3 (Xu et al., 2010b; Sankaran et al., 2011). GAPDH is a highly expressed housekeeping gene, widely used as a positive control for anti-H3K4me3 ChIP (Rugg-Gunn et al., 2010).
Fig. 3.1.10. Validation ChIPs in LCL 3CHT. (A) Comparison of H3K27me3, H3K4me3 and IgG quantities at p16<sup>INK4A</sup> exon1 and repressed γ-globin promoter in LCL 3CHT cultured with HT, after 30 days without HT and 20 days after re-adding HT. (B) Similar analysis comparing p16<sup>INK4A</sup> exon1 and active GAPDH promoter. Insignificant background is detected at all times by anti-IgG ChIP. H3K27me3 is correctly detected at the repressed γ-globin promoter but not at the active GAPDH promoter, while reverse holds true for H3K4me3. The H3K27me3 and H3K4me3 marks at γ-globin promoter or GAPDH promoter are not regulated in response to EBNA3C.
In order to test whether in proliferating WT LCL, when \(p16^{INK4A}\) locus is tightly repressed, EBNA3C is being recruited to the \(p16^{INK4A}\), a ChIP analysis with purified anti-EBNA3C antibody was performed to quantify EBNA3C at \(p16^{INK4A}\) locus. However, to date, a positive control for EBNA3C ChIP, i.e. a cellular or viral locus occupied by EBNA3C, has not been reliably identified. It remains unclear whether the anti-EBNA3C antibodies available work in ChIP assays. Therefore it is hoped that ChIP-validated anti-FLAG antibody could be used to assess the EBNA3C association with \(p16^{INK4A}\) locus in LCL produced using recombinant EBV-BAC viruses encoding strep-FLAG (TAP)-tagged EBNA3C (Gill Parker and Kostas Paschos, personal communication).

**EBNA3C-mediated regulation of \(p16^{INK4A}\) does not require Rb**

To avoid biases due to limited number of cell lines, I decided to confirm our findings using six newly established LCL 3CHT established using PBL from several single donors. It was immediately apparent that one of the cell lines did not arrest after the removal of HT from the medium (data not shown). In addition to western blots, which failed to detect Rb protein in this cell line (LCL 3CHT-E) cultured with or without HT, it was found using qPCR that LCL 3CHT-E expressed very low levels of Rb mRNA in comparison to other LCL 3CHT lines and WT LCL (Fig. 3.1.11A and B). It has been well established that functional Rb regulates \(p16^{INK4A}\) levels through a negative feedback loop and that Rb-negative tumours (such as carcinoma of cervix) express high levels of \(p16^{INK4A}\) (Little and Stewart, 2010). However, even in this Rb-reduced cell line, \(p16^{INK4A}\) was repressed in the presence of active EBNA3C, and this repression was relieved after EBNA3C inactivation. Furthermore, ChIP analysis confirmed that \(p16^{INK4A}\) locus was still epigenetically repressed by EBNA3C even when Rb was notably reduced. H3K27me3 and H3K4me3 distribution across the \(CDKN2A\) locus in LCL 3CHT-E cells following EBNA3C inactivation and reactivation showed chromatin remodelling identical to that seen in other LCL 3CHT lines (Fig. 3.1.11C).
and D). This reinforces the view that the epigenetic regulation of $p16^{INK4A}$ by EBNA3C is independent of Rb expression.

**Fig. 3.1.1.** EBNA3C-mediated regulation of $p16^{INK4A}$ does not require Rb. (A) Western blot analysis of whole cell lysates from two LCL 3CHT (-D and -E) cultured for 26 days with (+) or without (-) HT. Although Rb is undetectable in LCL 3CHT, when HT is removed from the growth medium, EBNA3C decreases and $p16^{INK4A}$ (p16) increases. E2F1 and γ-tubulin (γ-tub) levels do not alter. (B) qPCR of the steady-state levels of Rb mRNA in LCL 3CHT-E relative to 3 other LCL 3CHT and a WT LCL all cultured with HT. (C) ChIP analysis of H3K27me3 distribution across the CDKN2A locus in LCL 3CHT-E cells (expressing little or no Rb) cultured for 21 days with HT or without HT. (D) Similar ChIP analysis of H3K4me3. The $p16^{INK4A}$ locus is epigenetically regulated by EBNA3C even in Rb-reduced LCL 3CHT-E line.
EBNA3A also contributes to the regulation of p16\(^{\text{INK4A}}\)

A conditional EBV-BAC EBNA3A-HT fusion virus has been produced in our lab. However, BL31 cell line infected with EBNA3A-HT virus in the presence of HT failed to repress proapoptotic BIM which was used as a read-out for the efficiency of the system. Furthermore there was evidence of WT EBNA3A protein in LCL produced with EBNA3A-HT virus (data not shown). We decided not to use this EBNA3A-HT virus, but will consider constructing a new recombinant, with a more stable EBNA3A-HT fusion.

Recently, EBNA3A was shown to regulate p16\(^{\text{INK4A}}\) transcripts in a microarray study using EBNA3A KO LCL (Hertle et al., 2009). In our hands also, LCL carrying EBNA3A KO recombinant viruses can be established; however, the outgrowth of LCL produced with EBNA3A KO virus is much slower and less efficient in comparison to LCL produced with WT or revertant viruses (Rob White, personal communication). It is likely there is a strong selection for pro-proliferative mutations in cells during transformation with EBNA3A KO viruses, so EBNA3A KO LCL may not be an optimal system to study EBNA3A function. Nevertheless, EBNA3A KO LCL have produced data consistent with a role for EBNA3A in p16\(^{\text{INK4A}}\) regulation.

It has been demonstrated in our lab that EBNA3C and EBNA3A co-operate to regulate cellular genes (Paschos et al., 2009; White et al., 2010). Despite the pitfalls mentioned above, I was keen to investigate whether EBNA3A-associated p16\(^{\text{INK4A}}\) repression shows similarities to EBNA3C-mediated repression. Therefore two EBNA3A KO LCL lines produced in our lab were tested to determine whether our system is equivalent to the one described by Hertle et al. (2009). It was confirmed that in both EBNA3A KO LCL p16\(^{\text{INK4A}}\) protein was indeed elevated in comparison to LCL established from the PBL of the same donors (as used to produce EBNA3A KO LCL) with WT virus. However, this increased expression was modest compared to p16\(^{\text{INK4A}}\) accumulation in LCL 3CHT cultured without HT (Fig. 3.1.12A). A comparison of steady state levels of H3K27me3 and H3K4me3 at the
p16\textsuperscript{INK4A} locus revealed that in the absence of EBNA3A, the ratio of H3K27me3 to H3K4me3 associated with p16\textsuperscript{INK4A} exon 1 was reversed relative to LCL established with WT EBV BACs (Fig. 3.1.12B and C). Although the remodelling of chromatin at p16\textsuperscript{INK4A} in EBNA3A KO was not as pronounced as in the case of EBNA3C inactivation, the low level of H3K27me3 and high level of H3K4me3 were consistent with a more transcriptionally active locus and the higher levels of p16\textsuperscript{INK4A} protein detected in the EBNA3A KO lines. This suggests that EBNA3A, together with EBNA3C, is involved in the chromatin remodelling of p16\textsuperscript{INK4A}.

Fig. 3.1.12. EBNA3A contributes to the regulation of p16\textsuperscript{INK4A}. (A) Western blot analysis of three LCL established with virus derived from B95.8-EBV BAC (WT) and two established with EBNA3A KO recombinants. The steady state levels of p16\textsuperscript{INK4A} (p16) are elevated in the EBNA3A KO cells relative to the WT-EBV infected cells. An LCL 3CHT (3CHT) with (+) or without (-) HT is shown for comparison. WB shown is a representative of three separate experiments each including two EBNA3A KO LCL. (B) & (C) ChIP analysis of H3K27me3 and H3K4me3 distribution on p16\textsuperscript{INK4A} exon 1 (site C) and site A in the CDKN2A locus from an EBNA3A KO LCL (EBNA3A KO) and a WT LCL (WT). The chromatin at p16\textsuperscript{INK4A} exon 1 is remodelled in EBNA3A KO LCL to support transcription.
LCL expressing CtBP-binding mutants of EBNA3C and EBNA3A grow relatively poorly and express higher than normal levels of p16INK4A

It has been demonstrated that EBNA3C and EBNA3A mutant proteins that are unable to bind CtBP are inferior in their ability to transform primary rat embryo fibroblasts in cooperation with activated Ras in comparison to WT EBNA3C and EBNA3A (Touitou et al., 2001; Hickabottom et al., 2002). Moreover, recently Mroz et al. (2008) found that CtBP is involved in the repression of p16INK4A but not p14ARF locus in primary human fibroblasts and keratinocytes. This led me to hypothesize that interaction of EBNA3C and/or EBNA3A with CtBP might contribute to the repression of p16INK4A in proliferating LCL.

Recombinant EBV-BAC viruses in which the CtBP-binding sites of EBNA3A (3A\textsuperscript{CtBP}) or EBNA3C (3C\textsuperscript{CtBP}) or both EBNA3s (E3\textsuperscript{CtBP}) were mutated were produced and validated (see Materials and Methods, pp. 64-67). It was confirmed by immunoprecipitations that in CtBP-binding mutant LCL the interaction between respective EBNA3s and CtBP was abolished and that the expression of EBV latent proteins did not exhibit any consistent changes in comparison to the respective revertant LCL or WT LCL (Skalska et al., 2010).

Although it was possible to establish LCL using all three types of CtBP-binding mutant viruses, the rate of outgrowth of these LCL was considerably slower than of the respective revertant or WT LCL (Rob White, personal communication). Even once LCL were established, CtBP-binding mutant LCL continued to show a growth defect, exhibiting reduced rate of population growth relative to WT and revertant LCL. They also tended to have a much lower maximum cell density, with CtBP-binding mutants struggling to grow much beyond $0.7 \times 10^6$ cells/ml (Skalska et al., 2010; Rob White, personal communication).

When p16\textsuperscript{INK4A} transcripts and protein were quantified by qPCR and WB respectively (Fig. 3.1.13) it was apparent that all the mutants express more p16\textsuperscript{INK4A} mRNA and protein than either the WT or revertant LCL.
Fig. 3.1.13. Expression of p16$^{\text{INK4A}}$ in CtBP-binding mutant LCL relative to revertant and WT LCL. (A) The relative levels of CDKN2A transcripts quantified by qPCR in E3$^{\text{CtBP}}$, 3A$^{\text{CtBP}}$, 3C$^{\text{CtBP}}$ and two rev$^{\text{CtBP}}$ LCL harvested 28 days after the infection of PBL with the recombinant EBVs. (B) The relative levels of CDKN2A transcripts in RNA extracted from two established E3$^{\text{CtBP}}$ LCL, two 3A$^{\text{CtBP}}$ LCL, 3C$^{\text{CtBP}}$ LCL, a revertant (rev$^{\text{CtBP}}$) and a WT LCL. (C) Western blot analysis of protein extracts from two E3$^{\text{CtBP}}$ LCL, two 3A$^{\text{CtBP}}$ LCL and a 3C$^{\text{CtBP}}$ LCL all established from PBL of a single donor. A revertant (rev$^{\text{CtBP}}$) and a WT LCL are shown for comparison. Levels of p16$^{\text{INK4A}}$ (p16) are shown relative to γ-tubulin (γ-tub). p16$^{\text{INK4A}}$ is increased in all CtBP-binding mutant LCL early during the outgrowth and later in culture. Data are representative of three separate experiments including WT and CtBP-binding mutant LCL established from PBL of three different single donors (my data and Rob White, personal communication).

Although the CtBP-binding mutant LCL examined here expressed elevated levels of p16$^{\text{INK4A}}$ protein, the cells were clearly capable of cell division. Rb competence was therefore investigated. Rb protein and mRNA expression in the CtBP-binding mutant LCL were notably reduced and some E3$^{\text{CtBP}}$ LCL appeared to lose Rb expression completely (Fig. 3.1.14, also Rob White, personal communication).
Fig. 3.1.14. Reduction of Rb in CtBP-binding mutant LCL. (A) Western blot analysis showing the reduction of Rb protein expression in the established E3\(^{\text{CtBP}}\) and 3A\(^{\text{CtBP}}\) LCL in comparison to rev\(^{\text{CtBP}}\) and WT LCL. The levels of p16\(^{\text{INK4A}}\) are shown for comparison. \(\gamma\)-tubulin (\(\gamma\)-tub) was used as a loading control. (B) Steady-state levels of Rb mRNA in the RNA harvested from the same lines as in (A). Data are representative of two separate experiments including WT and CtBP-binding mutant LCL established from PBL of two different single donors (my data and Rob White, personal communication).

Interaction of EBNA3C and EBNA3A with CtBP is necessary for the chromatin remodelling associated with the repression of \(p16^{\text{INK4A}}\)

CtBP has been implicated in PcG-mediated repression (Sewalt et al., 1999; Atchison et al., 2003; Basu and Atchison, 2010); in addition CtBP-containing supercomplexes have been shown to contain multiple histone modifying enzymes including a H3K4 dimethyl and monomethyl demethylase LSD1 (Shi et al., 2003). We therefore hypothesised that CtBP might co-operate with EBNA3C and EBNA3A in epigenetic repression of \(p16^{\text{INK4A}}\). ChIP analysis revealed that all CtBP-binding mutant LCL (E3\(^{\text{CtBP}}\), 3A\(^{\text{CtBP}}\) and 3C\(^{\text{CtBP}}\) LCL) contain active chromatin at the \(p16^{\text{INK4A}}\) locus in comparison to WT LCL and the corresponding revertant (Fig. 3.1.15A and B). The E3\(^{\text{CtBP}}\) LCL profile resembled those of LCL-3CHT cells cultured without HT. In contrast, the WT and revertant profiles resembled LCL 3CHT
cultured in the presence of HT (Fig. 3.1.15C and D and Fig. 3.1.9A and B). This implies that the interaction of both EBNA3C and EBNA3A with CtBP is important for EBV-mediated chromatin remodelling and repression of $p16^{INK4A}$ in LCL.

Fig. 3.1.15. The interaction of EBNA3C and EBNA3A with CtBP is necessary for the chromatin remodelling associated with the repression of $p16^{INK4A}$. (A) & (B) ChIP analysis of H3K27me3 and H3K4me3 at $p16^{INK4A}$ exon 1 (two assays in similar location within exon 1) on an E3\textsuperscript{CtBP} LCL, a 3A\textsuperscript{CtBP} and a 3C\textsuperscript{CtBP} LCL. Similar analysis of a WT LCL is shown for comparison. (C) & (D) ChIP analysis of H3K27me3 and H4Kme3 distribution across the CDKN2A in an E3\textsuperscript{CtBP} LCL. Similar assays performed on revertant (rev\textsuperscript{CtBP}) and WT LCL are shown for comparison. Repressive chromatin is maintained at $p16^{INK4A}$ locus only when EBNA3C and EBNA3A are able to bind CtBP.
The regulation of overall EZH2 expression or p16<sub>INK4A</sub> promoter DNA methylation does not play a major role in the mechanism of EBNA3C-mediated p16<sub>INK4A</sub> repression.

H3K27me3 mark is established by SET-containing EZH2 subunit of the PRC2 complex (Margueron and Reinberg, 2011). PRC2 core members (EZH2, SUZ12 and EED) are interdependent; the withdrawal of EZH2 or other core components of PRC2 complex results in the genome-wide de-repression of the PRC2 target genes (Landeira and Fisher, 2011). To assess whether the overall degree of EZH2 expression in LCL 3CHT contributes to the regulation of p16<sub>INK4A</sub> locus in response to EBNA3C, four LCL 3CHT lines, including the Rb-reduced line (LCL 3CHT-E), were cultured for 26 days with or without HT and the expression of EZH2 was assessed by western blotting of whole cell lysates.

In all Rb-competent lines, the expression of EZH2, a direct E2F1 target (Bracken et al., 2003), was significantly decreased. However, in Rb-reduced LCL3CHT line (-E), proliferation of which was not influenced by EBNA3C inactivation, EZH2 levels remained similar before and after EBNA3C inactivation (Fig. 3.1.16). However, the p16<sub>INK4A</sub> locus was de-repressed and H3K27me3 mark was erased after EBNA3C inactivation in all LCL 3CHT, including LCL 3CHT-E, irrespective of EZH2 levels (Fig. 3.1.11). This suggests that epigenetic regulation of p16<sub>INK4A</sub> by EBNA3C is not primarily through regulation of overall EZH2 expression.

The expression of H3K27me3 demethylases JMJD3/KDM6B and UTX/KDM6A was also assessed in LCL 3CHT cultured with or without HT and no changes in expression in response to EBNA3C activity were detected (data not shown).
Fig. 3.1.16. EZH2 expression in LCL 3CHT. Western blot illustrating the EZH2 expression in four LCL 3CHT including the Rb-reduced line (-E) cultured 26 days with (+) or without (-) HT. EZH2 is reduced following EBNA3C inactivation in all three LCL 3CHT in which proliferation was inhibited (- B, -C, -D). In Rb-reduced line (-E), the expression of EZH2 remains stable. This is consistent with EZH2 being a direct E2F1 target gene and implies EBNA3C does not regulate p16INK4A locus via modulation of overall EZH2 expression. EBNA3C-HT fusion protein was quantified to demonstrate the consistent EBNA3C inactivation followed by its degradation in all LCL 3CHT lines and γ-tubulin (γ-tub) was used as a loading control. WB shown is a representative of two replicate experiments.

Next, I asked whether DNA methylation status at p16INK4A changes in response to EBNA3C. Genomic DNA from 2 newly established LCL 3CHT lines (i.e. 3 months from the day of infection) cultured for 33 days with or without HT was isolated, bisulphite converted and the degree of the CpG island methylation at p16INK4A promoter was quantitatively measured by qPCR using commercially available CDKN2A methylight assay (Qiagen, for details see Materials and Methods, p.77). DNA isolated from BL31, where p16INK4A promoter is known to be heavily methylated, was used as a control, in addition to commercially available completely unmethylated and methylated control DNA. p16INK4A promoter in LCL 3CHT was not methylated in proliferating cells cultured with HT at the start of the experiment (day 0), or after 33 days of culture with and without HT (Fig. 3.1.17). Consistent with the findings of Maruo et al. (2011), DNA methylation of the p16INK4A promoter did not contribute to the changes in p16INK4A expression in response to EBNA3C and H3K27me3 alone was sufficient for p16INK4A repression in LCL with active EBNA3C.
Fig. 3.1.17. \( p16^{\text{NK4A}} \) promoter DNA methylation in newly established LCL in response to EBNA3C. Methylation-specific qPCR (methylight) was used to assess the degree of \( p16^{\text{NK4A}} \) promoter methylation in two LCL 3CHT (-A and -C) harvested in day 0 and then again after 33 days of being cultured with or without HT. DNA from BL31 and commercially available control DNA were used as controls. \( p16^{\text{NK4A}} \) promoter is unmethylated in these relatively newly established LCL regardless of EBNA3C activity. Results are representative of two similar experiments.
3.1.2 The association of EBNA3C and EBNA3A with components of CtBP supercomplexes

Objectives

Even though I was unable to detect EBNA3C, EBNA3A and CtBP at the p16\textsuperscript{INK4A} locus (data not shown), I clearly showed the co-operation of these viral oncoproteins with CtBP is required in the epigenetic repression of p16\textsuperscript{INK4A}. In addition, in a microarray analysis performed using LCL and BL31 carrying CtBP-binding mutants of EBNA3C and/or EBNA3A or revertant and WT viruses; it was shown that the ability of EBNA3C and/or EBNA3A to bind CtBP was required for regulation of a subset of cellular genes (Rob White, personal communication). Furthermore, the epigenetic repression of another EBNA3C and EBNA3A-regulated gene BIM by H3K27me3 was modestly but significantly reduced in BL31 carrying CtBP-binding mutants of EBNA3C and/or EBNA3A (Kostas Paschos, personal communication). In order to gain further insight into the mechanics of epigenetic regulation of p16\textsuperscript{INK4A} locus and perhaps other genes by EBNA3C and EBNA3A, I decided to investigate the composition of EBNA3C and/or EBNA3A and CtBP-containing complexes in B-cell derived lines.

Results

**EBNA3C and EBNA3A are co-immunoprecipitated with several chromatin remodelling members of CtBP supercomplex**

First, the co-immunoprecipitation procedure was tested by confirming the known association of EBNA3C and EBNA3A with CtBP in protein extracts from WT LCL. As expected, anti-CtBP antibody pulled down both EBNA3C and EBNA3A, but did not pull
down EBNA3B, which does not contain any CtBP-binding sites. Next, I tested whether H3K4 demethylase LSD1, a known component of CtBP supercomplexes, associates with EBNA3C and/or EBNA3A. Anti-LSD1 antibody convincingly pulled down EBNA3C and EBNA3A but not EBNA3B in WT LCL (Fig. 3.1.18). The same findings were confirmed in several WT LCL lines and with an alternative anti-LSD1 antibody (data not shown).

In further co-immunoprecipitation experiments, a panel of other members of CtBP supercomplex including HDAC1/2, Co-REST, G9a, Ubc 9 and HPC2 (Chinnadurai, 2007) were tested for their ability to pull down EBNA3s. Anti-co-REST pulled down EBNA3A but not EBNA3B in WT LCL protein extracts (Fig.3.1.19A). Co-REST-EBNA3C pull down was not included for technical reasons (both anti-CoREST and anti-EBNA3C antibodies available were of the same species). Furthermore, anti-HDAC1 pulled down EBNA3C, EBNA3A and CtBP in WT LCL (Fig. 3.1.19B).

**Fig 3.1.18.** Co-immunoprecipitation with anti-CtBP and anti-LSD1 antibody in WT LCL. On the right, western blot of EBNA3s precipitated by anti-CtBP and anti-IgG antibody from the same input. On the left, western blot depicting the quantity of EBNA3s precipitated by anti-LSD1 and the corresponding anti-IgG antibody from the same input. Input represents 10% of the total cell extract used for the immunoprecipitation. In WT LCL, co-immunoprecipitation procedure was validated by CtBP pull-down which correctly showed that anti-CtBP pulls down EBNA3C and EBNA3A but not EBNA3B. Anti-LSD1 similarly pulls down EBNA3C and EBNA3A but not EBNA3B in WT LCL. Data shown are representative of at least three replicate experiments including different WT LCL lines.
Fig 3.1.19. Co-immunoprecipitation with anti-Co-REST and anti-HDAC1 antibody in WT LCL. Input represents 5% of the total cell extract used for the immunoprecipitation. (A) Western blot of EBNA3A and EBNA3B precipitated by anti-co-REST and the corresponding anti-IgG antibody from the same input. (B) Western blot of EBNA3C, EBNA3A and CtBP1 precipitated by anti-HDAC1 and anti-IgG antibody from the same input. Anti-HDAC1 and anti-co-REST pull-down EBNA3A, and anti-HDAC1 pulls down EBNA3C in WT LCL. WBs shown are representative of two (in A) or three (in B) separate experiments.

To verify that these co-immunoprecipitations are not an artefact of using a single cell line, similar experiments were performed in BL31 infected with WT EBV-BAC virus (WT BL31). Anti-LSD1 and anti-HDAC1 pulled down EBNA3C (and CtBP) also in the protein extracts from WT BL31 (Fig. 3.1.20).

Fig. 3.1.20. Co-immunoprecipitation with anti-HDAC1 and anti-LSD1 antibody in WT BL31. Western blot of EBNA3C and CtBP1 precipitated by anti-HDAC1, anti-LSD1 and corresponding anti-IgG antibodies from the same input. Input represents 10% of the total cell extract used for the immunoprecipitation. Anti-LSD1 and to a lesser degree anti-HDAC1 pull down EBNA3C and CtBP1 in WT BL31 protein extracts. WB shown is a representative of two separate experiments.
EBNA3C binding of CtBP seems to make its association with LSD1 more robust

A direct physical interaction between EBNA3C and HDAC1 was reported previously (Radkov et al., 1999), so it is very likely that EBNA3C can bind HDAC1 in both CtBP-dependent and -independent manner. I was interested to determine whether the co-immunoprecipitation of LSD1 with EBNA3C and EBNA3A is strictly dependent on the intact CtBP-binding sites of EBNA3C and EBNA3A and is likely to be mediated via CtBP.

Anti-LSD1 pulled down EBNA3C in WT LCL more efficiently than in LCL produced with viruses where the CtBP-binding sites of either EBNA3C (3C\text{CtBP} LCL) or EBNA3A (3A\text{CtBP} LCL) or both EBNA3s were mutated (E3\text{CtBP} LCL) (Fig. 3.1.21A and B). Similarly, anti-HDAC1 seemed to pull down more EBNA3C in WT LCL (Fig. 3.1.21B).

Since in 3A\text{CtBP} LCL, the pull down of EBNA3C by anti-LSD1 was also reduced, it is possible that EBNA3C and EBNA3A co-operate in binding LSD1 through CtBP. It is yet to be investigated whether anti-LSD1 pulls down EBNA3A in CtBP-binding LCL less efficiently than in WT LCL and whether in 3C\text{CtBP} LCL, co-immunoprecipitation of LSD1 with EBNA3A will be reduced.

Anti-LSD1 antibody seemed to pull down slightly more EBNA3C than the negative control anti-IgG in protein extracts from E3\text{CtBP} LCL (Fig. 3.1.21B). It therefore remains to be excluded whether EBNA3C and/or EBNA3A with mutated CtBP-binding site(s) might still associate with LSD1.

Finally, I confirmed that the physical associations detected were not influenced by the different availability of the CtBP supercomplex constituents in various cell lines. According to the microarray studies and protein quantification, CtBP, LSD1, HDAC1 and Co-REST are either well or robustly expressed in both LCL and BL31 lines. Their expression was not found to be regulated by EBNA3s (Hertle et al., 2009; White et al., 2010 and data not shown).
Fig. 3.1.21. Co-immunoprecipitation with anti-LSD1 (and anti-HDAC1) antibody in WT and CtBP-binding mutant LCL. Input represents 10% of the total cell extract used for the immunoprecipitation. (A) Western blot of EBNA3C precipitated with anti-LSD1 antibody in WT LCL, E3CtBP, 3CtBP and 3AtBP LCL. Anti-LSD1 antibody pulls down EBNA3C more efficiently in WT LCL than in any CtBP-binding mutant LCL. (B) Western blot of EBNA3C precipitated by anti-HDAC1, anti-LSD1 and anti-IgG in WT LCL and E3CtBP LCL. Anti-LSD1 and perhaps also anti-HDAC1 pull down EBNA3C more efficiently in WT LCL than in E3CtBP LCL. Data are representative of two separate experiments including WT and CtBP-binding mutant LCL established from PBL of two different single donors.

EBNA3C, EBNA3A, CtBP, HDAC1 and LSD1 are not detectable at the 16INK4A locus

In addition to previous effort to locate EBNA3C at the 16INK4A locus, ChIP experiments were carried out to search also for occupancy by EBNA3A, CtBP, HDAC1 and LSD1. At the time of writing, neither EBNA3 oncproteins nor the constituents of the CtBP supercomplex could be convincingly detected at 16INK4A locus (data not shown). Currently, the ChIP technique and the choice of antibodies are being optimized.
3.1.3 Other potential mechanisms of $p16^{\text{INK4A}}$ de-repression

**H2AX is phosphorylated in LCL following EBNA3C inactivation and in CtBP-binding mutant LCL**

In eukaryotes, DNA double strand breaks have been shown to trigger the phosphorylation of serine 139 at the carboxy terminus of histone H2AX mediated by ATM kinase. H2AX phosphorylation is important for recruitment and maintenance of the DNA repair machinery at the site of the break (Rogakou et al., 1998). The accumulation of DNA damage or the activation of DNA-damage response (DDR) can be monitored by the increase in phosphorylated H2AX (gamma-H2AX) either by western blotting or immunofluorescence. It has been shown recently that following EBV infection of primary B lymphocytes, activation of the host cell ATM/CHK2-mediated DDR pathway suppresses B cell transformation. However, EBNA3C appeared to disable the ATM/CHK2-mediated host defence and permitted B cell transformation (Nikitin et al., 2010).

The $p16^{\text{INK4A}}$ locus is known to be activated in response to various cellular stresses, including DNA damage (Shapiro et al., 1998). Therefore it was tested whether DNA damage occurs and/or DDR is activated following EBNA3C inactivation. The degree of H2AX phosphorylation was assessed by western blotting in the whole cell lysates of three LCL 3CHT cultured for 26 days with or without HT. A modest increase in phosphorylation of H2AX was detected in all three lines cultured without HT. Similarly, the majority of CtBP-binding mutant LCL seemed to exhibit more gamma-H2AX than the respective revertant or WT LCL (Fig. 3.1.22). The question of EBNA3C having either a protective effect against DNA damage and/or attenuating the DDR pathway in established LCL was therefore further addressed in additional set of experiments (see Chapter 3.2).
Fig. 3.1.22. Phosphorylation of H2AX in LCL 3CHT and in CtBP-binding mutant LCL. Western blot quantifying the γ-H2AX in three LCL 3CHT (-B, -C, -D) cultured for 26 days with (+) or without (-) HT and in CtBP-binding mutant LCL and respective revertant or WT LCL. γ-tubulin (γ-tub) was used as a loading control. The phosphorylation of H2AX is modestly increased following EBNA3C inactivation and in CtBP-binding mutant LCL. WBs shown are representative of two experiments.

EBNA3C inactivation does not lead to increase in p38 MAPK phosphorylation

Since it has been established that stress-induced p38 mitogen-activated protein kinase (p38 MAPK) acts downstream of MEK to mediate Ras-induced p16INK4A expression and senescence (Han and Sun, 2007; Freund et al., 2011), it was tested whether p38 MAPK could be similarly activated by phosphorylation following EBNA3C inactivation. However, only partial phosphorylation of p38 MAPK was detected in all LCL 3CHT irrespective of EBNA3C activity (Fig. 3.1.23).

Fig. 3.1.23. Phosphorylation of p38MAPK in LCL in response to EBNA3C. Western blot quantifying the p38 MAPK phosphorylation in three LCL 3CHT (-B, -C, -E) cultured for 26 days with (+) or without (-) HT and in protein lysates from supplier validated as a positive and negative control. γ-tubulin (γ-tub) was used as a loading control. Partial p38 MAPK phosphorylation was detected in all lines regardless of EBNA3C activity.
ANRIL expression is positively correlated with CDKN2A expression in LCL

Since the long non-coding RNA (ncRNA) ANRIL, transcribed antisense to the INK4-ARF-locus, has been implicated in $p16^{INK4A}$ repression mediated by PcG in prostate cancer (Yap et al., 2010), I asked whether a similar mechanism could contribute to EBNA3C and EBNA3A-mediated $p16^{INK4A}$ repression in LCL. Since in the prostate cancer model, ANRIL expression inversely correlated with $p16^{INK4A}$ transcription and the increased ANRIL levels were thought to facilitate repression of the $p16^{INK4A}$ locus, the expression of CDKN2A mRNA and ANRIL ncRNA was examined in LCL 3CHT cultured with or without HT. ANRIL ncRNA was co-regulated simultaneously with CDKN2A mRNA in response to EBNA3C. ANRIL transcription followed the same trend as CDKN2A, but to a lesser extent (Fig. 3.1.24).

![Graph showing the co-regulation of ANRIL ncRNA with CDKN2A (p16^INK4A + p14^ARF) mRNA in LCL 3CHT lines. ANRIL, CDKN2A and control ncRNA transcript Malat-1 were quantified in two LCL 3CHT. LCL 3CHT-A was cultured with HT (A +HT), 14 and 31 days without HT (A -14 and A -31) and 12 days after HT was re-added (A re-add HT). Similarly, LCL 3CHT-C was cultured with HT (C +HT) and 21 days without HT (C -21). ANRIL ncRNA was upregulated following EBNA3C inactivation, however, to a lesser degree than CDKN2A mRNA. Control ncRNA Malat-1 remained unaltered regardless of EBNA3C activity.](image-url)
Since the positive correlation of CDKN2A and ANRIL transcription in LCL 3CHT was the opposite to that seen in prostate cancer, I tested whether these transcripts were generally co-expressed in LCL. The analysis was performed on a number of samples (approximately 30) including WT LCL, EBNA3A KO LCL and corresponding revertant, E3\textsuperscript{CtBP} and rev\textsuperscript{CtBP} LCL, EBNA3B KO LCL and revertants and several LCL 3CHT cultured with or without HT. A positive correlation between CDKN2A and ANRIL expression in LCL was observed (Fig. 3.1.25).

**Fig. 3.1.25. Positive correlation between CDKN2A mRNA and ANRIL ncRNA in LCL carrying various EBV-BAC recombinant viruses.** qPCR analysis of about 30 LCL samples including WT LCL, EBNA3A KO LCL and corresponding revertant; E3\textsuperscript{CtBP} LCL, EBNA3B KO LCL and revertants and LCL 3CHT cultured for up to 31 days with or without HT. ANRIL ncRNA expression shows positive correlation with CDKN2A mRNA expression. The samples of LCL 3CHT cultured without HT are highlighted by arrows.

All quantifications described above were performed using ANRIL assay positioned across the exon 1/2 boundary. However, the ANRIL gene consists of 20 exons, and the ANRIL transcripts are differentially spliced into at least 8 variants. In a further experiment, an amplicon positioned at the opposite end of this long transcript, across 15/16 exon
boundary, that might be part of the different splice variants, was quantified in the same samples. Although the transcription trend was similar, the extent of regulation appeared to be much higher for the amplicons positioned across the 15/16 exon boundary (Fig.3.1.26).

Fig. 3.1.26. Comparison of the quantity of two amplicons positioned in the opposite ends of the ANRIL transcript in the same samples. ANRIL transcripts were quantified using two assays positioned either in exon1/2 or exon 15/16 boundary in WT BL31, LCL 3CHT cultured with HT (LCL-A +HT), 31 days without HT (LCL-A -31) and after HT was re-added for 12 days (LCL-A re-add HT). Prostate cancer cell line (PC3) was used as a positive control. The regulation of the amplicons followed the same trend, however, the extend of the regulation was greater in case of the amplicon located across the exon 15/16 boundary.
3.1.4 Regulation of \( \text{p14}^{\text{ARF}} \) by EBNA3C in LCL 3CHT

Objectives

Although \( \text{p14}^{\text{ARF}} \) is transcribed from a separate promoter, it shares second and third exons with \( \text{p16}^{\text{INK4A}} \). As a result, \( \text{p14}^{\text{ARF}} \) and \( \text{p16}^{\text{INK4A}} \) can be regulated either separately or co-regulated in human cells and tissues depending on the context. I therefore tried to assess whether \( \text{p14}^{\text{ARF}} \) (in addition to \( \text{p16}^{\text{INK4A}} \)) is regulated by EBNA3C in LCL and investigate the functional significance of such regulation.

Results

EBNA3C might repress \( \text{p14}^{\text{ARF}} \); however \( \text{p14}^{\text{ARF}} \) de-repression following EBNA3C inactivation is not convincingly reflected at the level of protein or in the activation of the p53-p21\(^{\text{CIP1}}\) pathway

In LCL 3CHT cultured without HT, \( \text{p14}^{\text{ARF}} \) transcripts generally increased at the same time as \( \text{p16}^{\text{INK4A}} \); however, \( \text{p14}^{\text{ARF}} \) de-repression was not entirely consistent between cell lines and between experiments. The degree of \( \text{p14}^{\text{ARF}} \) mRNA upregulation varied greatly and was at times of small magnitude or entirely undetectable (Fig. 3.1.27 LCL 3CHT-C cell line). After the reactivation of EBNA3C by re-adding HT, \( \text{p14}^{\text{ARF}} \) mRNA gradually decreased with kinetics similar to \( \text{p16}^{\text{INK4A}} \) (Fig. 3.1.27).
Fig. 3.1.27. Repression of p14\textsuperscript{ARF} mRNA following EBNA3C reactivation. (A) After 14 days without HT (0), total RNA was extracted from aliquots from two LCL 3CHT populations (-A and -C). HT was re-added to the remaining cells and further RNA samples were taken at the times indicated. qPCR was performed to quantify p14\textsuperscript{ARF} transcripts. The histogram corresponds to p14\textsuperscript{ARF} mRNA relative to that in control cycling populations of each LCL 3CHT. (B) Similar assays to those described in (A) after 33 days without HT. p14\textsuperscript{ARF} transcripts are at times de-repressed following EBNA3C inactivation; however, not consistently in all lines and experiments.

It has been noted previously (O’Nions and Allday, 2004), that p14\textsuperscript{ARF} protein is very poorly expressed in WT LCL and is usually undetectable by western blotting. In LCL 3CHT cultured with HT, p14\textsuperscript{ARF} protein was barely detectable but increased to a detectable level within 14 days of culture without HT (Fig. 3.1.28A). The subtle increase of p14\textsuperscript{ARF} was further confirmed in other LCL 3CHT lines following EBNA3C inactivation (Fig. 3.1.28B); however this required the use of concentrated anti-p14\textsuperscript{ARF} antibody and an increased amount of protein extract loaded on the separating gel indicating that the expression level of p14\textsuperscript{ARF} in LCL with or without HT was indeed very low. Subsequently, it was tested whether p14\textsuperscript{ARF}-p53-p21\textsuperscript{CIP1} pathway is engaged in the absence of active EBNA3C. For up to 24 days following EBNA3C inactivation, the level of p53 protein did not increase (Fig. 3.1.28C) and p21\textsuperscript{CIP1} transcriptional up-regulation was not observed (Fig. 3.1.28D).
Fig. 3.1.28. **p14<sup>ARF</sup>-p53-p21<sup>CIP1</sup> axis in LCL 3CHT after inactivation of EBNA3C** (A) Western blot depicting the p14<sup>ARF</sup> protein levels following EBNA3C inactivation. After 14 and 33 days without HT, faint bands can be detected if concentrated anti-p14<sup>ARF</sup> antibody and higher amounts (60ug) of LCL 3CHT total protein are used. (B) Western blot of p14<sup>ARF</sup> protein in three LCL 3CHT (A, B, E) cultured for 26 days with (+) or without (-) HT. Subtle upregulation of p14<sup>ARF</sup> protein is visible in all three lines following EBNA3C inactivation. (C) Western blot monitoring p53 protein levels following inactivation of EBNA3C. No increase in p53 protein is observed up to 24 days without HT. (D) qPCR monitoring p21<sup>CIP1</sup> transcripts following inactivation of EBNA3C. The levels of p21<sup>CIP1</sup> mRNA are stable for at least 24 days without HT. Data are representative of three separate experiments including at least three different LCL 3CHT lines.
In CtBP-binding mutant LCL, p14\(^{ARF}\) also seemed to be modestly upregulated at the level of transcription in comparison to the revertant and WT LCL (Fig 3.1.29A). However, p14\(^{ARF}\) protein could not be easily detected in CtBP-binding mutants (data not shown), and the p53-p21\(^{CIP1}\) axis did not appear to be activated (Fig. 3.1.29B and data not shown).

Fig. 3.1.29. p14\(^{ARF}\) and p21\(^{CIP1}\) transcripts in CtBP-binding mutant LCL. (A) The relative levels of p14\(^{ARF}\) transcripts in RNA extracted from two established E3\(^{CIBP}\) LCL, two 3A\(^{CIBP}\) LCL, 3C\(^{CIBP}\) LCL, a revertant (rev\(^{CIBP}\)) and a WT LCL. p14\(^{ARF}\) transcripts are upregulated in all CtBP-binding mutant LCL in comparison to revertant and WT LCL. (B) The relative levels of p21\(^{CIP1}\) transcripts in RNA extracted from three WT LCL, two established E3\(^{CIBP}\) LCL and two 3A\(^{CIBP}\) LCL. p21\(^{CIP1}\) transcripts do not consistently increase in CtBP-binding mutant LCL.

p14\(^{ARF}\) promoter contains only background levels of H3K27me3 in LCL regardless of EBNA3C activity or the ability of EBNA3C and EBNA3A to bind CtBP

To date it is not well understood whether the repression of p14\(^{ARF}\) in human pre-senescent or tumour cells is mediated by polycomb-based H3K27me3 owing to the low quantities of H3K27me3 detectable at p14\(^{ARF}\) promoter, and the absence of a ‘peak’ of H3K27me3 when p14\(^{ARF}\) is repressed. Intriguingly, distinct H3K4me3 peak has been found associated
with the $p14^{ARF}$ promoter even when $p14^{ARF}$ is repressed (Bracken et al., 2007; Kia et al., 2008; Barradas et al., 2009; Li et al., 2009).

Consistent with the published findings, in LCL 3CHT lines cultured with or without HT and in CtBP-binding mutant and revertant LCL, in the context of H3K27me3 distribution across the entire CDKN2A locus, only low levels of H3K27me3 could be detected at the $p14^{ARF}$ promoter at any time (Fig. 3.1.30B and D). In two LCL 3CHT lines, the $p14^{ARF}$ promoter was not found to be convincingly regulated via H3K27me3 in a time-course following EBNA3C switch (Fig.3.1.30A). Intriguingly, the distinct peak of H3K4me3 detected at the $p14^{ARF}$ promoter was quantitatively equivalent to that localized at $p16^{INK4A}$ exon1 and was stable regardless of EBNA3C activity or the ability of EBNA3C and EBNA3A to bind CtBP (Fig. 3.1.30C and E).
Fig. 3.1.30. ChIP–qPCR analysis of H3K27me3 and H3K4me3 at the p14\textsuperscript{ARF} promoter in LCL.

(A) ChIP analysis of H3K27me3 distribution on p14\textsuperscript{ARF} promoter (using two independent qPCR assays) in LCL 3CHT-A cultured for up to 33 days without HT and after HT was re-added for 16 days (sample annotated +). (B) ChIP analysis of H3K27me3 distribution across the entire CDKN2A locus including p14\textsuperscript{ARF} promoter (p14) in LCL 3CHT cultured with HT, after 30 days without HT and 20 days after re-adding HT. (C) Similar ChIP analysis of H3K4me3 distribution as in (B). (D) & (E) Similar analysis as in (B) and (C) in E3\textsuperscript{CtBP} and rev\textsuperscript{CtBP} or WT LCL. Histograms show very low levels of H3K27me3 and a distinct peak of H3K4me3 at p14\textsuperscript{ARF} promoter in all lines.
Summary key points

- EBNA3C and EBNA3A together repress $p16^{\text{INK4A}}$ at the level of transcription
- EBNA3C inactivation or the absence of EBNA3A reset the epigenetic status of $p16^{\text{INK4A}}$ locus to support transcription
  - repressive H3K27me3 is diminished
  - activation-associated H3K4me3 moderately increases
- Interaction between EBNA3C and EBNA3A and CtBP is required to maintain repressive chromatin at the $p16^{\text{INK4A}}$ locus
- LSD1, Co-REST and HDAC1 are found in complexes containing EBNA3C and EBNA3A
- ANRIL expression is positively co-regulated with CDKN2A in LCL
- EBNA3C might repress $p14^{\text{ARF}}$, however not via H3K27me3 and without impacting on the p53-p21$^{\text{CIP1}}$ axis

Discussion

DNA viruses often infect cells within their human or animal hosts that are differentiated and have left the cell cycle. They therefore evolved multiple strategies to force the infected cells back into the cell cycle and gain access to the replication machinery (Ferrari, Berk and Kurdistani, 2009). It is common for the oncoviruses to inactivate simultaneously both Rb and p53 pathways. Oncoproteins such as adenoviral E1A, papillomaviral E7 and E6 and SV40 large T antigen target cell cycle regulators Rb and/or p53 directly to disrupt cell cycle checkpoints (Howley and Livingston, 2008). However, the evidence for direct association
between an EBV protein and Rb or p53 is not as convincing as in other tumour viruses and it seems that EBV might use a more distinctive strategy to facilitate B cell proliferation.

**EBV in regulating epigenetic processes**

Like other herpesviruses, EBV epigenetically regulates its own genome to control the establishment and maintenance of latency and to evade the host immune system (Günther and Grundhoff, 2010; Takacs et al., 2010; Tempera et al., 2010). Similarly, through epigenetic mechanisms, EBV regulates host transcription to ensure a favourable environment for its persistence by modifying genes to prevent apoptosis or growth arrest (White et al., 2010). Recently, our lab has demonstrated that EBNA3C and EBNA3A co-operate to epigenetically repress the proapoptotic regulator BIM by trimethylation of H3K27 and subsequent DNA methylation at the *BIM* promoter. EBNA3C and EBNA3A inhibit productive BIM transcription in BL31 by two distinct but perhaps related mechanisms. Firstly, EBNA3C and EBNA3A are both essential for the complete assembly of PRC2 on the *BIM* promoter. Secondly, EBNA3C and EBNA3A activity (either directly or indirectly) prevents the phosphorylation of Ser 5 on Pol II on a previously H3K4me3-marked *BIM* promoter (Paschos et al., 2009; Paschos et al., under revision). On the whole, there is accumulating evidence that EBNA3C and EBNA3A modulate the expression of cellular genes by epigenetic mechanisms, specifically by regulating the establishment and maintenance of H3K27me3 at specific promoters.

**EBNA3C and EBNA3A co-operation**

Although EBNA3C and EBNA3A have probably originated by a duplication event in an ancestral EBV genome, there is no evidence to suggest that their functions are broadly redundant. However, they share two important features, the ability to bind RBPJk/CBF1 and to bind CtBP. EBNA3C and EBNA3A are also likely present in the cell in the same
complexes. They were reproducibly co-immunoprecipitated in protein extracts from LCL and EBV positive BL31 lines (M. Hickabottom and K. Paschos, personal communication) and their physical association has recently been confirmed by a very reliable anti-FLAG pull-down in the protein extracts from LCL carrying EBNA3C strep-FLAG (TAP)-tagged virus (Oak Watanatanasup and Gill Parker, personal communication). In a microarray study, apart from genes regulated separately by either EBNA3C or EBNA3A, a large subset of cellular targets was found to be co-regulated by both EBNA3s (White et al., 2010). Since inactivation of either EBNA3C or EBNA3A in LCL was reported to result in similar growth inhibition (Maruo et al., 2003 and 2006), it does not come as a surprise that both EBNA3C and EBNA3A contribute to $p16^{\text{INK4A}}$ repression in LCL and that they achieve this by directly or indirectly remodelling the chromatin at $p16^{\text{INK4A}}$ locus.

**Conditional EBNA3C**

EBNA3C appears to be absolutely essential for primary B cell immortalization and can only be inactivated conditionally once the LCL have been established. Due to the strict requirement for EBNA3C function in transformation, successful establishment of LCL 3CHT means that modified EBNA3C retains the functions needed in transformation. In addition, by dividing the lines and studying simultaneously the cells cultured without HT alongside those cultured with HT, the system provides a very reliable isogenic control. Importantly, the expression of LMP1 does not decrease following EBNA3C inactivation in LCL 3CHT (Fig. 3.1.2 and Maruo et al., 2006). LMP1 has been shown previously by others to repress $p16^{\text{INK4A}}$ (Ohtani et al., 2003) and LMPs are currently emerging as important regulators of the cellular epigenome in EBV-associated epithelial tumours (Paschos and Allday, 2010). It is therefore crucial that an indirect mechanism of $p16^{\text{INK4A}}$ regulation via LMP1 in LCL 3CHT system could be excluded.
Putative mechanisms of EBNA3C and EBNA3A-mediated p16\(^{\text{INK4A}}\) epigenetic repression

I have shown that EBNA3C inactivation initiates chromatin remodelling that resets the epigenetic status of the p16\(^{\text{INK4A}}\) promoter. In the presence of active EBNA3C the p16\(^{\text{INK4A}}\) promoter region displays repressive chromatin and the locus is closed for transcription. In contrast when EBNA3C is inactivated the chromatin is remodelled into an active state supporting transcription (Fig. 3.1.9). LCL carrying EBNA3A KO virus show similar chromatin remodelling changes at the p16\(^{\text{INK4A}}\) locus as in the case of EBNA3C inactivation, perhaps to a lesser extent (Fig. 3.1.12B and C).

EBNA3C and EBNA3A might be acting on chromatin at the p16\(^{\text{INK4A}}\) locus either directly or indirectly. Although *Paramecium bursaria* Chlorella virus 1 encodes its own viral H3K27me3 methyltransferase (Mujtaba *et al.*, 2008), surprisingly, this is the only H3K27 methyltransferase identified to date in addition to PcG EZH1 and EZH2. Structural and biochemical properties of EBNA3C and EBNA3A were extensively studied in the past (Jiang, Cho and Wang, 2000; West *et al.*, 2004; West, 2006). Neither EBNA3C nor EBNA3A were judged to possess any enzymatic activity, but they certainly could facilitate the activity of other cellular chromatin remodelling enzymes, most notably PcG, at the p16\(^{\text{INK4A}}\) locus in several ways. Even though EBNA3C and EBNA3A cannot bind DNA directly, they may target PRC complexes to the p16\(^{\text{INK4A}}\) locus through interaction with p16\(^{\text{INK4A}}\) promoter-binding proteins or binding of sequence-specific non-coding RNA. Alternatively, EBNA3C and EBNA3A might be necessary for the maintenance of the repressive complexes at 16\(^{\text{INK4A}}\) locus, fine-tuning their composition or stability or might be required for the transmission of the epigenetic marks at p16\(^{\text{INK4A}}\) locus during the cell division. Of course, these viral proteins might not be present at p16\(^{\text{INK4A}}\) locus or physically engaged in targeting but instead transcriptionally regulate chromatin-modifying machinery or sequence-specific elements engaged at p16\(^{\text{INK4A}}\) locus. The co-operation of a cellular co-factor CtBP with these viral oncoproteins will be discussed later.
The nature of the proliferative arrest and the reversible phenotype

It is generally unknown whether B cells ‘senesce’ similar to fibroblasts and other cell types and whether following EBNA3C inactivation, some LCL could undergo senescence per se, a durable block of cell proliferation that becomes independent of p16\(^{INK4A}\) expression or Rb hypophosphorylation (Dai and Enders, 2000). Although following EBNA3C inactivation, LCL upregulate p16\(^{INK4A}\) and accumulate in G1 phase of the cell cycle, there are no apparent changes in the cell morphology and beta-galactosidase accumulation is not detectable (data not shown). It has not been tested whether the proliferative arrest is accompanied with expression of known components of senescence-associated secretory phenotype.

The p16\(^{INK4A}\)-Rb pathway in LCL, although under suppression, remains intact and can be activated if EBNA3C function is withdrawn (Fig. 3.1.6 and Fig.3.1.7). I have shown that both the de-repression of the p16\(^{INK4A}\) locus and the proliferative arrest following EBNA3C withdrawal in LCL 3CHT system are reversible at the level of total cell population after EBNA3C is reactivated by re-adding HT (Fig. 3.1.4B and C and Fig. 3.1.6 and Fig. 3.1.9). It is possible that either the elevated p16\(^{INK4A}\) levels are re-repressed in the majority of cells following EBNA3C reactivation allowing the entry into the cell cycle (i.e. a genuine reversal of proliferative arrest) or a small non-arrested subpopulation of cells (i.e. the portion of cell which retained p16\(^{INK4A}\) repression and kept proliferating) comes to dominate the culture following EBNA3C reactivation.

Further evidence acquired using a system of continuously proliferating LCL 3CHT where the selection based on proliferative advantage has been removed (described in detail in Chapter 3.2.1) indicated that, although both of the above scenarios remain possible, the gradual p16\(^{INK4A}\) repression and the reversal of the proliferative arrest following EBNA3C reactivation are unlikely to be driven by the pro-proliferative selection pressures in cell culture, since even when EBNA3C is reactivated in continuously proliferating LCL that have
not been arrested, EBNA3C reactivation still leads to gradual epigenetic repression of \( p16^{\text{INK4A}} \) (described in detail in Chapter 3.2.1).

**Bivalent domain and stability of H3K27me3**

A narrow well-defined peak of H3K4me3 is present at the \( p16^{\text{INK4A}} \) exon 1 even when LCL are cultured with HT, EBNA3C is active and \( p16^{\text{INK4A}} \) expression is repressed (Fig. 3.1.9B). The co-existence of H3K4me3 and H3K27me3 peaks on the \( p16^{\text{INK4A}} \) exon 1 represents a bivalent domain and enables the expression flexibility of this locus. In the event of aberrant pro-proliferative signal or anti-proliferative signals (such as oncogenic stress, oxidative stress or DNA damage), the \( p16^{\text{INK4A}} \) locus is prepared for efficient rapid activation.

H3K27me3 is known to cause local formation of heterochromatin which is labile or readily reversible; however, this type of histone methylation can facilitate methylation of DNA of the same region, particularly in the development of cancer (Baylin, 2005). DNA methylation represents a more stable modification and can ‘fix’ the repression of the locus. It has been shown that PcG interact with DNMT and recruit them to chromatin. The sites of PcG binding which are largely unmethylated in normal tissues serve during tumorigenesis as a map to direct methylation. CpG islands de novo methylated in cancer have often been previously marked by the presence of PcG proteins and H3K27me3 (Cedar and Bergman, 2009). CpG methylation of the \( p16^{\text{INK4A}} \) promoter frequently occurs in high-grade non-Hodgkin lymphoma, mucosa-associated lymphoid tissue lymphoma and most BL (Klangby, et al., 1998; Platt, Carbone and Mittnacht, 2002). Oncoviruses frequently cause the same modifications or mutations as seen in sporadic cancers of non-viral aetiology, only with much greater efficiency. DNA methylation of the \( p16^{\text{INK4A}} \) promoter could represent a ‘hit and run’ mechanism of permanent oncogenic alteration established by the transient activity of the EBNA3C and EBNA3A viral oncoproteins. The stable, initiation factor-independent repression of \( p16^{\text{INK4A}} \) by promoter DNA methylation could contribute to the
lymphomagenesis even in EBV-associated tumours that no longer express the latency III programme that includes EBNA3C and EBNA3A.

**Rb independence**

An LCL 3CHT cell line in which Rb protein is undetectable, regardless of the presence of functional EBNA3C, was produced. In this operationally Rb-negative cell line, p16$^{\text{INK4A}}$ was epigenetically repressed in the presence of functional EBNA3C and de-repressed after EBNA3C inactivation (Fig. 3.1.11). Therefore, p16$^{\text{INK4A}}$ regulation by EBNA3C in LCL 3CHT appears to be Rb-independent. The repression of p16$^{\text{INK4A}}$ in Rb-negative LCL 3CHT-E is functionally irrelevant. The observation that EBNA3C epigenetically repressed p16$^{\text{INK4A}}$ expression even when no additional proliferation advantage could be gained implies that repression of p16$^{\text{INK4A}}$ by EBNA3C is specific, although not necessarily direct.

**EBNA3C and EBNA3A co-operation with CtBP supports tumorigenesis**

CtBP was discovered by virtue of its binding to adenoviral oncoprotein E1A. Binding of E1A to CtBP antagonizes the function of CtBP; E1A mutants unable to bind CtBP show enhanced transformation efficiency (Schaeper et al., 1995). In contrast, EBNA3C and EBNA3A mutants unable to bind CtBP have been less effective in immortalizing and transforming primary rat embryo fibroblasts in cooperation with oncogenic Ras (Touitou et al., 2001; Hickabottom et al., 2002). In addition, LCL produced by transformation with CtBP-binding mutant viruses grow out much slower than WT LCL and fail to repress p16$^{\text{INK4A}}$. Taken together, binding of EBNA3C and EBNA3A to CtBP seems to augment the transformation efficiency and LCL outgrowth. Similarly, Marek’s disease virus, a herpesvirus which induces T cell lymphoma in chickens, requires its nuclear oncoprotein Meq to interact with CtBP for tumorigenesis (Brown et al., 2006).
Although the outgrowth of CtBP-binding mutant LCL was delayed and the established mutants still exhibited a partial growth defect in comparison to revertant or WT LCL, they were still capable of proliferation despite the elevated p16\textsuperscript{INK4A} levels. It is therefore not surprising that CtBP-binding mutant LCL generally contained reduced overall Rb protein levels (Fig. 3.1.14). In addition, decreased Rb mRNA has been reported by others in EBNA3A KO LCL (Hertle \textit{et al.}, 2009). Since Rb loss confers a common proliferative advantage, it is probable that elevated p16\textsuperscript{INK4A} in CtBP-binding mutant LCL as well as in EBNA3A KO LCL creates a selection pressure during transformation and outgrowth leading to the loss or decreased expression of Rb.

**Contribution of CtBP in EBNA3C and EBNA3A-mediated epigenetic repression of cellular genes**

Throughout the duration of this project, others have reported that EBNA3C and EBNA3A seem to modulate their target genes mainly through H3K27me3 (White \textit{et al.}, 2010; Paschos \textit{et al.}, under revision; Ian Groves, personal communication). I have shown in this study, that co-operation of EBNA3C and EBNA3A with CtBP is required for repression of p16\textsuperscript{INK4A} via H3K27me3 and perhaps its interplay with H3K4me3 (Fig. 3.1.15). In addition, in BL31 infected with EBV-BACs carrying CtBP-binding mutants of EBNA3C and EBNA3A, the repression of another EBNA3C and EBNA3A-target gene BIM by H3K27me3 is modestly but significantly reduced (Kostas Paschos, personal communication). Furthermore, in an microarray analysis comparing the gene sets regulated by EBNA3C and/or EBNA3A in B-cell derived lines with the cellular genes regulated in the cell lines carrying the CtBP-binding mutants of EBNA3C and/or EBNA3A, CtBP was shown to be important for the regulation of a subset of cellular targets of EBNA3C and/or EBNA3A (Rob White, personal communication).

However, the same microarray analysis also clearly identified a large group of EBNA3C and/or EBNA3A-regulated genes, expression of which was not influenced by the ability of
EBNA3C and/or EBNA3A to bind CtBP (Rob White, personal communication). Although CtBP is known to have a role in PcG-mediated repression (Atchison et al., 2003; Basu and Atchison, 2010), I propose a model in which EBNA3C and EBNA3A possess a CtBP-independent ‘core’ ability to recruit PcG, through as yet uncharacterized domain(s) outside of the CtBP-binding site, which is important for the regulation of majority of EBNA3C and EBNA3A target genes (Fig. 3.1.31).

In addition, EBNA3C and EBNA3A can associate with CtBP which might augment the repression of a distinct subset of cellular genes. Interaction of CtBP with EBNA3C and EBNA3A might mediate association with targeting sequence-specific elements (TF or non-coding RNA), stabilize assembly of PRC complexes at the EBNA3C and/or EBNA3A-target loci, recruit chromatin remodelling enzymes other than PcG that enforce the repression (such as HDACs or G9a) and recruit LSD1 which can act in H3K4 demethylation and in the maintenance of bivalency.

EBNA3C and/or EBNA3A might interact with several other cellular co-factors or TFs such as RBPJk/CBF1 to regulate distinct gene sets or allow for different context-dependent transcriptional outcome. Interaction of EBNA3C and EBNA3A with RBPJk/CBF1 was shown to be essential for the maintenance of LCL proliferation (Maruo et al., 2005 and 2009). The role of RBPJk/CBF1 in the EBNA3C and/or EBNA3A-mediated epigenetic regulation of cellular genes (e.g. in the recruitment of PRC2 complex to the promoters of EBNA3C and/or EBNA3A-regulated target genes) remains to be investigated.
EBNA3C, EBNA3A, CtBP and macromolecular complexes

In eukaryotic cells, multidimensional protein networks consisting of numerous macromolecular protein assemblies carry out most cellular processes (Gavin et al., 2002). The varying modes of co-operation and communication of the protein subunits in macromolecular assemblies enable complex fine-tuned cellular processes but are very challenging to dissect.

CtBP is known to have the potential to recruit multiple different chromatin remodelers depending on the cellular context. Incorporation of EBNA3C and EBNA3A in CtBP co-repressor supercomplexes might alter either stability or composition of these assemblies at EBNA3C and/or EBNA3A-regulated loci. EBNA3C and EBNA3A might bridge the
chromatin remodelling enzymes and other constituents within the CtBP supercomplex or with other cofactors and enzymes. More than 30 different transcription factors and DNA-binding factors have been reported to recruit CtBP to mediate the transcriptional repression of various target genes, mostly through the PLDLS binding motifs (Kuppuswamy et al., 2008). Although neither EBNA3C nor EBNA3A appear to have a direct DNA-binding activity, this does not exclude the possibility that they have an active role in gene targeting. It has been shown that non-DNA-binding factors can enhance DNA-binding specificity of transcriptional regulatory complexes or even generate a novel specificity (Siggers et al., 2011).

LSD1, Co-REST and HDAC1 were found to co-immunoprecipitate with EBNA3C and with EBNA3A, which indicates they might be a part of the same complex within a cell (Fig. 3.1.18, Fig. 3.1.19 and Fig. 3.1.20). LSD1, a part of a new class of histone demethylating enzymes, removes the activating H3K4 mono- and dimethylation mark but can also remove the repressive H3K9 dimethylation (H3K9me2) mark when complexed with the androgen receptor. In addition, LSD1 is essential for the maintenance of global DNA methylation through demethylation of a non-histone substrate, DNMT1 (Nicholson and Chen, 2009).

Co-immunoprecipitation of EBNA3C and also EBNA3A with LSD1 (even if mediated via CtBP, Fig. 3.1.21) is of particular interest since LSD1 functions in the maintenance of bivalent promoters. In human embryonic stem cells, LSD1 acts in the silencing of several developmental genes by regulating the balance of H3K4 and H3K27 methylation at their promoters (Adamo et al., 2011). In a similar manner, LSD1 can promote tumour development by maintaining the undifferentiated phenotype in cancer through aberrant silencing of a subset of tumour suppressor genes (Schulte et al., 2009).

Furthermore, LSD1/Co-REST was shown to be co-recruited to DNA with members of PRC2 complex by long intergenic non-coding (linc) RNA HOTAIR (Tsai et al., 2010). HOTAIR physically bridges LSD1 and PRC2 complexes and this mechanism was proposed to
couple histone H3K27 trimethylation and H3K4 demethylation and act in the maintenance of bivalent domains at the promoters. LincRNAs may provide a widespread mechanism for coordinating the activity of several histone-modifying enzymes. EBNA3C and EBNA3A could (via an interaction with LSD1/Co-REST) partake in the assembly of such complex structures of chromatin modifiers on the promoters of their target genes (Fig. 3.1.32).

Fig. 3.1.32. Synchronized recruitment of PRC2 and LSD1/Co-REST to cellular gene promoters by linc RNAs (modified from Kaikkonen, Lam and Glass, 2011). EBNA3C and EBNA3A through associating with LSD1/Co-REST could partake in the assembly of such complexes at the promoters of their target genes.

ANRIL

ANRIL was found to be expressed at a relatively low level in WT LCL (data not shown). However, the majority of ncRNAs (with some notable exceptions) are low-abundance transcripts when compared to messenger RNAs and many are only present in certain cell types, developmental stages, or growth conditions. Even very low abundance transcripts might be functionally important. For example, any mechanism involving a high-affinity interaction of RNA with DNA sites might proceed with only a few transcripts per cell (Pontig and Belgard, 2010). In theory, no more than two copies of ANRIL transcripts per cell would be necessary to mediate epigenetic repression in cis at a single gene locus. Recently, two such models of ANRIL-mediated epigenetic repression were described, ANRIL’s function
being the recruitment of polycomb complexes to the promoters of neighbouring genes. In diploid fibroblasts, ANRIL was bound to PRC2-component SUZ12 and was required for the repression of the \( p15^{INK4B} \) locus. In prostate cancer cell lines and tissues, ANRIL bound CBX7, one of five mammalian orthologues of Drosophila Polycomb, within the PRC1 and was associated with the recruitment of the polycomb proteins to the \( p16^{INK4A} \) locus. In addition, both CBX7 and ANRIL were found at elevated levels in prostate cancer tissues (Kotake et al., 2010; Yap et al., 2010).

In contrast to the prostate cancer model, a positive correlation between ANRIL and CDKN2A expression was found in LCL (Fig. 3.1.24 and Fig. 3.1.25). Such co-regulated expression of CDKN2A and ANRIL has been previously described in several genome-wide association studies (Cunnington et al., 2010; Pasmant et al., 2010). CDKN2A and antisense-localized ANRIL genes are transcribed divergently from transcription start sites that are in close proximity. Transcription of DNA across or in the surrounding area of the CDKN2A promoters can influence CDKN2A expression in several ways. Although the ANRIL promoter is currently not characterised, it may share promoter elements with CDKN2A that allow for co-regulation (Cunnington et al., 2010). Alternatively, antisense transcription might facilitate removal of nucleosomes and allow easier access of transcription factors to the surrounding DNA elements. Furthermore, epigenetic changes are not always confined into discrete loci, but frequently span large regions encompassing both coding and non-coding sequences, e.g. large organized chromatin K9 modifications (LOCKs) span up to several megabases (Wen et al., 2009). Genes co-localized in the same part of the chromosome might be co-regulated by the expanding chromatin modifications and contribute to the phenotype. I can therefore also hypothesise that CDKN2A is the critical locus specifically regulated by EBNA3C and EBNA3A, while ANRIL is a bystander gene co-regulated due to its localization.
**p14<sup>ARF</sup> regulation by EBNA3C**

The participation of p14<sup>ARF</sup> in the phenotype that results from EBNA3C inactivation has been a matter of controversy. In functional studies performed by Maruo *et al.* (2011) using shRNAs against either p16<sup>INK4A</sup> or p14<sup>ARF</sup> or both, silencing of p16<sup>INK4A</sup> alone only partially rescued the growth of LCL 3CHT in the absence of HT. Preventing the expression of both proteins arising from *CDKN2A* locus was necessary to fully restore the growth rate. My data show that even in the instances of p14<sup>ARF</sup> transcriptional upregulation after EBNA3C inactivation, p14<sup>ARF</sup> protein expression is very low, p53 stability is not increased and p21<sup>CIP1</sup> is not transcriptionally upregulated (Fig. 3.1.28). A similar situation occurs in CtBP-binding mutant LCL; p14<sup>ARF</sup> seems to be somewhat upregulated at the level of transcription in the CtBP-binding mutant LCL in comparison to the revertants; however, this still translates to only barely detectable protein in the cell (Fig. 3.1.29 and data not shown).

It is not entirely understood whether (and how) the p14<sup>ARF</sup> promoter in human pre-senescent and tumour cells is repressed by polycomb and H3K27me3. At the p14<sup>ARF</sup> promoter region in human cells, primary and tumour cells alike, PcG and H3K27me3 can be detected, but these are present only in low quantities even when p14<sup>ARF</sup> transcription is repressed and do not form a distinct ‘peak’ (Bracken *et al.*, 2007; Kia *et al.*, 2008 and Barradas *et al.*, 2009). The same appears to be true in LCL regardless of the EBNA3C activity or the ability of EBNA3C and EBNA3A to bind CtBP. Strikingly, there is a peak of H3K4me3 present at the p14<sup>ARF</sup> promoter during its repressed state (Fig. 3.1.30). So currently, it is not clear which mechanisms or chromatin modifications are repressing p14<sup>ARF</sup> promoter in the WT LCL or the functional significance of its regulation.
**Limitations of the study**

Although, there is no doubt that LCL 3CHT have already greatly enhanced our knowledge of EBNA3C functions, there are two main limitations of LCL 3CHT system. Firstly, even in the presence of HT, the repression of p16$^{\text{INK4A}}$ is not complete. The levels of p16$^{\text{INK4A}}$ are higher than in WT LCL, almost equivalent to the accumulation seen in EBNA3A KO LCL and CtBP-binding mutant LCL. The presence of elevated p16$^{\text{INK4A}}$ levels (relative to those in WT LCL) and of p130 in LCL 3CHT in the presence of HT might indicate that EBNA3C activation is not sufficient in a subpopulation of cells and these exit from the cell cycle. Alternatively, fusion of EBNA3C with the rather large HT domain might slightly compromise some EBNA3C functionality in all the cells in the population.

The protein-based regulation of HT-fusion proteins should allow for more immediate control than systems based on transcriptional induction. However, EBNA3C disappears from whole cell lysates following HT withdrawal and is most likely targeted for proteasomal degradation. Therefore, following HT re-addition, in addition to activation of the remaining available EBNA3C protein, which should be immediate, the bulk of the protein needs to be re-synthesized, folded and post-translationally modified. Therefore the functional ‘switch’ is not as rapid as initially expected and there might be delays especially in instances when the intracellular concentration of EBNA3C is important.

I have attempted to investigate the composition of EBNA3C and/or EBNA3A and CtBP-containing complexes in B-cell derived lines. However, co-immunoprecipitation experiments indicate only the potential of various proteins to bind and interact physically within the protein extract. Successful co-immunoprecipitation represents a biochemical but not necessarily functional link between the proteins. Such protein binding may not occur *in vivo* and even if the co-immunoprecipitated proteins are indeed constituents of one complex *in vivo*, the precise localization and actual function of the complex is not being
assessed. The function should therefore be further verified by ChIP experiments, showing association of the members of the CtBP co-repressor complex with the promoters of EBNA3C and EBNA3A-regulated genes. Another elegant alternative to gain insight into the EBNA3C- and EBNA3A-associated macromolecular assemblies is the tandem affinity purification (TAP) technology combined with mass spectrometry. Strep-FLAG (TAP) tag fused with either EBNA3C or EBNA3A would allow for purification of EBNA3C or EBNA3A-associated proteins and their identification by mass spectrometry analysis. This technique would provide a ‘bigger picture’, simultaneously examining multiple elements of EBNA3C and/or EBNA3A-containing complexes rather than binary interactions.

EBNA3C, EBNA3A and CtBP co-repressor complex (CtBP, LSD1, HDAC1) ChIPs at \( p16^{\text{INK4A}} \) locus were unsuccessful, most likely due to issues related to antibody quality. Although ChIP is a powerful tool to detect direct association of proteins with chromatin, ChIP captures just a snapshot of a dynamic process, while the co-repressors, chromatin remodelers, EBNA3C and EBNA3A might associate with chromatin transiently. Large multiprotein complexes might assemble on chromatin and the proteins that associate with chromatin through intermediates would be more difficult to detect by ChIP. Lastly, although the \( p16^{\text{INK4A}} \) locus and promoter region is well characterized, EBNA3C, EBNA3A, CtBP and the chromatin remodelers may bind only to a very discrete and relatively small region within the locus.

Lastly, in this study I have shown a positive correlation between the expression of CDKN2A and ANRIL transcripts in LCL. However, at least 8 ANRIL splice variants have been indentified to date and it is possible that various splice variants have different tissue distributions and functions. The qPCR assays used in this study are not comprehensive and could not distinguish between these multiple splice variants.
Future work

- EBNA3C and EBNA3A ChIP at $p16^{INK4A}$ locus using LCL carrying EBNA3C or EBNA3A strep-FLAG (TAP)-tagged recombinant viruses which have been produced by Gill Parker (personal communication). The feasibility of EBNA3C and EBNA3A ChIP would greatly enhance the study of EBNA3C and EBNA3A function not only in $p16^{INK4A}$ repression but more generally in other aspects of EBV-associated lymphomagenesis.

- Co-immunoprecipitation to test LSD1 association with EBNA3A in CtBP-binding mutant LCL. LSD1-GST pull-downs to probe the direct interaction of LSD1 with EBNA3C, EBNA3A and their deletion mutants (which have been constructed by Gill Parker, personal communication). Simultaneously, probe the function of LSD1 in EBNA3C and EBNA3A–mediated gene repression using either an LSD1 inhibitor such as trans-2-phenylcyclopropylamine derivatives (Mimasu, et al., 2010) or virally transduced LSD1 shRNA.

- RNA immunoprecipitations (RIP) in LCL using antibodies against strep-FLAG (TAP)-tagged EBNA3C and EBNA3A followed by qPCR or profiling of all pulled RNAs using deep-sequencing technologies (RNA-seq analysis) to investigate whether ncRNA (including ANRIL), which may serve as scaffold for chromatin remodelling enzymes or target the epigenetic machinery to the DNA, physically associate with EBNA3C and/or EBNA3A.
3.2 The functional significance of $p16^{\text{INK4A}}$ repression by EBNA3C

3.2.1 $p16^{\text{INK4A}}$ functionally null LCL 3CHT system

Objectives

In the course of this study, several reports were published by others using a similar system, in which conditional EBNA3C was expressed in LCL. These describe the contribution of various EBNA3C-mediated alterations of cellular gene expression to the LCL transformed phenotype. Zhao et al. (2011a) reported that 550 genes are regulated by EBNA3C in LCL and Maruo et al. (2011) found that EBNA3C and EBNA3A maintain LCL growth by repressing $p14^{\text{ARF}}$ expression in addition to $p16^{\text{INK4A}}$.

Therefore I decided to re-address the functional importance of $p16^{\text{INK4A}}$ repression by EBNA3C (and EBNA3A) in LCL proliferation and B cell transformation and immortalization by using an elegant system in which $p16^{\text{INK4A}}$ function is entirely abrogated.

Results

Production of $p16^{\text{INK4A}}$ functionally null (p16-null) LCL 3CHT

We received PBL from an individual homozygous for a 19bp germline deletion in the second exon of $\text{CDKN2A}$ as a kind gift from Gordon Peters (London Research Institute) and Alison Sinclair (University of Surrey). LCL produced by immortalizing these cells with WT EBV were characterized previously (Hayes et al., 2004). Since exon 2 is shared by both $p16^{\text{INK4A}}$ and $p14^{\text{ARF}}$, neither of the two WT proteins is produced. Instead, two
chimerical proteins originate from the locus: p16/X, comprising the first 74 residues of p16\textsuperscript{INK4A} followed by 64 amino acids specified by the +1 reading frame and p14/p16, in which the amino-terminal 88 residues of p14\textsuperscript{ARF} are fused to the last 76 residues of p16\textsuperscript{INK4A} (Fig. 3.2.1). The p14/p16 chimera retains all the known functions of p14\textsuperscript{ARF}; however neither protein exhibits any of the known functions of p16\textsuperscript{INK4A} (Brookes et al., 2002; Hayes et al., 2004).

Fig. 3.2.1. CDNK2A transcripts in normal cells and in individual with a 19bp deletion in the gene (reproduced from Hayes et al., 2004). The coding sequence of p16\textsuperscript{INK4A} is shown in stipples boxes, p14\textsuperscript{ARF} coding sequence in grey boxes, the deletion is shown as a black box highlighted by an arrow. Normal p16\textsuperscript{INK4A} and p14\textsuperscript{ARF} transcripts are depicted above the two chimerical transcripts p16/X and p14/p16.

Here, PBL containing the above described deletion were infected with recombinant BAC-based EBV viruses expressing the EBNA3C-HT fusion in the presence of the activating ligand HT (see Material and Methods, pp. 64-65 and p.67). After the outgrowth of p16-functionally null LCL 3CHT, cultures were divided in half and cultured in parallel either with or without HT in all subsequent experiments. Similar to p16-competent LCL 3CHT lines, in four p16-null LCL 3CHT lines (A1, A2, C1, C2) cultured without HT, EBNA3C expression declined with time, probably due to proteasomal degradation in the cytoplasm (Fig. 3.2.2.
and Fig. 3.2.3B and C). The expression of other EBV latent proteins in p16-null lines was similar as in p16-competent lines and was not consistently impacted by EBNA3C inactivation, with the exception of EBNA2 and LMP1 which seemed to be modestly increased in several LCL 3CHT cultured over prolonged time without HT (Fig. 3.2.2). A modest increase in EBNA2 and LMP1 was already observed previously during validation of p16-competent LCL 3CHT (Fig. 3.1.2) in some but not all cases.

**Fig. 3.2.2.** Expression of EBV latent proteins in p16-null and p16-competent LCL 3CHT with or without HT. p16-competent lines LCL 3CHT-A and LCL 3CHT-C were previously used in Chapter 3.1. p16-null LCL 3CHT-A corresponds to clone A2, and p16-null LCL 3CHT-C to the clone C2. In LCL cultured 30 days without HT, EBNA3C is degraded. The expression of other EBV latent proteins does not consistently change, apart from modest EBNA2 and LMP1 increase. p16-competent LCL 3CHT in this blot appear to express little EBNA-LP; however, EBNA-LP expression in LCL is generally highly variable and EBNA-LP did not appear to be consistently reduced in p16-competent LCL 3CHT (Fig. 3.1.2). γ-tubulin (γ-tub) was used as a loading control.
Next, two monoclonal antibodies were used to visualize the proteins that arise from CDKN2A locus in p16-null and p16-competent LCL cultured 21 days with or without HT. The mAb JC8 detects an epitope at the N-terminus of WT p16\textsuperscript{INK4A}, while DCS50 mAb was raised against an epitope at C-terminus of WT p16\textsuperscript{INK4A} (Fig. 3.2.3A). The mAb JC8 was used to detect p16/X fusion protein which appeared as a double band, with the upper part of similar size or modestly smaller as the WT p16\textsuperscript{INK4A}, and the bottom part distinctly smaller. As observed previously (Hayes et al., 2004) the p16/X product was consistently more difficult to detect than WT p16\textsuperscript{INK4A}, suggesting that it may be less stable. The p14/p16 product, recognized by the DCS50 mAb, was larger than WT p16\textsuperscript{INK4A} (Fig. 3.2.3C).

As expected, in p16-competent cells, robust p16\textsuperscript{INK4A} de-repression was detected by both JC8 and DCS50 mAb following EBNA3C inactivation. In p16-null LCL 3CHT, no WT p16\textsuperscript{INK4A} protein was expressed regardless of EBNA3C activity. The p14\textsuperscript{ARF} function-carrying p14/p16 chimera was consistently marginally upregulated following EBNA3C inactivation (Fig. 3.2.3C), while the non-functional p16/X was upregulated only in some cases (Fig. 3.2.3B and C).

Since the deletion is localized in the second exon of the CDKN2A locus, a qPCR assay detecting an amplicon in p16\textsuperscript{INK4A} exon 1 could be used to assess the quantity of exon 1-comprising transcripts (see Fig. 3.1.5 for the localization of the assay). In p16-competent LCL 3CHT, the amount of functional p16\textsuperscript{INK4A} transcripts was tightly controlled - very low in the cells cultured with HT and upregulated around 7-fold in the same lines cultured 21 days without HT. In the p16-null LCL 3CHT, exon 1-containing transcripts accumulated up to 30-fold above the levels found in p16-competent cells. Modest increase after EBNA3C inactivation was visible; however, most likely due to the accumulation of these non-functional transcripts, the difference in their abundance in p16-null LCL 3CHT cultured with or without HT was not as obvious as in the p16-competent lines (Fig. 3.2.4).
Fig. 3.2.3. Characterization of the p16-null LCL 3CHT. (A) Schematic representation of the WT p16<sup>INK4A</sup> protein produced in p16-competent LCL and the two chimerical proteins produced in p16-null LCL 3CHT lines. The epitopes detected by both JC8 and DCS50 anti-p16<sup>INK4A</sup> antibodies are depicted. (B) Western blot illustrating expression of EBNA3C-HT and the non-functional p16/X chimera in four p16-null LCL 3CHT (A1, A2, C1, C2) cultured 21 days with (+) or without (-) HT. After 21 days without HT, EBNA3C is degraded while p16/X is either unchanged or increased. (C) Western blot comparing expression of EBNA3C and proteins arising from CDKN2A locus in two p16-null LCL 3CHT (corresponding to A2 and C2 in Fig. 3.2.3B) and two p16-competent LCL 3CHT (-A and -C) cultured 21 days with (+) or without (-) HT. Data are representative of two independent experiments including four p16-null and two p16-competent LCL 3CHT lines.
Fig. 3.2.4. Quantification of $p16^{\text{INK4A}}$ exon1-comprising transcripts in p16-null and competent LCL 3CHT with or without HT. (A) Uncalibrated relative quantities of the amplicon located in the $p16^{\text{INK4A}}$ exon1 in four p16-null LCL 3CHT (A1, A2, C1 and C2) and two p16-competent LCL 3CHT-A and -C (ctrl A and ctrl C) cultured for 21 days with or without HT. p16-null lines contain markedly increased quantities of $p16^{\text{INK4A}}$ exon1-comprising transcripts in comparison to their p16-competent counterparts. (B) Values for each cell line are calibrated to the samples cultured with HT. The $p16^{\text{INK4A}}$ transcripts are upregulated about 7-fold in p16-competent LCL 3CHT cultured for 21 days without HT, while the exon1 amplicon is increased only about 2-fold in majority of p16-null lines.

**EBNA3C inactivation in p16$^{\text{INK4A}}$ functionally null LCL does not impair cell cycle progression**

It was immediately apparent that the proliferation of p16-null LCL 3CHT cultured without HT was not inhibited in comparison to the same lines cultured with HT (i.e. the p16-null LCL 3CHT cultured without HT reached similar cell densities and were split with the same frequency as the p16-null LCL 3CHT controls grown with HT). To precisely quantify the proliferation rate, the p16-null and -competent LCL 3CHT cultured for up to 30 days with or without HT were incubated at day 14, 21, 27 and 30 for 1 hour in the medium containing BrdU, fixed, stained with anti-BrdU-FITC and PI and analyzed with flow cytometry. The p16-null LCL 3CHT cultured with HT proliferated about 20% more rapidly than the p16-
competent lines. As expected, the proliferation rate of p16-competent lines progressively declined following EBNA3C inactivation. In stark contrast, proliferation rate of all four p16-null LCL 3CHT remained unchanged for up to 30 days after EBNA3C inactivation (Fig. 3.2.5 and Fig. 3.2.6).

<table>
<thead>
<tr>
<th>S-phase (R3)</th>
<th>22 days with HT</th>
<th>22 days without HT</th>
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<tr>
<td></td>
<td>Mean (%)</td>
<td>SD</td>
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<tr>
<td>p16-competent</td>
<td>30.85</td>
<td>2.61</td>
</tr>
<tr>
<td>p-16-null</td>
<td>38</td>
<td>4.24</td>
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<tr>
<td></td>
<td>11.6</td>
<td>0.56</td>
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<td>41.5</td>
<td>2.12</td>
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Fig. 3.2.5. Proliferation rate of p16-null and -competent LCL 3CHT lines following EBNA3C inactivation. (A) Representative FACS analysis of cells cultured for 22 days with or without HT, then incubated for 1 hour with BrdU and stained with anti-BrdU-FITC/PI. The gated population comprises the cells that have undergone DNA replication while incubated with BrdU (cells that have DNA content between 2N and 4N and highly incorporate BrdU). (B) The percentage of gated cells for each line and condition in (A). The mean and SD of two biological replicates are shown. While the cell population in S phase decreases by about two thirds in p16-competent LCL 3CHT-A cultured for 22 days without HT, in 16-null LCL 3CHT (A2) the degree of proliferation remains unaltered. (C) Comparison of the proliferation of four p16-null LCL 3CHT (C1, C2, A1, A2) and two p16-competent LCL 3CHT (-C and -A, line A in replicate experiment), quantified by BrdU incorporation, in the time-course of up to 30 days following removal of HT. The BrdU incorporation in every cell line cultured without HT was normalized in each time-point by the values measured in respective cell line cultured with HT. The proliferation rate of the p16-competent LCL 3CHT cultured without HT progressively declines with time, while the proliferation of the p16-null LCL 3CHT without HT remains unaltered.
Fig. 3.2.6. Alternative representation of the data summarized in Fig. 3.2.5C. Proliferation rate of multiple p16-null LCL 3CHT cultured with or without HT and in various time-points following EBNA3C inactivation. (A) Proliferation of three p16-null LCL 3CHT lines (A1, A2 and C2) cultured for 27 days with or without HT that were incubated with BrdU, stained and analyzed as described previously. (B) Proliferation of p16-null LCL 3CHT line (C1) cultured for 21, 27 and 30 days with or without HT. The proliferation is not impacted by EBNA3C inactivation in neither of the p16-null LCL 3CHT. No difference is detectable between the proliferation of p16-null LCL 3CHT cultured with or without HT for up to 30 days. Error bars represent the standard deviation from the mean of two technical replicates.

EBNA3C inactivation does not lead to activation of the p16<sup>INK4A</sup>-Rb-E2F1 axis and provides a system to study EBNA3C-regulated genes in a cell cycle-independent manner

In p16-competent LCL 3CHT following EBNA3C inactivation, Rb was hypophosphorylated and the overall levels of Rb were decreased. Consistent with proliferative arrest, p130 was upregulated and p107 downregulated (Fig. 3.2.7 and Fig. 3.1.7). In contrast, no changes in the quantity or phosphorylation of the pocket proteins were detected following EBNA3C inactivation in p16-null LCL 3CHT, confirming that p16<sup>INK4A</sup>-Rb-E2F1 axis was not activated (Fig.3.2.7).
Fig. 3.2.7. Quantity and phosphorylation of the pocket proteins in p16-null and competent LCL 3CHT lines with or without HT. Anti-pan-Rb antibody (pan-Rb) used in this WB detects all forms of Rb from hypophosphorylated (lower bands annotated pRb) to hyperphosphorylated (upper bands annotated ppRb). Anti-phospho-Rb antibody (Rb-PO$_4$) detects only the hyperphosphorylated forms. γ-tubulin (γ-tub) was used as a loading control. In p16-competent LCL 3CHT cultured for 21 days without HT, Rb is downregulated and hypophosphorylated; p130 upregulated and p107 downregulated. In p16-null LCL 3CHT (corresponding to A2 and C2 in Fig. 3.2.3B), no difference in the quantity of Rb, p130 and p107 nor Rb phosphorylation is detectable regardless of EBNA3C activity. Data are representative of two independent experiments each including at least two p16-null LCL 3CHT lines.

Since the E2F1-mediated transcription was not blocked in p16-null LCL 3CHT after EBNA3C inactivation and the proliferation rate was not influenced, I was able to test the regulation of cellular genes by EBNA3C independently of proliferation and E2F1-mediated transcription, helping clarify the cause-effect relationship between the gene regulation and the change of LCL phenotype in response to EBNA3C. For example, the SET methyltransferase EZH2, a known direct E2F1 target, was downregulated in p16-competent LCL 3CHT cultured without HT, while there was no regulation in the p16-null counterparts (Fig. 3.2.8). This is consistent with my previous findings (Fig. 3.1.16) and indicates that the regulation of EZH2 expression does not causally contribute to the p16$^{INK4A}$ upregulation and cell cycle inhibition following EBNA3C inactivation in LCL but is on the contrary associated with reduced proliferation of LCL.
Similarly, the increase in phosphorylation of H2AX observed after EBNA3C inactivation in p16-competent lines was not detected in the p16-null proliferating LCL 3CHT (Fig. 3.2.8). In a recent study, it was shown that EBNA3C suppresses DDR pathway mediated by ATM/CHK2 in the initial phases of primary B cell transformation by EBV (Nikitin et al., 2010). I also found that in the p16-competent LCL 3CHT, a modest increase in H2AX phosphorylation is detectable following EBNA3C inactivation. A similar modest increase was found in all CtBP-binding mutant LCL in comparison to revertant or WT LCL (Fig. 3.1.22). However, since no change in H2AX phosphorylation could be detected in the proliferating p16-null lines following EBNA3C inactivation, I conclude that EBNA3C does not directly block H2AX phosphorylation (or the upstream events leading to H2AX phosphorylation) in the established proliferating LCL and that it is rather the proliferative arrest in p16-competent LCL 3CHT cultured without HT or in the CtBP-binding mutant LCL that is associated with increased H2AX phosphorylation. Nevertheless, it is possible that EBNA3C might be important for suppression of DDR or prevention of DNA damage during the initial phases of primary B cell transformation by EBV or when the established LCL are challenged by DNA-damage causing agents.

Fig. 3.2.8. Proliferation- and E2F1-dependent modulation of genes in p16-competent LCL 3CHT. Western blot of EZH2 and γ-H2AX in two p16-null LCL 3CHT (corresponding to A2 and C2 in Fig. 3.2.3B) and two competent LCL 3CHT (-A and -C) cultured for 21 days with (+) or without (-) HT. γ-tubulin (γ-tub) was used as a loading control. Downregulation of EZH2 and phosphorylation of H2AX (γ-H2AX) are detected only in p16-competent lines cultured without HT. Data are representative of two independent experiments each including at least two p16-null LCL 3CHT lines.
p14<sup>ARF</sup>-p53-p21<sup>CIP1</sup> pathway is not activated following EBNA3C inactivation in p16-null LCL 3CHT

The p14/p16 chimerical protein that exhibits all the known functions of p14<sup>ARF</sup> was marginally but consistently upregulated in p16-null LCL 3CHT after EBNA3C inactivation (Fig. 3.2.3C). I tested whether the p14<sup>ARF</sup>-p53-p21<sup>CIP1</sup> pathway was activated (perhaps in a compensatory manner after the abrogation of p16<sup>INK4A</sup>-Rb-E2F1 axis) in p16-null LCL 3CHT cultured without HT. However, p53 protein was not consistently stabilized after EBNA3C inactivation, and neither p21<sup>CIP1</sup> transcripts nor protein were upregulated (Fig. 3.2.9).

Fig. 3.2.9. p14<sup>ARF</sup>-p53-p21<sup>CIP1</sup> pathway is not activated in p16-null LCL 3CHT following EBNA3C inactivation. (A) Western blot of p53 and p21<sup>CIP1</sup> in four p16-null LCL 3CHT cultured for 21 days with (+) or without (-) HT. No consistent changes in expression of p53 or p21<sup>CIP1</sup> are observed. (B) qPCR of p21<sup>CIP1</sup> transcripts in four p16-null LCL 3CHT (A1, A2, C1, C2) and two p16-competent LCL-3CHT-A and -C (ctrl A and ctrl C) cultured for 20 days with or without HT.

The p16<sup>INK4A</sup> locus is epigenetically regulated by EBNA3C even in the absence of p16<sup>INK4A</sup> function

The system with non-functional p16<sup>INK4A</sup> provided a unique opportunity to test whether the regulation of p16<sup>INK4A</sup> locus by EBNA3C can be separated from the production of the
functional p16\textsuperscript{INK4A} protein. The composition of chromatin at the p16\textsuperscript{INK4A} locus could be assessed in the cells with active or inactive EBNA3C, proliferating at the exactly same rate. The polycomb-imposed repressive H3K27me3 was convincingly diminished at the p16\textsuperscript{INK4A} locus following EBNA3C inactivation in all p16-null LCL 3CHT tested (Fig. 3.2.10B).

**Fig. 3.2.10.** Epigenetic regulation of p16\textsuperscript{INK4A} locus by EBNA3C in p16-null LCL 3CHT. (A) A scheme of the CDKN2A locus depicting the location of the assays used in ChIP-qPCR. Assays C is located in p16\textsuperscript{INK4A} exon 1 and the histone modifications in this region have the major impact on p16\textsuperscript{INK4A} transcription. The details of the primer position and sequence are listed in Table 2.7 and Table 2.8. (B) ChIP-qPCR analysis quantifying the H3K27me3 across CDKN2A locus in two p16-null LCL 3CHT (corresponding to C1 and C2 in Fig. 3.2.3B) cultured 27 days with or without HT. The peak of H3K27me3, centred at p16\textsuperscript{INK4A} exon 1, is detected in p16-null LCL 3CHT cultured with HT and is diminished in the lines cultured without HT. H3K27me3 mark at the control γ-globin promoter remains stable regardless of EBNA3C activity.
The levels of H3K27me3 were stable at the control γ-globin locus regardless of EBNA3C activity demonstrating that H3K27me3 was not diminished genome-wide after EBNA3C inactivation but was specifically erased at the p16\textsuperscript{INK4A} locus (Fig. 3.2.10). H3K27me3 is catalyzed by SET domain of EZH2. Although EZH2, as a direct E2F1 target (Bracken et al., 2003), decreased considerably in p16-competent LCL 3CHT during the proliferative arrest following EBNA3C inactivation, it remained constant in p16-null LCL 3CHT lines (Fig. 3.1.16 and Fig. 3.2.8). Since the p16\textsuperscript{INK4A} locus was differentially regulated by EBNA3C in p16-null lines despite the stable expression of EZH2, I can confirm the findings obtained previously from Rb-reduced LCL 3CHT-E (Fig. 3.1.11 and Fig. 3.1.16) and convincingly show that the regulation of H3K27me3 mark at the p16\textsuperscript{INK4A} locus by EBNA3C is independent of the overall EZH2 expression.

A peak of activation-related H3K4me3 is co-localized with the repressive H3K27me3 at p16\textsuperscript{INK4A} exon 1, forming a bivalent domain. I showed previously that H3K4me3 was modestly increased after EBNA3C inactivation in p16-competent LCL 3CHT (Fig.3.1.9B). In p16-null LCL 3CHT, the levels of H3K4me3 did not substantially change after EBNA3C inactivation (Fig. 3.2.11).

![ChIP-qPCR analysis quantifying the H3K4me3 across CDKN2A locus in p16-null LCL 3CHT](image)

**Fig. 3.2.11.** ChIP-qPCR analysis quantifying the H3K4me3 across CDKN2A locus in p16-null LCL 3CHT. The peaks of H3K4me3 centred at p16\textsuperscript{INK4A} exon 1 detected in two p16-null LCL 3CHT (C1 and C2) cultured for 27 days with or without HT do not considerably differ. Analyzing the GAPDH promoter serves as a positive control.
The kinetics of epigenetic regulation of $p16^{INK4A}$ locus by EBNA3C are similar in p16-null and p16-competent LCL 3CHT

After 30 days of culture without HT, HT was re-added to the p16-null LCL 3CHT and the kinetics of $p16^{INK4A}$ repression were monitored. Three days after re-adding HT, a subtle but significant H3K27me3 increase could be detected at $p16^{INK4A}$ exon 1 in the total cell population. By day 14 after re-adding HT, an H3K27me3 peak at $p16^{INK4A}$ exon 1 could be detected, although perhaps not yet fully developed (i.e. there seemed to be an increased H3K27me3 deposition in the control upstream region A and the difference between the upstream region -A- and $p16^{INK4A}$ exon1 -C - had reached only 2-fold) (Fig. 3.2.12A). The H3K4me3 mark was not significantly regulated after re-adding HT (Fig. 3.2.12B).

As reported previously, in p16-competent LCL 3CHT, the peak of H3K27me3 at the $p16^{INK4A}$ locus was fully restored 20 days after re-adding HT (Fig. 3.1.9). Surprisingly, the repressive mark at $p16^{INK4A}$ in p16-null lines was reconstituted in a similar time-scale, requiring weeks rather than days. This strongly implies, that the elapsed time and/or the high number of cell divisions required for $p16^{INK4A}$ repression by EBNA3C are related directly to the mechanism of repression and not to the outgrowth of selected populations.

Since the p14/p16 protein was consistently modestly upregulated in the p16-null LCL 3CHT following EBNA3C inactivation (Fig. 3.2.3C), the chromatin status at the $p14^{ARF}$ promoter was also assessed. Consistent with the findings in p16-competent LCL 3CHT (Fig. 3.1.30), in p16-null LCL 3CHT, only low quantities of H3K27me3 mark were detectable at $p14^{ARF}$ promoter in cells cultured with HT, when EBNA3C was active and $p14^{ARF}$ locus repressed; the mark perhaps further slightly decreased after 30 days following EBNA3C inactivation (Fig. 3.2.13A). However, 21 days following EBNA3C reactivation in two p16-null LCL 3CHT, the H3K27me3 mark at $p14^{ARF}$ promoter, in contrast to $p16^{INK4A}$ exon1, did not increase (Fig. 3.2.13B). H3K4me3 formed a peak at $p14^{ARF}$ promoter, quantitatively comparable to that present at $p16^{INK4A}$ exon1 (assay C), irrespectively of EBNA3C activity.
(3.2.13A). I conclude that similar to p16-competent LCL 3CHT, in p16-null LCL 3CHT p14<sup>ARF</sup> did not seem to be regulated by H3K27me3 or H3K4me3 in response to EBNA3C activity.

Fig. 3.2.12. The kinetics of epigenetic modulation of p16<sup>INK4A</sup> locus by EBNA3C. (A) ChIP-qPCR analysis quantifying the H3K27me3 mark across the CDKN2A locus in p16-null LCL 3CHT (A2) cultured with HT, 30 days without HT or 72h or 14 days after HT was re-added. The peak of H3K27me3 centred at p16<sup>INK4A</sup> exon 1 detected in p16-null LCL 3CHT cultured with HT is diminished in lines cultured without HT and subsequently gradually restored following the readdition of HT. (B) Similar analysis quantifying H3K4me3. H3K4me3 does not change significantly across the CDKN2A locus in p16-null LCL 3CHT in response to EBNA3C activity.
Fig. 3.2.13. Histone modifications at CDKN2A locus including p14ARF promoter in p16-null LCL 3CHT. (A) H3K27me3 and H3K4me3 quantified by ChIP-qPCR at CDKN2A locus including p14ARF promoter (p14) in p16-null LCL 3CHT (A2) cultured for 30 days with or without HT. (B) H3K27me3 quantified by ChIP-qPCR at CDKN2A locus including p14ARF promoter (p14) in two p16-null LCL 3CHT (A2 and C2) cultured for 30 days without HT or 21 days after HT was re-added. Neither of the modifications at p14ARF promoter appears regulated by EBNA3C.
The cumulative nature of H3K27me3 restoration at p16\textsuperscript{INK4A} exon 1 following EBNA3C reactivation

Since the epigenetic repression of p16\textsuperscript{INK4A} by EBNA3C requires a surprising length of time, the kinetics were studied and modelled in detail. Firstly, time-course ChIP experiments performed on p16-null and -competent LCL 3CHT following HT removal or readding were compared. The time required for the loss and restoration of the H3K27me3 mark in response to changes in EBNA3C activity was similar in multiple lines (Fig. 3.2.14A, Fig. 3.1.8B and C and data not shown). Approximately 14 days were required for a convincing decrease or increase in H3K27me3, while by 21 days there was a pronounced loss of H3K27me3 at p16\textsuperscript{INK4A} exon 1 if EBNA3C was inactivated or a well-defined H3K27me3 peak at p16\textsuperscript{INK4A} exon 1 if EBNA3C was re-activated. It is possible that in p16-null lines, the H3K27me3 mark was fully restored in a slightly shorter period of time (i.e. 14 days) following EBNA3C reactivation than in p16-competent lines (Fig. 3.2.12A and 3.2.14A). In addition, since H3K27me3 was progressively erased at p16\textsuperscript{INK4A} exon 1 with increasing time of culture without functional EBNA3C, the time required for restoration of H3K27me3 at p16\textsuperscript{INK4A} exon 1 after EBNA3C reactivation seemed partially dependent on the time the line was cultured without functional EBNA3C; the longer time EBNA3C was inactivated, the longer (in days) it would take to restore H3K27me3 at p16\textsuperscript{INK4A} exon1. This is in agreement with the previous data showing that the period needed for the full repression of p16\textsuperscript{INK4A} and restoration of proliferation after reactivation of EBNA3C in p16-competent LCL 3CHT was dependent on the time the cells were previously cultured without functional EBNA3C (Fig. 3.1.4 and Fig. 3.1.6).

Strikingly, the process of H3K27me3 deposition at the p16\textsuperscript{INK4A} locus was cumulative and very gradual. As an example, following prolonged EBNA3C inactivation (35 days) in p16-null LCL 3CHT, the repressive H3K27me3 was erased from exon 1 of p16\textsuperscript{INK4A} even to levels below the surrounding upstream and downstream regions. Early after re-adding HT (3 days), the first signs of H3K27me3 restoration at the p16\textsuperscript{INK4A} exon1 could be observed.
(Fig. 3.2.14B), and the accumulation of H3K27me3 continued over weeks to form a mature peak (Fig. 3.2.14C).

**Fig. 3.2.14. Cumulative nature of H3K27me3 restoration at \( p16^{INK4A} \) locus following EBNA3C reactivation in p16-null LCL 3CHT.**
(A) The illustration of the configuration of H3K27me3 mark at \( p16^{INK4A} \) locus in p16-null lines cultured for various times with or without HT. p16-competent lines with or without active/WT EBNA3C are added for comparison. Although the results of several independent ChIP experiments were combined in this figure, the distribution of H3K27me3 across \( p16^{INK4A} \) locus (i.e. the shape of the peaks) is directly comparable. (B) ChIP-qPCR mapping H3K27me3 at the \( p16^{INK4A} \) locus in p16-null LCL 3CHT (A2) cultured for 35 days without HT and for 3 days after HT was re-added. (C) Similar ChIP-qPCR analysis depicting the well-defined H3K27me3 peak at \( p16^{INK4A} \) locus in two p16-null LCL 3CHT (A2 and C2) 21 days after re-adding HT.
EBNA3A is required for additional functions in the maintenance of LCL proliferation apart from p16\(^{\text{INK4A}}\) repression

Functionally p16-null EBNA3A KO and revertant LCL were produced by infecting the p16-deleted PBL with respective recombinant EBV-BAC viruses. However, due to the limited number of these rare PBL, only one pair of EBNA3A KO and revertant LCL was established and the results presented below were acquired using this single pair.

As in p16-null LCL 3CHT, there was no WT p16\(^{\text{INK4A}}\) produced in p16-null EBNA3A KO or revertant LCL; however, the non-functional p16/X and the p14\(^{\text{ARF}}\) function-carrying p14/p16 chimerical proteins were expressed. Both chimeras were marginally upregulated in p16-null EBNA3A KO LCL in comparison to the revertant (Fig 3.2.15).

Fig. 3.2.15. Characterization of the p16-null EBNA3A KO and revertant LCL. Western blot illustrating the expression of EBNA3A and proteins arising from the CDKN2A locus in p16-null and – competent EBNA3A KO and revertant LCL. p16\(^{\text{INK4A}}\), detected by both N-terminal and C-terminal mAb, is upregulated in p16–competent EBNA3A KO line in comparison to the revertant. In p16-null lines, no WT p16\(^{\text{INK4A}}\) is detectable. The p16/X chimerical protein detected by N-terminal mAb and p14/p16 chimera detected by C-terminal mAb both appear modestly upregulated in EBNA3A KO line in comparison to the revertant. WB shown is representative of two experiments performed using the only available p16-null EBNA3A KO line.
The proliferation of the newly established p16-null EBNA3A KO and revertant LCL, i.e. three months after infection, was assessed by BrdU incorporation and compared with their p16-competent counterparts which spent approximately equal time in culture following infection with EBV. I have reported previously that p16-competent EBNA3A KO LCL (similarly to CtBP-binding mutant LCL) seem to undergo a selection in culture for the loss of Rb expression and might recover the proliferation rate similar to the respective revertants. Here, I show p16-competent EBNA3A KO LCL early after outgrowth (three months after infection). The proliferation rate, assessed by BrdU incorporation, in LCL lacking EBNA3A was decreased by two thirds in comparison to the respective revertant. In contrast to the unaltered proliferation of the p16-null LCL 3CHT cultured without HT, the proliferation of p16-null EBNA3A KO LCL was also reduced, suggesting that additional functions of EBNA3A other than p16$^{\text{INK4A}}$ repression were required for maintenance of LCL proliferation (Fig. 3.2.16). Although only one pair of p16-null EBNA3A KO and revertant LCL was available, the reduction of proliferation in EBNA3A KO was reliably reproduced in several independent measurements.

Similar to p16-null LCL 3CHT, the $p16^{\text{INK4A}}$ locus was epigenetically regulated by EBNA3A even in the absence of p16$^{\text{INK4A}}$ function. H3K27me3 was decreased at the $p16^{\text{INK4A}}$ locus in a p16-null EBNA3A KO LCL in comparison to the revertant, although to a lesser extent than in the case of EBNA3C inactivation (Fig. 3.2.17). It is nevertheless clear that EBNA3A contributes to the epigenetic regulation of $p16^{\text{INK4A}}$ locus in LCL.
Fig. 3.2.16. Proliferation rate of p16-null and -competent EBNA3A KO and revertant LCL. (A) One representative FACS analysis (of three similar measurements) of cells incubated for 1 hour with BrdU and stained with anti-BrdU-FITC/PI. The gated population (R3) comprises the cells that have undergone DNA replication while incubated with BrdU (the cells which have DNA content between 2N and 4N and highly incorporate BrdU). (B) The mean and SD of percentage of cells in the S phase for the same lines in three separate measurements. The population in S phase decreases by almost two thirds in p16-competent EBNA3A KO LCL and by almost half in p16-null EBNA3A KO LCL in comparison to the respective revertants.
Fig. 3.2.17. Epigenetic regulation of $p16^{INK4A}$ locus by EBNA3A in p16-null LCL. ChIP-qPCR analysis quantifying the H3K27me3 mark across CDKN2A locus in EBNA3A KO and revertant p16-null LCL. The peak of H3K27me3 centred at $p16^{INK4A}$ exon 1 in the revertant is visibly diminished in EBNA3A KO.
3.2.2 Expression of p16\textit{INK4A} during the transformation of primary B cells by EBV

Objectives

The induction of p16\textit{INK4A} was shown to robustly repress LCL proliferation and there is no doubt that a strong requirement for very tight control of p16\textit{INK4A} exists in established LCL. EBV seems to have evolved several proteins dedicated to p16\textit{INK4A} repression (i.e. EBNA3C, EBNA3A, LMP1). This led me to hypothesize that p16\textit{INK4A} induction might present a barrier not only in maintenance of LCL growth but also during the transformation of B cells by EBV. In order to test this, the expression of p16\textit{INK4A} in the early stages of B cell transformation was assessed.

The results presented in this section should be considered only preliminary, since the experiments did not contain all necessary controls (see discussion) that would allow definitive conclusions to be drawn.

Results

\textbf{CDKN2A/p16\textit{INK4A} expression is not maintained at the basal levels found in primary B cells, but is increased after EBV infection}

CDKN2A mRNA, quantified by an assay targeting the exon 2/3 boundary shared by p16\textit{INK4A} and p14\textit{ARF}, was expressed at basal, barely detectable levels in primary B cells purified by anti-CD19 coated MACS micro-beads. Surprisingly, after EBV infection,
CDKN2A transcripts were not maintained in this highly repressed state and increased with time reaching up to 6-fold by 14 days post-infection. (Fig. 3.2.18).

**Fig. 3.2.18. CDKN2A transcripts after EBV infection of purified primary B cells.** CDKN2A transcripts quantified by qPCR at day 0 (two separate samples) 2, 4, 7 and 14 following EBV infection of primary B cells. CDKN2A transcripts increase up to 6-fold following EBV-infection of primary B cells.

Transcripts of other EBNA3C or EBNA3C and EBNA3A-regulated genes (BIM EL, TGFβR2 and NOTCH2) and a non-regulated gene (RPLP0 and possibly Rb) on the level of transcription were assessed in the same samples as CDKN2A transcripts. BIM EL, TGFβR2 and NOTCH2 play essential roles in the B lymphocyte function and are modulated in B cell-derived malignancies. BH3-only protein BIM, a member of the Bcl-2 family, regulates B lymphocyte apoptosis (Clybouw et al., 2005; Biswas et al., 2007). NOTCH2 is preferentially expressed in mature B cells; conditionally targeted deletion of NOTCH2 results in the defect of marginal zone B cells and their precursors (Witt et al, 2003; Saito et al., 2003). Depending on cancer-type, NOTCH2 can serve as both oncogene and tumour suppressor (O’Neill et al., 2007; Chu et al., 2009). TGFβ induces apoptosis in BL cell lines and in primary human B lymphocytes (Spender and Inman, 2009). TGFβR2 mediates the tumour suppressor activity of the TGFβ signalling pathway and is inactivated in several cancers (Kaklamani and Pasche, 2004). BIM EL and TGFβR2 are expressed at very low levels in established LCL lines, while NOTCH2 is well expressed; however, in established LCL all three are repressed by EBV at the level of transcription, specifically by either EBNA3C or by co-operation of EBNA3C and EBNA3A (White et al., 2010 and unpublished...
data). *RPLP0* encodes a ribosomal protein that is a component of the 60S subunit; RPLP0 transcription is not regulated by EBNA3C or EBNA3A (data not shown) and was used throughout this entire study to normalize gene expression. The transcriptional regulation of Rb by EBNA3C, EBNA3A or EBV has not been previously excluded; the Rb transcription was estimated following infection of primary B cells with EBV due to the possible interplay of Rb and p16<sup>INK4A</sup> expression (Rb-p16<sup>INK4A</sup> feedback loop) (see Introduction, p.31).

Bim EL, NOTCH2 and TGFβR2 were found robustly down-regulated in the early stages of B cell transformation while RPLP0 and Rb transcripts were not markedly altered (Fig. 3.2.19).

**Fig. 3.2.19. Control transcripts repressed or unaltered after EBV infection of purified primary B cells.** BIM EL, NOTCH2, TGFβR2, RPLP0 and Rb transcripts quantified by qPCR at day 0 (two separate samples) 2, 4, 7 and 14 following EBV infection of purified primary B cells. BIM EL, NOTCH2 and TGFβR2 transcripts are soon repressed, while RPLP0 and Rb transcripts remain mostly unaltered in the early stages of B cell transformation.
In a further experiment, PBL were used instead of purified B cells. A small number of B cells was purified from PBL by anti-CD19 coated MACS micro-beads at the start of the experiment to serve as a day 0 control. The first harvesting point was set only at day 7 post-infection when the majority of PBL other than B cells should be eliminated from the culture (i.e. T cells and NK cells will be eliminated, while macrophages/monocytes will still be present). PBL infected with EBV were separated at day 7, 14 and 25 using Ficoll-Paque gradient (see Materials and Methods Fig. 2.2) and the transcripts were quantified in RNA isolated from both normal- and high-density fractions. Normal-density fraction consists of the subpopulation of the normal, live cells while the high-density fraction comprises cells that are most likely apoptotic (Czene et al., 2002; Belloni et al., 2008). p16\textsuperscript{INK4A} mRNA expression was assessed in the normal-density fraction of EBV-infected PBL. Consistent with the previous experiment (Fig. 3.2.18), p16\textsuperscript{INK4A} mRNA increased following EBV infection (Fig. 3.2.20).

![Figure 3.2.20.](image)

**Fig. 3.2.20.** p16\textsuperscript{INK4A} transcripts in the normal-density cell population after EBV infection of PBL. The p16\textsuperscript{INK4A} transcripts quantified by qPCR at day 0, 7, 14 and 25 were upregulated following EBV infection of PBL.
The accumulation of growth-inhibitory CDKN2A/p16\textsuperscript{INK4A} transcripts seems to be more pronounced in the high-density fraction

Analysis of the relative quantities of CDKN2A, p16\textsuperscript{INK4A} and Rb transcripts in the normal and high-density fraction of EBV-infected PBL was performed on the group of about 20 samples harvested at various time-points (between day 7 and day 25) following EBV infection of PBL in two independent experiments. Interestingly, higher amounts of CDKN2A and p16\textsuperscript{INK4A} transcripts seemed to be found in high-density population (Fig. 3.2.21).

![Fig. 3.2.21. Accumulation of p16\textsuperscript{INK4A} and CDKN2A transcripts in the high-density fraction of EBV-infected PBL.](image)

<table>
<thead>
<tr>
<th>Separation</th>
<th>p16\textsuperscript{INK4A} mRNA</th>
<th>CDKN2A mRNA</th>
<th>Rb mRNA</th>
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<tbody>
<tr>
<td>EBV infected high density</td>
<td>14.13</td>
<td>4.04</td>
<td>1.25</td>
</tr>
<tr>
<td>EBV infected normal density</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</tbody>
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The histogram shows relative quantities of p16\textsuperscript{INK4A}, CDKN2A and control Rb transcripts in the high and normal-density fractions of PBL infected with EBV in 20 samples from two independent experiments. The table shows the increase in expression in high-density fractions as a fold difference relative to the respective normal-density fractions.
Summary key points

- Repression of p16\textsuperscript{INK4A} transcripts is the major function of EBNA3C in EBV-driven LCL proliferation
- The p16\textsuperscript{INK4A} locus is specifically targeted and repressed by EBNA3C even in the absence of functional p16\textsuperscript{INK4A} protein
- The p16-null LCL 3CHT system allows the study of EBNA3C-regulated genes in a proliferation–independent manner
- p16\textsuperscript{INK4A} is induced as naive B cells are activated to proliferate by EBV

Discussion

EBNA3C is a multifunctional protein and regulates an entire subset of cellular genes at the level of transcription (White et al., 2010; Zhao et al., 2011a). Functional analysis performed previously by Maruo et al. (2011), using RNA interference to decrease the expression of p16\textsuperscript{INK4A} and p14\textsuperscript{ARF} separately and in combination indicated that p14\textsuperscript{ARF} contributes to the cell cycle arrest following EBNA3C inactivation. Others have also reported several EBNA3C-mediated alterations in expression of genes such as TCL1 expected to contribute to the EBNA3C-dependent maintenance of LCL proliferation (Lee et al., 2009; Zhao, et al., 2011a). Surprisingly, I obtained unambiguous data proving that the absence of p16\textsuperscript{INK4A} abrogates the need for EBNA3C activity in the maintenance of proliferation in established LCL (Fig. 3.2.5 and Fig.3.2.6). I show that p16\textsuperscript{INK4A} repression is the main and the only necessary function of EBNA3C in EBV-driven LCL proliferation.

In addition, EBNA3C can cause epigenetic modification of p16\textsuperscript{INK4A} even in a system where the regulation is separated from the functional outcome and the selection based on the
proliferative advantage does not play any role (Fig. 3.2.10 and Fig. 3.2.12). This finding puts forward convincing evidence that EBNA3C can directly or indirectly modify histones at the specific cellular locus and alter the cellular identity towards a malignant phenotype.

**Kinetics of p16\(^{INK4A}\) regulation by EBNA3C**

At the level of cell population – when a total population of p16-null LCL 3CHT is analyzed at various time-points with or without HT rather than a single cell - the kinetics of \(p16^{INK4A}\) regulation is relatively slow (Fig. 3.2.12A and Fig. 3.2.14). The H3K27me3 peak on \(p16^{INK4A}\) is fully reconstituted only after a period of at least 14 days following EBNA3C reactivation. Before the establishment of p16-null LCL, it could have been argued that the epigenetic modifications of \(p16^{INK4A}\) occur as a few stochastic events followed by the selection after EBNA3C reactivation. However, the modification of \(p16^{INK4A}\) regardless of its function implies that this locus is specifically targeted for repression by EBNA3C and precludes the acquisition of the \(p16^{INK4A}\) repression in the cell population as a consequence of selection. Therefore the relatively slow kinetics of \(p16^{INK4A}\) modulation by EBNA3C seem to be genuinely related to the mechanism of its regulation.

I and others (Maruo et al., 2006 and Zhao et al., 2011a) failed to identify any phenotypic changes early in the hours or first days after EBNA3C reactivation in p16-competent LCL 3CHT. The first detectable (subtle) increase in proliferation -at the level of the whole cell population- correlated with a decrease in \(p16^{INK4A}\) expression and occurred only 4-6 days after EBNA3C reactivation (Fig. 3.1.4). The loss and re-establishment of H3K27me3 at \(p16^{INK4A}\) locus in p16-competent and -null LCL 3CHT is gradual and appears almost passive (Fig. 3.1.8B and C, Fig. 3.2.12A and Fig. 3.2.14). Furthermore, another well-defined EBNA3C and EBNA3A-regulated promoter – \(BIM\), has been shown to be de-repressed in BL31 3CHT following EBNA3C inactivation with similar kinetics as seen for \(p16^{INK4A}\) (Paschos et al., under revision). At the level of the cell population, the complete loss or re-
appearance of the H3K27me3 mark in response to EBNA3C reproducibly occurs only after a rather high number of cell divisions (estimated to be about 15-20).

A possible explanation for the delayed H3K27me3 regulation is the combination of the slow EBNA3C inactivation or reactivation and stability of H3K27me3 mark. The EBNA3C fusion protein is degraded following its inactivation and time is required for the newly synthesised protein to form and fold after re-adding HT. H3K27me3 arises from monomethylation of H3K27me2 and is thought to be one of the most stable post-translational histone modifications. The average half-maximal time of fully labelled H3K27me3 histones in proliferating HeLa cells, measured by combining stable isotope labeling of amino acids in cell culture (SILAC) pulse with quantitative mass spectrometry-based proteomics, is estimated to be as long as 3.1 days (Zee et al., 2010).

In such a scenario, when re-adding HT to the population of p16-null LCL 3CHT, which have been previously cultured without HT for 21-30 days and have lost the H3K27me3 peak at p16\(^{\text{INK4A}}\) exon 1, an initial latency of about 24-72h following readdiction of HT would be expected while EBNA3C-HT protein is synthesised and the levels usually found in cells cultured with HT are reached. However, the remaining non-degraded EBNA3C-HT molecules should be reactivated shortly after readdiction of HT, so some EBNA3C activity should be present immediately after HT is re-added. According to current data on H3K27me3 turnover (Zee et al., 2010) and the timing of the regulation of the p16\(^{\text{INK4A}}\) locus by H3K27me3 in human fibroblasts (Barradas et al., 2009) at the level of cell population, the peak of H3K27me3 equivalent to that found in LCL 3CHT cultured with HT would be expected to form in maximum 6-7 days after EBNA3C reactivation, if the H3K27me3-establishing complexes (i.e. PRC2) were available and active at p16\(^{\text{INK4A}}\) locus in all cells simultaneously. Taken together, if the p16\(^{\text{INK4A}}\) locus in all cells in the population was actively modified by PRC2 following readdiction of HT, a more rapid restoration of the H3K27me3 peak at p16\(^{\text{INK4A}}\) locus would be expected over the time period of 6 to 9 days (Fig. 3.2.22). My current data do not fit this model, since surprisingly, even in the cycling
and exponentially dividing population of p16-null LCL 3CHT, a longer period of about 14 to 21 days following readdition of HT is needed- at the level of cell population- for the reconstitution of H3K27me3 at $p16^{INK4A}$ exon 1 to the quantities observed in controls cultured with HT.

Fig. 3.2.22. The model of the expected timing of H3K27me3 restoration at $p16^{INK4A}$ exon 1 following readdition of HT on p16-null LCL 3CHT cultured previously without HT. After the initial latency of about 3 days, which allows for synthesis and posttranslational modification of the EBNA3C-HT protein (depleted in the absence of HT), the peak of H3K27me3 would be expected to fully form in about 6-7 days. However, this model is not consistent with my current data, which show that the H3K27me3 at $p16^{INK4A}$ locus is, at the level of cell population, deposited in a gradual manner following readdition of HT. The full peak of H3K27me3 at $p16^{INK4A}$ locus is restored slower than expected, after about 20 cell divisions.

The de-repression of both $p16^{INK4A}$ and BIM after EBNA3C inactivation implies that the initiating factor needs to persist in LCL and BL31, in the absence of DNA methylation, in order to maintain the epigenetic repression. Modelling of the epigenetic repression at $p16^{INK4A}$ locus by EBNA3C illustrates the gradual cumulative nature of the process at the level of cell population (Fig. 3.2.14), which can be interpreted as increasing number of cells acquiring repressive H3K27me3 mark at $p16^{INK4A}$ locus with each cell division. These
findings imply that the process is mitosis-dependent (i.e. dependent on the number of mitotic divisions) even when the potential selection pressure in culture has been eliminated. It is therefore possible that EBNA3C (perhaps in co-operation with EBNA3A) plays a role in the transmission of H3K27me3 at the target loci throughout the cell division.

Several models of H3K27me3 transmission - the maintenance of the H3K27me3 mark in proliferating cells - have been proposed so far, but the mechanism of propagation of this and other histone modifications is largely unknown (Bonasio, Tu and Reinberg, 2010; Zhu and Reinberg, 2011). During cell division, chromatin undergoes transient genome-wide disruption followed by restoration on the DNA of the daughter cells. Histone modifications could be either copied according to a parental template or re-established in a DNA replication-coupled process (Jasencakova and Groth, 2010; Zhu and Reinberg, 2011).

Combination of the crystal structure of the methyllysine histone-binding WD40 domain from the EED subunit in complex with H3K27me3 peptides and in vitro histone methylation assays has demonstrated that the binding of H3K27me3 to EED allosterically stimulates PRC2 and promotes formation of new H3K27me3 marks (Margueron et al., 2009). Similar to DNA methylation maintenance, the reading of existing H3K27me3 marks by EED is thought to lead to the PRC2-mediated writing of the new ones (Margueron et al., 2009; Xu et al., 2010a). EBNA3C could assist in directing such templated copy event by acting like a locus-specific methylation reader and it would therefore be of interest to assess the quantitative binding of H3K27me3 peptides to EBNA3C. Such a mechanism of H3K27me3 transmission by templated copying is feasible, provided the histones are divided in a semi-conservative manner, when H3-H4 tetramer split into two copies with each daughter cell containing one of the original H3-H4 dimers and one newly deposited. This has not been convincingly proven; on the contrary the recent findings argue that the H3-H4 tetramer splitting is generally infrequent and the majority of H3-H4 tetramers segregate in a conservative manner, resulting in nucleosomes bearing exclusively old or new H3–H4 tetramers. This would exclude histone modifications transmission based on
copying preexisting modifications within the same nucleosome (Xu and Zhu, 2010; Katan-Khaykovitch Y, Struhl, 2011; Zhu and Reinberg, 2011).

Another potential mechanism of epigenetic inheritance of histone modifications is replication-dependent re-establishment of histone modifications at the nascent chromatin. It was suggested that either transcription factors or transactivators are retained at the locus on mitotic chromosomes and tag the locus for subsequent fast activation or repression (Zaidi et al., 2010 and 2011; Zhao et al., 2011b). The information about the functional state of the gene is so conveyed to the daughter cells and can be readily reproduced post-mitotically in both daughter cells. This concept was termed gene bookmarking and is currently being explored using in vivo real-time imaging systems at high temporal resolution (Zaidi et al., 2010; Zhao et al., 2011b). Once the EBNA3C ChIP assay has been established using the strep-FLAG (TAP) tagged EBNA3C proteins, it would be of great interest to see, not only whether EBNA3C can be detected at the \( p16^{\text{INK4A}} \) locus, but also whether it remains associated with the locus during cell division or more generally whether EBNA3C is retained on mitotic chromosomes.

In addition, the requirement for a rather high number of cell divisions in the epigenetic regulation of \( p16^{\text{INK4A}} \) might also stem from different availability or activity of EBNA3C throughout the cell cycle. EBNA3C might be posttranslationally modified or the complexes containing EBNA3C might be disrupted or assembled in association with cell division.

Lastly, I still have not excluded the possibility that EBNA3C regulates \( p16^{\text{INK4A}} \) indirectly and there could be a number of molecular events intercalated between EBNA3C inactivation or reactivation and the transcriptional modulation of \( p16^{\text{INK4A}} \).
**p16^{INK4A} as a driver locus**

Cancers are thought to arise as a result of an accumulation of genetic and epigenetic abnormalities within a cell (Hanahan and Weinberg, 2000; Billaud and Santoro, 2011). Although numerous genetic anomalies and extensive epigenetic reprogramming have been identified in cancerous cells, there seems to be a hierarchy in such alterations in regards to their contribution to the transformed phenotype. Certain mutations and epimutations appear to be crucial drivers of transformation while others are more auxiliary. It has been shown that a modulation of a few ‘driver’ loci or in an extreme case a single locus could induce or reverse a very profound malignant phenotype challenging the paradigm of multi-step tumorigenesis (Jain et al., 2002; Felsher, 2004; Jonkers and Berns, 2004; Shachaf and Felsher, 2005; Geyer, 2010). For example, c-myc, once its proapoptotic functions had been abrogated, could alone induce carcinogenesis in β-islets of pancreas (Greenwood, 2002, Pelengaris, Khan and Evan, 2002). Strikingly, this process could be reversed even at the stage of extensive angiogenic tumours invading local blood vessels and draining lymph nodes, solely by inactivating c-myc (Greenwood, 2002, Pelengaris, Khan and Evan, 2002).

In LCL 3CHT, 550 genes were found to be differentially regulated 7 days after EBNA3C inactivation, before the p16^{INK4A}-Rb axis was engaged. Furthermore, p14^{ARF} in addition to p16^{INK4A} is thought to contribute to the proliferative arrest following EBNA3C inactivation (Maruo et al., 2011; Zhao et al., 2011a). However, in p16-null lines, the proliferation rate in the absence of this single gene remained constant following EBNA3C inactivation despite the regulation of approximately 200 cellular genes (microarray by Rob White, personal communication). The repression of p16^{INK4A} by EBNA3C thus seems to be an overriding regulatory event in LCL. In addition, it is known that cell cycle checkpoint defects tend to make cells more reliant on the remaining intact pathways and checkpoints (Dixon and Norbury, 2002; Reinhardt et al., 2007). Therefore it would be reasonable to expect that the p14^{ARF} pathway or other proliferation-controloing genes/pathways regulated by EBNA3C might take over p16^{INK4A} function and inhibit the proliferation in p16-null LCL 3CHT.
following EBNA3C inactivation. However, this is clearly not the case, since none of the EBNA3C-regulated genes in LCL could compensate for or, at least partially, substitute for the p16$^{\text{INK4A}}$ function (Fig. 3.2.5, Fig. 3.2.6 and Fig.3.2.9). This is yet another demonstration of the notion that not all gene modifications are equally functionally significant and that regulation of certain driver loci is highly important in particular contexts.

**p16$^{\text{INK4A}}$ is specifically important for B cell resistance to transformation**

Increases in p16$^{\text{INK4A}}$ and p14$^{\text{ARF}}$ in lymphoid progenitors were found to be causally associated with age-related decreases in B lymphopoiesis and increased resistance to transformation (Signer et al., 2008). A B lineage-specific ablation of p16$^{\text{INK4A}}$ in mice was associated with an increased incidence of systemic, high-grade B cell neoplasms (Liu et al., 2011). This is in contrast to other tissues; e.g. T-lineage specific deletion of p16$^{\text{INK4A}}$ was not associated with increased tumour formation (Liu et al., 2011). Although these studies were performed on B cell progenitors as opposed to differentiated B lymphocytes, they further reinforce the view that p16$^{\text{INK4A}}$ tumour suppressor mechanism might be particularly important for the B cell lineage.

**p16$^{\text{INK4A}}$ as a barrier to B cell transformation and immortalization by EBV**

In the experiments presented in this study, PBL or purified primary B cells were infected with EBV and the transcripts of CDKN2A/p16$^{\text{INK4A}}$ and several control genes were quantified for up to 25 days. The timescale of 25 days was chosen to assess the dynamic changes rather than a snapshot of cellular transcription. By the final time-point (25th day), EBV-infected cell cultures could be considered as emerging transformed LCL; they were actively proliferating and comprising cell clumps.

CDKN2A/p16$^{\text{INK4A}}$ transcription was not maintained at its original very low level after EBV infection of primary B cells or PBL, but increased several fold (Fig. 3.2.18 and Fig. 3.2.20). Nikitin et al. (2010) showed in a similar experiment that EBV infection of primary B cells
leads to hyperproliferation and replication stress, resulting in DNA double-strand breaks that induce the DDR. After EBV infection, the host cell activates protective ATM/CHK2 mediated DDR signalling pathways to suppress B cell transformation. However, EBNA3C appears to disable ATM/CHK2-mediated host defence and permits B cell proliferation (Nikitin et al., 2010). If my data regarding an increase in CDKN2A/p16\textsuperscript{INK4A} transcripts following EBV infection of primary B cells prove to be reproducible, they would suggest that CDKN2A/p16\textsuperscript{INK4A} could act, in an analogous manner to ATM/CHK2 pathway, as a barrier to transformation. Although CDKN2A/p16\textsuperscript{INK4A} transcripts increased in the normal-density subpopulation, considerably higher levels were found in the high-density fraction (Fig. 3.2.21). Even if CDKN2A/p16\textsuperscript{INK4A} transcription was not silenced in the live transformed cells, we may hypothesize that it was retained below a critical threshold that permits proliferation. EBNA3C (and EBNA3A) might be important, in addition to blocking the host cell DDR, to control the induction of p16\textsuperscript{INK4A} during primary B cell transformation by EBV.

It has been reported previously (Maruo et al., 2011) that the p16\textsuperscript{INK4A} locus in LCL contains lower levels of H3K27me3, relative to resting B cells, and these are further reduced following EBNA3C inactivation. This supports the notion that p16\textsuperscript{INK4A} expression increases during transformation of B cells, and in the newly formed LCL, a new equilibrium in p16\textsuperscript{INK4A}-Rb pathway is established, with steady-state p16\textsuperscript{INK4A} expression higher than in resting B cells but tightly controlled by EBNA3C and EBNA3A. Once the equilibrium in p16\textsuperscript{INK4A}-Rb pathway in LCL is set, even relatively small increase in p16\textsuperscript{INK4A} expression (e.g. 2 or 3-fold) following EBNA3C inactivation already inhibits LCL proliferation (Fig. 3.1.4 and Fig.3.1.6 – the measurement of LCL 3CHT proliferation and p16\textsuperscript{INK4A} transcript levels in the same samples).

Previously, transformation/immortalization experiments were performed by comparing the outgrowth of p16-competent and p16-functionally null B cells infected with the WT B95-8 strain of EBV (Hayes et al., 2004). Outgrowth occurred simultaneously for both sets of cells
by 14-days post-infection. The authors concluded that p16\textsuperscript{INK4A} status does not have a major impact on the rate of outgrowth of early established LCL, although subtle changes in the outgrowth in the initial stages of transformation could not be excluded. I would offer an alternative interpretation of the outcomes of Hayes \textit{et al.} (2004) study, specifically the facts that the p16-competent cells could be transformed similarly to p16-null B cells, and that the proliferation of established LCL in normal conditions, were unaffected by the p16\textsuperscript{INK4A} status. I suggest that rather then p16\textsuperscript{INK4A} lacking its crucial tumour suppressive functions in B cells, EBV has evolved sufficient mechanism to overcome the barrier to transformation posed by activation of the p16\textsuperscript{INK4A}-Rb pathway, by keeping CDKN2A/p16\textsuperscript{INK4A} expression within the range that allows transformation, most likely with a combination of several very efficient mechanisms (EBNA3C, EBNA3A, possibly LMP1).

The benefit of p16\textsuperscript{INK4A} repression for EBV

The control of an inappropriate induction of p16\textsuperscript{INK4A} is advantageous for EBV in the initial stages of EBV infection when it is crucial for the virus to force a subset of resting naive B cells into the cell cycle. It is probable that p16\textsuperscript{INK4A} repression has a ‘physiological’ function also later in a latently infected cell useful to the persistence of the virus. I hypothesise that it is cell rejuvenation. EBV, similarly to other \textit{herpesviridea}, requires very close contact between its human hosts for transmission. If a pathogen depends on the host for mobility, if it needs to be spread by host to another host, the mild pathogens are favoured by natural selection (Ewald, 2004). In the evolution towards lower pathogenicity, EBV was likely to develop mechanisms to ensure the survival and longevity of the target cells for persistence in its host. In their seminal work, Thorley-Lawson and his team described how EBV achieves persistence by gaining entrance into the long-lived compartment of memory B cells (Thorley-Lawson and Gross, 2004). However, it is possible that EBV might contribute to the extended life-span of the cells it resides in. EBV latent infection might modify the pathways involved in cell longevity and impose modifications that extend cellular lifespan (i.e. H3K27me3 at p16\textsuperscript{INK4A} locus preventing significant p16\textsuperscript{INK4A} increase). The repression
of $p16^{\text{INK4A}}$ in infected B cells could be maintained by latent EBV to ensure these cells stay ‘biologically younger’. It is generally accepted that EBV transiently drives proliferation of the naive B cells and after their maturation takes advantage of the intrinsic long-lived properties of the memory B cell. However, EBV latency genes may also act directly to promote more long-lived B cells through modulations of genes having anti-senescence and antiapoptotic functions. The repression of $p16^{\text{INK4A}}$ would be advantageous for EBV in both of the scenarios.

In summary, although EBNA3C seems to directly or indirectly govern a reprogramming of the entire subset of cellular genes, in this work I have identified the repression of $p16^{\text{INK4A}}$ by EBNA3C in LCL as the major phenotype-determining event, the switch between the benign or senescent phenotype and the malignant proliferative phenotype. The absence of this ‘driver’ locus in LCL overrides the phenotypic change otherwise observed after EBNA3C inactivation and renders EBNA3C dispensable for proliferation of established LCL.

In addition, the p16-null LCL 3CHT system enables the study of cellular genes regulated by EBNA3C without the interference of E2F1- or otherwise cell cycle-mediated transcription (Fig. 3.2.5 and Fig.3.2.7). Since the LCL proliferation remains unchanged regardless of EBNA3C activity, the selection pressure based on faster outgrowth of advantageously modified subset of cells is removed. This system therefore bypasses several problems associated with the study of EBNA3C and should allow new insights into EBNA3C function.
Limitations of the study

The 19bp germline deletion in CDKN2A was identified in Leiden University Medical Centre within the families with hereditary occurrence of melanoma of the skin and other cancers, e.g. pancreatic cancer (de Vos tot Nederveen Cappel, 2003; de Snoo, 2008). The vast majority of the carriers contain a heterozygous germline deletion in the locus; however, at least two patients with homozygous germline deletion have been identified so far (Gruis et al., 1995; Brookes, 2002), including the patient that supplied the blood used in this study (Gordon Peters and Alison Sinclair, personal communication). The patient included in this study was a male in his 20s with the clinical history of multiple atypical naevi and several superficially spreading malignant melanomas (Gordon Peters and Alison Sinclair, personal communication). It cannot be excluded that the patient carrying the homozygous germline defect in CDKN2A does not carry additional mutations in genes regulating cell proliferation. However, Brookes et al. (2002) investigated the karyotype and the functionality of the p53 pathway in p16-null fibroblasts of the same individual, and excluded any gross genetic instability, pre-existing cytogenetic aberrations or defects in p53 function. Furthermore, I have observed 50% reduction in proliferation of p16-null EBNA3A KO LCL in comparison to the respective p16-null EBNA3A revertant LCL (Fig.3.2.16), which implies that p16-null LCL are not generally resistant to the inhibition of proliferation.

Findings from studies using in vitro LCL systems should only be carefully extrapolated into in vivo situations. The EBV transformation efficiency in vitro is much greater than the ability of EBV to cause tumours in vivo. About 95% of the world adult population has been infected with EBV and, following primary infection, remains lifelong carriers of the virus. In some cases, tumours characterized by the presence of multiple extrachromosomal copies of the viral genome in tumour cells and the expression of part of the EBV genome arise in carriers. However, the development of the EBV-related tumour is a relatively rare event. Latent EBV infection creates an environment permissive for transforming events, but
usually further aberrant pro-proliferative or anti-differentiation lesions are necessary for lymphomagenesis. The loss of p16\textsuperscript{INK4A} function due to repressive modifications mediated by EBV is likely to facilitate survival of EBV-infected cells following other EBV-related and random EBV-unrelated oncogenic events. The H3K27me3 mark might direct DNA methylation of the p16\textsuperscript{INK4A} locus which would further stabilize the repression. Such heritable modifications could occur in other loci as well, e.g. of proapoptotic genes such as BIM. These non-neoplastic but epigenetically disrupted persisting precursor cells could create a reservoir with an epigenetic environment favouring neoplastic transformation.

While studying the primary B cell transformation and immortalization by EBV, in order to make meaningful conclusions, the phenotype of EBV-infected B-cells needs to be accurately assessed – in regards to the rate of apoptosis (by eg. annexin V with 7-AAD flow cytometry), activation (eg. anti-CD23 flow cytometry) and proliferation (using BrdU incorporation or CFSE proliferation-tracking dye). In addition, the more precise isolation of cells into the pure subpopulations than by Ficoll-Paque gradient could be achieved by flow cytometry assessing EBNA2 or CD23 expression (sorting for EBV-infected subpopulation), or using proliferation-tracking dye CFSE (sorting for proliferating cells). Furthermore, in order to distinguish the changes in the gene expression related either to activation of resting B cells into B blasts or specifically to EBV, EBV-infected cells can be compared to cells activated by soluble CD40L in the presence of interleukin-4. This combination of signals mimics B cell activation by CD4+ T cells and efficiently induces B cell proliferation for up to ten weeks \textit{in vitro} (O’Nions and Allday, 2004).
Future work

- Re-introduce p16\textsuperscript{INK4A} into p16-null LCL 3CHT by p16\textsuperscript{INK4A}-expressing lentivirus. Inhibition of proliferation of p16-null LCL 3CHT following enforced p16\textsuperscript{INK4A} expression would demonstrate that p16-null LCL 3CHT are responsive to WT p16\textsuperscript{INK4A} and do not contain defects down-stream of p16\textsuperscript{INK4A}.

- Microarray of cellular transcripts of p16-null LCL 3CHT cultured 30 days with and without HT, and cultured 30 days without HT and after HT was re-added for 30 days, have already been performed (Rob White, personal communication). The data will be compared with the previously reported sets of genes regulated by EBNA3C in p16-competent LCL 3CHT (Zhao \textit{et al}., 2011a) and in BL31 infected with WT and EBNA3C KO recombinant EBV-BAC (White \textit{et al}., 2010). In addition, to determine the role of EBNA3C-CtBP co-operation in regulation of cellular genes other than p16\textsuperscript{INK4A}, the gene set regulated by EBNA3C in p16-null LCL 3CHT will be compared with the cellular genes regulated in LCL carrying the CtBP-binding mutant of EBNA3C (3C\textsuperscript{CtBP} LCL) (Rob White, personal communication).

- Attempt to immortalize p16-null B lymphocytes with EBNA3C KO virus (assess whether the control of p16\textsuperscript{INK4A} expression is the single reason why EBNA3C is essential for transformation).
3.3 EBNA3C and/or EBNA3A-regulated cellular microRNAs

Objectives

Recent genome-wide analyses have shown that virtually all DNA nucleotides of the human genome are, to some degree, transcribed into RNA. Only 2% of the mammalian genome encodes mRNAs that are translated into proteins, while the vast majority is transcribed into regulatory non-protein-coding RNAs, including miRs, small interfering RNAs, PIWI-interacting RNAs and various classes of long ncRNAs (Taft et al., 2010). Thousands of ncRNAs are regulated in response to acute virus infection (Peng et al., 2010) and during cancer development (Braconi et al., 2011a and 2011b). Since tools for robust functional analysis of ncRNA are only just becoming available (e.g. siRNA libraries), functions of the majority of ncRNA are currently uncharacterized.

In the past, identification of the cellular targets of viral oncoproteins led to the discovery of several important growth and cell cycle regulators (Ferrari, Berk and Kurdistani, 2009). In this study, I investigated whether EBNA3C and EBNA3A in addition to their well-known role in regulating cellular protein-coding transcripts (White et al., 2010) also modify the expression of ncRNAs, in particular miRs.

All of the results listed below were acquired using the p16-competent LCL expressing the WT p16\textsuperscript{INK4A}, unless otherwise indicated.
Results

**EBNA3C and EBNA3A regulate expression of several cellular miRs in LCL**

In order to determine whether EBNA3C and EBNA3A can trigger changes in cellular miR expression, the expression of 377 human biologically active mature miRs was examined using Taqman real-time qPCR low density arrays (TLDA) to analyse two LCL 3CHT lines cultured for 28 days with or without HT and two EBNA3A KO LCL and their respective revertants. The TaqMan® Array Human MicroRNA A Card v 2.0 contained a selection of cellular miRs that are functionally defined, broadly expressed and/or highly expressed. Three ncRNA endogenous controls were available on the card to aid in data normalization, including U6snRNA assay which was repeated four times on each card. The RNA preparations used in the screen were enriched for miRs and small RNAs up to 200bp and analyzed directly by TLDA without pre-amplification (see Materials and Methods, pp.75-76).

Several cellular miRs were found to be regulated by either EBNA3A or EBNA3C or both (data not shown). The data acquired were screened for positive leads to be followed-up by further qPCR measurements and was not statistically analyzed for several reasons. As the result of the high complexity of the miR pool, it was reported that fold change measurements of some assays within the card may be less than the true value. Furthermore, the expression of many cellular miRs is strictly context and tissue-specific, and a large proportion of miRs in LCL are intrinsically expressed at low levels. Due to simultaneous high background from a subset of assays within the card, it was difficult to set meaningful cut-off values. In addition, since the effectiveness of miRs largely depends on the quantity of their regulated cellular mRNA targets, it would be problematic to set a uniform level of miR differential expression that could be biologically significant.

The positive leads from the screen were followed-up using the single Taqman microRNA expression assays. The oncogenic growth-promoting miR-221 was found to be regulated by both EBNA3C and EBNA3A. MiR-221 was induced approximately 2-3 fold by EBNA3C
and 6-20 fold by EBNA3A (Fig. 3.3.1A). MiR-221 was relatively well expressed in LCL and even the 2.5-fold increase therefore translates to a considerable change in the miR-221 copy number in a cell (Fig. 3.3.1B).

**A**

**Fig. 3.3.1. Regulation of miR-221 by EBNA3C and EBNA3A in LCL.** (A) qPCR of miR-221 in two LCL 3CHT (-A and -C) cultured for 28 days with (+HT) or without HT (-HT) and in EBNA3A KO (3AKO) and revertant (rev) LCL. MiR-221 is induced about 2-3 fold in LCL 3CHT cultured with HT and 6-20 fold in EBNA3A revertant LCL. (B) Comparison of the absolute amounts of miR-221 and U6snRNA quantified by qPCR in miR-enriched RNA preparation from LCL 3CHT-A and -C cultured for 28 days with or without HT. MiR-221 belongs to miRs with medium abundance in LCL.
MiR-138, a tumour suppressor miR, was found robustly repressed by EBNA3A alone. EBNA3A KO LCL expressed 12-24 fold more miR-138 than the respective revertants (Fig. 3.3.2A). Similar to miR-221, miR-138 was well expressed in LCL in comparison to U6snRNA and other miRs (Fig. 3.3.2B and data not shown). Mir-138 expression was found unchanged in LCL 3CHT cultured for 28 days with or without HT (Fig. 3.3.2A).

Fig. 3.3.2. Regulation of miR-138 by EBNA3A in LCL. (A) qPCR of miR-138 in two EBNA3A KO (3AKO) and revertant (rev) LCL and two LCL 3CHT (-A and -C) cultured for 28 days with or without HT (+HT or -HT). miR-138 is upregulated about 12-24 fold in EBNA3A KO LCL. (B) Comparison of the absolute amounts of miR-138 and U6snRNA quantified by qPCR in miR-enriched RNA preparation from EBNA3A KO and revertant LCL. Mir-138 belongs to miRs with medium abundance in LCL.
EBNA3C and EBNA3A repress the anti-proliferative miR cluster mir-143/145

MiR cluster 143/145 has emerged in recent years as a major universal tumour suppressor across a variety of tissues. MiR-143/145 are down-regulated in a wide range of cancers, including B cell malignancies. They co-operatively promote differentiation and repress proliferation in several cancer and primary cell lines and are both up-regulated during senescence in human fibroblasts (Elia et al., 2009; Kent et al., 2010; Bonifacio and Jarstfer, 2010).

MiR143/145 were found almost undetectable in the LCL containing WT and active EBNA3C and EBNA3A, but their expression was released once either EBNA3C or EBNA3A activity was withdrawn (Fig. 3.3.3A). Mature miR-143 and miR-145 were upregulated about 8-12 fold 28 days following EBNA3C inactivation in LCL 3CHT and about 20-50 fold in EBNA3A KO LCL in comparison to the respective revertants (Fig. 3.3.3B).
Fig. 3.3.3. Regulation of mature miR-143 and miR-145 by EBNA3C and EBNA3A. (A) Absolute amounts of miR-143 and miR-145 quantified by qPCR in miR-enriched RNA preparations from two EBNA3A KO (3AKO) and revertant (rev) LCL and two LCL 3CHT (-A and -C) cultured for 28 days with or without HT (+HT or -HT). MiR-143/145 are barely detectable in the presence of active EBNA3C and EBNA3A and even after their repression is released by EBNA3C inactivation or in the absence of EBNA3A, they still belong to low-expressed group of miRs in LCL. (B) qPCR - representative of three independent experiments- of miR-143 and miR-145 in two LCL 3CHT (-A and -C) cultured for 28 days with or without HT and in two EBNA3A KO and revertant LCL. MicroRNA 143/145 cluster is upregulated in LCL after EBNA3C inactivation or in the absence of EBNA3A.
The expression of the miR\(^*\)-143/145 carrier strand is consistent with miR-143/145 guide strand

The functional significance of the carrier strand of the miR duplex, i.e. the strand that is not incorporated into RNA-induced silencing complex (RISC), is currently the topic of ongoing investigations (Guo and Lu, 2010). It has been proposed that the carrier strands are not merely a vehicle for the active guide strand of the miR duplex but that at least in some cases possess their own regulatory functions (Okamura et al., 2008; Guo and Lu, 2010). MiR\(^*\) carrier strands frequently mimic the expression of the guide strand. The regulation of the carrier strands is typically less pronounced since they tend to be degraded faster.

The quantities of the carrier strands complementary to miR-143/145 were assessed in LCL 3CHT cultured for 28 days with or without HT and in EBNA3A KO and revertant LCL. The expression of the carrier miR\(^*\)-143/145 was similar to the expression of the guide strand, although perhaps in some cases, such as miR\(^*\)-145 in EBNA3A KO LCL, the de-repression was less pronounced (Fig. 3.3.4).

![Fig. 3.3.4. Regulation of the carrier miR\(^*\)-143 and miR\(^*\)-145 by EBNA3C and EBNA3A. qPCR of miR\(^*\)-143/145 in two LCL 3CHT (-A and -C) cultured for 28 days with or without HT (+HT or -HT) and in EBNA3A KO (3AKO) and revertant (rev) LCL. The expression of the microRNA\(^*\) 143/145 is analogous to the expression of the respective guide strands.](image-url)
EBNA3C and EBNA3A repress the long non-coding RNA precursors of miR 143/145 cluster

According to Iio et al. (2010), repression of miR-143/145 in human cancer cell lines and in cancer tissues is caused by the down-regulation of host gene expression, specifically the 11, 7.5, and 5.5 kb long transcripts that are the precursors of miR-143/145 (NCR-143/145, see Introduction, pp.47-48). It was therefore tested whether EBNA3C and/or EBNA3A repress miR-143/145 in LCL via downregulation of NCR-143/145.

NCR 143/145 were quantified in LCL 3CHT cultured with or without HT using qPCR assays developed by Iio et al. (2010) and annotated as loc 10 and loc 3. Localization of the assays is depicted in Fig. 3.3.5A. In LCL 3CHT cultured with HT, NCR 143/154 were barely detectable; however, their expression increased progressively with time of culture without HT. By day 31 without HT, both amplicons were upregulated about 20 to 30-fold in comparison to the controls cultured with HT. When HT was re-added, the accumulation of NCR 143/145 was reversed (Fig. 3.3.5B).

Next, I assessed whether the expression of the mature miRs and their precursors correlated in the same samples. The expression was very well matched in both LCL 3CHT cultured for 28 days with or without HT (Fig. 3.3.6A) and in EBNA3A KO and revertant LCL (Fig. 3.3.6B).
Fig. 3.3.5. NCR-143/145 expression in LCL 3CHT cultured with or without HT. (A) Scheme of the chromosomal region 5q33 encoding the long non-coding precursor of miR-143/145. Position of loc3 and loc10 amplicons within the long precursors of miR-143/145 is depicted (adapted from Iio et al., 2010). (B) qPCR of loc3 and loc10 amplicons in two LCL 3CHT lines cultured with or without HT. LCL 3CHT-A was cultured with HT (A +HT), 14 and 31 days without HT (A -14 and A -31) and 12 days after HT was readded (A re-add HT). Similarly, LCL 3CHT-C was cultured with HT (C +HT) and 21 days without HT (C -21). NCR 143/145 are progressively de-repressed with time following EBNA3C inactivation; however, the process is reversed after EBNA3C reactivation.
Fig. 3.3.6. The correlation of mature miR-143/145 and their long non-coding precursors in the same LCL samples. (A) miR-143/145 and NCR143/145 were assessed by qPCR in two LCL 3CHT (-A and -C) cultured for 28 days with or without HT (+HT or -HT). The de-repression of NCR 143/145 correlated well with that of the mature miRs in LCL following EBNA3C inactivation. (B) Similar analysis in EBNA3A KO (3AKO) and revertant (rev) LCL.
The regulation of the NCR 143/145 by EBNA3C and EBNA3A is less pronounced in p16-null LCL

Since the expected function of miR-143/145 in LCL is inhibition of proliferation, the regulation of both mature miRs and their precursors by EBNA3C was assessed in the proliferation-independent p16-null LCL 3CHT system. EBNA3A KO and revertant p16-null lines were included for completeness.

NCR 143/145 were quantified simultaneously in p16-null and p16-competent LCL 3CHT cultured for 21 days with or without HT and in EBNA3A KO and revertant LCL. As expected, in the p16-competent lines lacking EBNA3C or EBNA3A function, the NCR-143/145 were de-repressed. The de-repression of the NCR 143/145 transcripts in the p16-competent lines seemed about 2 to 2.5-fold greater than reported previously (Fig. 3.3.5B and 3.3.6), this was probably due to the use of a different set of housekeeping genes in the normalization procedure.

In the p16-null lines, the NCR-143/145 transcripts were also found to be upregulated following EBNA3C inactivation and in EBNA3A KO cells; however, the de-repression seemed to be less pronounced than in the p16-competent cell lines (Fig. 3.3.7).
Fig. 3.3.7. Regulation of NCR 143/145 by EBNA3C and EBNA3A in p16-null LCL. (A) qPCR of NCR 143/145 in p16-competent (ctrl A and ctrl C) and p16-null (A1, A2, C1, C2) LCL 3CHT cultured for 21 days with or without HT. NCR143/145 are de-repressed in p16-null lines following EBNA3C inactivation; however, to a lesser degree than in p16-competent lines. (B) Similar qPCR analysis performed using p16-competent EBNA3A KO (ctrl 3AKO), revertant (ctrl rev) and their p16-null counterparts.

At the moment, insufficient data is available to make similar meaningful comparisons between p16-null and -competent cell lines at the level of mature miR-143/145. The miRs were quantified in both p16-competent and p16-null LCL; however not alongside each other in a single measurement (using the same RNA isolation protocol and the same endogenous controls). In p16-null lines, miR 143 was de-repressed to a lesser degree than observed in p16-competent LCL after EBNA3C inactivation or in the absence of EBNA3A (Fig. 3.3.8 and Fig. 3.3.3B or Fig. 3.3.6). However, miR-145 seemed to be similarly regulated by both EBNA3C and EBNA3A in p16-null lines as in their p16-competent counterparts (Fig. 3.3.8 and Fig. 3.3.3B or Fig. 3.3.6).
Fig. 3.3.8. Regulation of the mature miR-143/145 by EBNA3C and EBNA3A in p16-null LCL.

qPCR of mature miR-143/145 in three p16-null LCL 3CHT (A2, C1 and C2) cultured for 27 days with or without HT and in p16-null EBNA3A KO (3AKO) and respective revertant (rev) LCL. The p16-competent lines were not available for direct comparison. MiR-143 might be regulated to a lesser degree by EBNA3C and EBNA3A in p16-null LCL.

Several miR-143/145 putative targets are regulated in LCL in response to EBNA3C and EBNA3A

In the preparation for functional studies, I assembled a set of putative miR-143/145 target genes in B cell-derived lines using miR target prediction software (miRDB) and including miR-143/145 target genes validated by others in a variety of cancer models. EBV microarray data (www.ebv.org.uk) containing expression profiles of WT BL31 and BL31 infected with various EBNA3 mutants (White et al., 2010) as well as WT LCL and EBNA3A KO LCL (Hertle et al., 2009) were then searched for the putative miR-143/145 target genes regulated by EBNA3C and/or EBNA3A. In the next step, qPCR and WB analysis of the selected putative miR-143/145 targets was performed in LCL 3CHT cultured with or without HT and in EBNA3A KO and revertant LCL. The preliminary data showed that several miR-143/145 target genes were regulated at the transcript and protein level by EBNA3C and/or EBNA3A in LCL, specifically the signalling molecule RTKN and epigenetic regulator DNMT3A.
RTKN (rhotekin) is a scaffold protein that interacts with GTP-bound Rho proteins; binding of this protein inhibits the GTPase activity of Rho proteins (Ito et al., 2005). DNMT3A is a DNA methyltransferase that functions in de novo methylation (Gao et al., 2011). In an EBV microarray database, RTKN transcripts were found modestly decreased in EBNA3C KO BL31 lines in comparison to the WT BL31 (Fig. 3.3.9A). DNMT3A transcripts were found to be modestly downregulated in EBNA3A KO LCL in comparison to the respective revertant; however, they were stable in LCL 3CHT cultured with and without HT (Fig. 3.3.9B).

**Fig. 3.3.9.** Regulation of RTKN and DNMT3A transcripts by EBNA3C and/or EBNA3A in B cell-derived lines. (A) Microarray dot plot illustrating the quantity of RTKN mRNA in BL31 infected with WT, EBNA3C KO and EBNA3A KO recombinant EBV viruses. RTKN transcripts are modestly downregulated in EBNA3C KO BL31 (White et al., 2010). (B) qPCR of DNMT3A transcripts in two LCL 3CHT (-A and -C) cultured for 28 days with or without HT (+HT and -HT) and in EBNA3A KO (3AKO) and revertant (rev) LCL. DNMT3A transcripts seem to be stable following EBNA3C inactivation and modestly downregulated in EBNA3A KO LCL.

At the level of protein, both RTKN and DNMT3A were regulated by EBNA3C and EBNA3A in multiple LCL; the decrease in these proteins was modest following EBNA3C inactivation in LCL 3CHT (Fig. 3.3.10A) but pronounced in LCL lacking EBNA3A (Fig. 3.3.10B). The expression of several other predicted targets of miR-143 and/or miR-145, i.e. ERK5, fascin
and K-Ras was not changed in LCL following EBNA3C inactivation or in the absence of EBNA3A (Fig. 3.3.10A and data not shown).

**Fig. 3.3.10. Regulation of RTKN and DNMT3A protein by EBNA3C and EBNA3A in LCL.** (A) Western blot depicting the quantities of RTKN, DNMT3A and two other putative miR-143/145 targets (ERK and fascin) in four LCL 3CHT (-B, -C, -D, -A) cultured for 31 days with or without HT. The quantities of RTKN and DNMT3A protein modestly decrease in all lines following EBNA3C inactivation, as opposed to ERK and fascin which expression remains unaltered. (B) Similar analysis in two EBNA3A KO (3AKO) and revertant (rev) LCL. RTKN and DNMT3A are robustly decreased in EBNA3A KO lines while the control γ-tubulin (γ-tub) expression remains stable. WBs shown are representative of two similar experiments.
Summary key points

- Several cellular miRs are regulated by either one or both EBNA3C and EBNA3A in LCL.
- The anti-proliferative miR 143/145 cluster is robustly repressed by both EBNA3C and EBNA3A in LCL.
- EBNA3C and EBNA3A repress the mature miR-143/145 by down-regulating their long non-coding precursors NCR 143/145.
- MiR-143/145 targets in LCL might include DNTM3A and RTKN.

Discussion

Regulation of transcription and posttranscriptional gene silencing represent two major pathways determining gene expression. Posttranscriptional silencing is transient and depends on the continued presence of the miR effector molecules, whereas transcriptional gene regulation occurs at the level of DNA via chromatin changes at the gene promoters/regulatory regions and can lead to long-term change in gene expression (Turner and Morris, 2010). I have shown that in addition to epigenetically regulating production of the protein-coding transcripts (e.g. p16\textsuperscript{INK4A}), EBNA3C and EBNA3A also regulate the expression of several miRs and in extension participate in posttranscriptional regulation of cellular genes.

As shown recently in a study that combined microarrays and shotgun proteomics to quantify absolute mRNA and protein levels for over 1000 genes in a tumour cell line, transcriptional regulation accounts for only about half of protein abundance. The remaining regulation is posttranscriptional, including miRs (Vogel et al., 2010). MiRs negatively regulate the stability and translation of target messenger RNAs and offer a flexible mode of
regulation by circumventing the need for translation. EBV itself encodes at least 40 miRs that are expressed in latently infected cells (Amoroso et al., 2011; Barth, Meister and Grasser, 2011). In addition, EBV might modulate processing of viral and cellular miRs via its own miR BART6 that inhibits production of endoribonuclease DICER, an enzyme necessary to cleave dsRNA and pre-miR into 20-25nt long fragments (Iizasa et al., 2010). EBV has been shown previously to induce changes in the transcription of cellular miRs in infected cells (Imig et al., 2010; Linnstaedt et al., 2010); however, the regulation of cellular miRs by EBNA3C and/or EBNA3A has not yet been explored. I screened the response of 377 cellular miRs to the withdrawal of EBNA3C or EBNA3A function by real-time qPCR low-density miR array. Several cellular miRs were found to be regulated by either one or both EBNA3C and EBNA3A and the miR 143/145 cluster was selected for further study.

**MiR-143/145 abundance in LCL**

MiR-221 and miR-138 were well expressed in LCL and therefore even a small fold difference in expression would translate into a considerable change in the miR copy number in the cell (Fig. 3.3.1B and Fig. 3.3.2B). However, miR-143/145 and their precursors were barely detectable in WT LCL and expressed moderately in LCL lacking functional EBNA3C or EBNA3A - in the range of ten(s) of copies per cell (Fig. 3.3.3A and data not shown). Although the cellular concentration of miR is critical to their function, the downregulation of the target mRNA by miR is also the function of the target’s abundance (Arvey et al., 2010). Low abundance miRs are effective in regulating low-abundance transcripts and about 11 thousand different messages in the cell are expressed in the 5-15 copies per cell range.
**Regulation of miR-143/145 locus**

I found that both EBNA3C and EBNA3A contribute to the repression of mature miR-143/145 and their precursor NCR-143/145 in LCL. The mechanisms of miR processing are accountable in large part for the final miR abundance; however, a primary level of control of miR expression is thought to be transcriptional (Cullen, 2004). According to the study by Iio, *et al.* (2010), expression of miR-143/145 in normal and cancer tissues accurately correlates with the expression of their precursor long ncRNAs. This holds true also in LCL with or without active EBNA3C or EBNA3A (Fig. 3.3.6).

The mechanism of *miR*-143/145 promoter regulation by the two viral proteins in LCL is yet to be investigated. Several intriguing modes of regulation have been proposed in other cancer models. The *miR*-143/145 promoter was shown to be induced by Notch signalling activity in vascular smooth muscle cells, and a RBPJk/CBF1 site was necessary for this induction (Boucher *et al.*, 2011). Oncogenic Ras repressed the *miR*-143/145 promoter in both human and murine cells through the Ras-responsive element-binding protein (RREB1) (Kent *et al.*, 2010). Furthermore, different epigenetic marks were found at the *miR*-143/145 promoter in various contexts. In human colon cancer cell lines, treatment with DNMT or HDAC inhibitors did not upregulate miR-143/145 expression (Akao *et al.*, 2007). However, miR-145 was silenced through DNA hypermethylation in laser capture microdissected prostate tissues (Suh *et al.*, 2011) and miR-143 was epigenetically repressed by promoter hypermethylation in MLL–AF4 positive B-cell ALL, but not in normal bone marrow cells or MLL–AF4-negative primary blasts (Dou *et al.*, 2011). Since the precise localization of the regulatory regions of *miR*-143/145 promoter has now been well defined by others, it should facilitate our own studies of *miR*-143/145 regulation by EBNA3C and EBNA3A in LCL.
Functional significance of miR-143/145 regulation by EBNA3C and EBNA3A in LCL

Since the functional outcome might depend not only on the degree of miR-143/145 upregulation in the absence of EBNA3C or EBNA3A function but also on other factors including miR-143/145 final quantity within a cell, it will be crucial to establish whether upregulation of miR-143/145 causally contributes to the growth inhibition in LCL. During senescence of human fibroblasts, miR-143/145 were upregulated and transfecting a synthetic mimic of miR-143 was able to inhibit the proliferation of fibroblasts in a dose-dependent manner (Bonifacio and Jarstfer, 2010). Gain-of-function and loss-of-function studies in other tumour cell lines also implicated miR-143/145 in direct proliferation inhibition (Chen et al., 2010b; Kent et al., 2010; Zhang et al., 2010; Peng et al., 2011).

In this study, miR-143/145 and their precursor NCR 143/145 were upregulated also in p16-null LCL 3CHT where EBNA3C inactivation does not lead to any detectable change in LCL proliferation. However, the regulation of the NCR-143/145 seemed to be less pronounced in p16-null lines than in their p16-competent counterparts (Fig. 3.3.7). The mature miRs in p16-null and -competent lines have not yet been directly compared.

The pattern of expression of miR-143/145 and their precursors in LCL is similar to p16^{INK4A} mRNA. They are repressed in WT LCL, upregulated in EBNA3A KO LCL and in LCL3CHT without HT but with considerable delay, and again repressed with similar delay following HT re-adding (Fig. 3.3.3B and Fig. 3.3.5B). Furthermore, miR-143/145 are both upregulated during senescence in primary human fibroblasts (Bonifacio and Jarstfer, 2010). It is possible that there might be a cross-talk between the miR-143/145 pathway and functional p16^{INK4A}-Rb pathway. Therefore the de-repression of miR-143/145 in the proliferating p16-null LCL in the absence of EBNA3C or EBNA3A activity, whether or not less pronounced than in the p16-competent lines, should not discourage the notion that these miRs can indeed directly inhibit LCL proliferation.
Limitations of the study and future work

According to the Comparative Genomic Hybridization database, the mir-143/145 (5q33) locus is frequently involved in chromosome copy number loss in various types of cancers including non-small cell lung cancer and gastric cancer (Iio et al., 2010). Although the integrity of this genomic locus in B cell derived lines used in this study was not investigated, the LCL 3CHT conditional system by-passes this concern and allows for convincing reversible de-repression of the host gene and its residing miRs.

The data presented in this chapter represent a preliminary finding of miR regulation by EBNA3C and/or EBNA3A in LCL, but are limited to correlations rather than direct functional studies. More work will be needed to assess the role of miR143/145 in LCL proliferation and to validate the selected protein targets by direct gain-of-function and loss-of-function studies. Re-expression of miR143/145 in WT LCL or their inhibition in LCL after EBNA3C inactivation or in LCL lacking EBNA3A could also dissect the possible cross-talk between the miR-143/145 miR pathway and p16INK4A-Rb pathway. Furthermore, it will be of great interest to investigate the chromatin status at the miR-143/145 locus in WT LCL and in LCL without EBNA3C or EBNA3A function and determine whether the regulation of the miR-143/145 locus by these proteins is similar to the epigenetic regulation of the previously studied p16INK4A and BIM loci.
Chapter 4
Thoughts and Final Conclusions
The functional significance of EBNA3C and EBNA3A in LCL proliferation and B cell transformation and immortalization by EBV

Around 20% of cancers world-wide are of infectious aetiology, with leading agents being viruses of hepatitis B and C, herpesviruses EBV, KSHV and the retrovirus HTLV-1 (Parkin, 2006; Wang et al., 2007b). EBV latently persists in more than 90% of world population and is associated with approximately 1% of tumours worldwide (Delecluse et al., 2007). A complex interaction between virally encoded proteins and B cell-specific cellular factors contributes to lymphomagenesis.

In the in vitro model of EBV-mediated lymphomagenesis, intact EBNA3C is essential for transformation of primary B cells into LCL and EBNA3A greatly enhances the efficiency of B cell transformation. EBNA3C and EBNA3A are both required for the maintenance of LCL proliferation (Maruo et al., 2003 and 2006). Integration of cellular transcript microarray and ChIP analysis of histone modifications at cellular promoters in B cell-derived lines has shown that EBNA3C and EBNA3A co-operate in the epigenetic regulation of a subset of cellular genes, most notably the negative inhibitor of the cell cycle progression p16\(^{INK4A}\) and the pro-apoptotic protein BIM.

In this work, I have found that the main and perhaps only necessary role of EBNA3C in EBV-driven LCL proliferation lies in the repression of p16\(^{INK4A}\). This finding reinforces the view that EBNA3C and EBNA3A are required in LCL proliferation and most likely also B cell transformation to repress cellular tumour suppressor defences. EBNA3C and EBNA3A thus complement the action of EBNA2 which acts as an activated oncogene, driving the B cell activation and hyperproliferation. Replication stress is sensed by cellular surveillance systems and activates protective pathways to suppress uncontrolled proliferation. These consist of mediators of apoptosis and oncogene-induced senescence or growth arrest. However, EBNA3C and EBNA3A via suppression of BIM (Anderton et al., 2007; Paschos...
et al., 2009), p16\textsuperscript{INK4A} (Skalska et al., 2010 and this study) microRNA 143/145 (this study) and the ATM/CHK2 DDR (Nikitin et al., 2010) disable cellular defences and permit further cell survival and proliferation (Fig. 4.1).

![Diagram](image)

**Fig. 4.1.** Scheme of the roles of EBNA3C and EBNA3A in the maintenance of LCL proliferation and in B cell immortalization. EBNA3C and EBNA3A suppress the host cell defences (OIS, DDR and apoptosis) and allow the survival and proliferation of EBV-infected B cells. The major function of EBNA3C in maintenance of LCL proliferation and likely also in B cell immortalization is repression of p16\textsuperscript{INK4A}.

In vivo, the transforming potential of EBV is much lower than in vitro and EBV acts as a co-factor alongside other transformation events such as chromosomal translocations. Extrapolating from in vitro findings, EBNA3C- and EBNA3A-primed inactivation of p16\textsuperscript{INK4A}, BIM and microRNAs with tumour suppressor functions in vivo is likely to substantially decrease the threshold for the number of subsequent mutagens and/or inappropriate growth stimuli required to promote oncogenesis.
Mechanism of epigenetic modification of cellular genes by EBNA3C and EBNA3A

Previous work in our lab has established that EBNA3C and EBNA3A-mediated changes in the expression of cellular genes are accompanied by simultaneous changes in the chromatin structure (and sometimes DNA methylation) at the cellular promoters in B cell-derived cell lines (Paschos et al., 2009; White et al., 2010). Using p16-null LCL 3CHT, I have shown that epigenetic regulation of p16\textsuperscript{ink4a} locus by EBNA3C is truly specific and independent of the functional outcome.

Although there is accumulating evidence showing that EBNA3C, probably in co-operation with EBNA3A, can directly or indirectly modify histones at the regulatory regions of cellular genes and direct its activities specifically to certain crucial cellular loci, several key questions regarding this mechanism remain unanswered and need further investigation.

Do EBNA3C and EBNA3A trigger a cascade of transcription-regulating events or do they directly regulate multiple (hundreds of) cellular promoter regions for the benefit of the virus?

A small number of well-defined factors are sufficient to reprogram a differentiated cell into induced pluripotent stem cell (Takahashi and Yamanaka, 2006) and the unscheduled expression of a single transcription factor can cause the transdifferentiation of a mature differentiated cell into another cell type, e.g. forced expression of C/EBPa leads to conversion of B lymphocytes into macrophages (Masip et al., 2010). Similarly, EBNA3C and EBNA3A could achieve the change of cellular expression profile by modifying the expression of a single or a few cellular master regulators.

Zhao et al. (2011a) performed a network-centred analysis of EBNA3C-regulated genes in LCL 3CHT lines cultured with HT, without HT and without HT but complemented with WT EBNA3C. They have identified 550 genes that were at least 1.5-fold up- or down-regulated with false discovery rates < 0.01. In a Bayesian network analysis RAC1, LYN and TNF
were found regulated upstream of other EBNA3C-regulated genes. RAC1 is a GTPase which belongs to the Ras superfamily of small GTP-binding proteins and regulates multiple cellular events, including cell growth, cytoskeletal reorganization, and the activation of protein kinases (Rathinam, Berrier and Alahari; 2011). LYN, a member of the Src family of protein tyrosine kinases, regulates cell activation through signalling cascades in B lymphocytes and myeloid cells (Scapini et al., 2009). TNF consists of a group of cytokines that have a key role in the regulation of apoptosis and inflammation, as well as proliferation, invasion and angiogenesis (Mathew et al., 2009). EBNA3C was suggested to modulate RAC1 signalling, down-regulate LYN and up-regulate TNF. This genome-wide analysis provided bioinformatic correlations but lacked direct functional experimentation. Nevertheless, the implication of these findings is that by direct regulation of only several loci, EBNA3C could indirectly control multiple diverse cellular processes.

Alternatively, EBNA3C and/or EBNA3A could directly regulate large sets of cellular promoters, similarly to adenoviral small E1A or the latency-associated nuclear antigen LANA of KSHV. Although small E1A does not bind directly to DNA, during E1A-mediated cellular transformation, E1A associates with different subsets of cellular promoters in defined temporal order causing either transcriptional activation or repression (Ferrari et al., 2008). Similarly, LANA was shown to associate directly with several cellular promoters (Shamay et al., 2006; Su et al., 2011). In both cases, the viral oncoprotein recruits and targets a component of the epigenetic machinery to the occupied cellular promoters.

In our lab, the question which cellular promoters are regulated directly by EBNA3C and/or EBNA3A will be addressed by ChIP-seq using BL and LCL carrying strep-FLAG (TAP)-tagged EBNA3C and EBNA3A-containing recombinant EBV-BACs.
In what way is the epigenome of BL and LCL with WT active EBNA3C and/or EBNA3A different from the epigenome of the same lines after EBNA3s have been inactivated or mutated?

It remains to be confirmed whether EBNA3C and/or EBNA3A regulate cellular gene expression predominantly via H3K27me3 or whether modifications of different type and position are altered on histones as a result of EBNA3C and/or EBNA3A activity. Integration of mRNA microarray data and ChIP-chip or ChIP-seq data mapping the histone modification in the same cell lines containing WT or inactivated EBNA3C or EBNA3A should show whether the transcriptional profile following EBNA3 inactivation overlaps that of differentially H3K27me3-regulated promoters. Since both EBNA3C and EBNA3A can bind CtBP and thus potentially associate with a number of chromatin remodelers, it is possible that EBNA3C and/or EBNA3A activity will be associated not just with H3K27me3 regulation but rather a more complex combinatorial chromatin signature or a variety of epigenetic states dictated by the context of EBNA3C and/or EBNA3A binding.

What roles do EBNA3C and/or EBNA3A have in modulating the cellular epigenome?

The establishment and maintenance of epigenetic information requires several linked processes: the targeting of the modification to the specific promoters or regulatory regions, the actual establishment of the mark by the chromatin remodelling enzymes, the transmission of the mark to the daughter cells in the next cell division and finally the signalling that connects the establishment of the epigenetic marks with the environmental cues. EBNA3C and/or EBNA3A could be modulating the cellular epigenome in several ways:
EBNA3C and EBNA3A do not bind DNA directly (Young et al., 2008; Saha et al., 2011) and do not possess any obvious intrinsic enzymatic activity (Jiang, Cho and Wang, 2000; West, 2006). Although the attempts to localize EBNA3C and EBNA3A at the promoters of their target genes BIM and p16\textsuperscript{INK4A} failed using the antibodies against the WT endogenous EBNA3s, the direct binding of EBNA3C and/or EBNA3A at these loci will now be re-addressed in the cell lines carrying the strep-FLAG (TAP)-tagged EBNA3C or EBNA3A recombinant EBV-BACs. EBNA3C and EBNA3A are required for establishment and maintenance of H3K27me3 at p16\textsuperscript{INK4A} and BIM locus. In addition, both viral proteins are required for PRC2 recruitment and block phosphorylation of Pol II on ser 5 at the BIM promoter (Paschos et al., 2009; Skalska et al., 2010; Paschos et al., under revision). It is possible that EBNA3C and EBNA3A physically associate with their target loci through a DNA-binding intermediate to carry out the above functions. EBNA3C and/or EBNA3A could facilitate PcG-mediated gene repression by influencing the stoichiometry or stabilizing the repressive complexes at EBNA3C and/or EBNA3A-regulated loci. In addition, both viral proteins associate with the cellular co-repressor of transcription CtBP and, via CtBP, could recruit a number of chromatin remodelers in addition to PcG (such as LSD1, HDACs and G9a) (Fig.4.2).
Fig. 4.2. Direct association of EBNA3C and/or EBNA3A with the chromatin remodelling complex at the target cellular promoter. (A) Legend annotating the symbols used in subsequent figures. (B) Physical participation of EBNA3C and/or EBNA3A at the target locus. EBNA3C and/or EBNA3A could, through an intermediate DNA-binding element, associate with the promoter of the target gene and either recruit, in a CtBP-dependent or –independent manner, the chromatin remodelers or impact on the stability or stoichiometry of the repressive complexes at the locus.
In the targeting of chromatin remodelling complexes (enzymes) to specific loci

EBNA3C and/or EBNA3A could target the chromatin remodelers to the cellular promoters directly through association with DNA-binding elements such as ncRNA or sequence-specific TF (Fig. 4.3A). For example, EBNA3C and EBNA3A both bind the sequence-specific TF RBPJk/CBF1. In ChIP experiments performed in BL31 infected with recombinant EBV-BAC carrying WT EBNA3C and EBNA3A, RBPJk seems to be present at the BIM promoter close to the transcription start site when BIM is repressed (Kostas Paschos, personal communication).

EBNA3C and/or EBNA3A molecules could undergo different posttranslational modifications in diverse cellular contexts which might give them the ability to bind various TFs, co-factors or ncRNAs and modulate distinct subsets of cellular genes in a context-specific manner. Alternatively, EBNA3C and/or EBNA3A could up- or down-regulate the expression of sequence-specific TFs or ncRNAs (Fig. 4.3B).

By 'bookmarking' the target genes

Since 16-null LCL 3CHT in culture proliferate at the same rate regardless of EBNA3C activity, most or all of the selection pressures based on proliferative advantage following EBNA3C reactivation have been removed. However, the removal or acquisition of H3K27me3 peak at the p16$^{INK4a}$ exon1 at the level of cell population following EBNA3C switch is gradual and requires approximately 20 cell divisions. Similar kinetics of H3K27me3 removal has been observed for another PcG- and H3K27me3-regulated EBNA3C- and EBNA3A-target gene BIM (Paschos et al., under revision). It is possible, that EBNA3C and EBNA3A act in the transmission of the existing repressive marks at the target loci in the proliferating cell population. EBNA3C and EBNA3A could maintain the association with target loci during the cell division and ensure the restitution of the epigenetic mark in the daughter cells at the exact same location (Fig. 4.4). The population
of cells containing the target gene in the repressed state would be maintained in the proliferating LCL or BL containing WT active EBNA3C and/or EBNA3A, or would be gradually lost following the inactivation of EBNA3C and/or EBNA3A.

Fig. 4.3. Direct and indirect targeting of chromatin remodelling complexes to the cellular promoters by EBNA3C and/or EBNA3A. (A) EBNA3C and/or EBNA3A could directly associate with sequence-specific TFs or ncRNA and simultaneously (in CtBP-dependent or –independent manner) with chromatin remodelers and thus facilitate targeting of the chromatin remodelers to the promoters of EBNA3C and/or EBNA3A-regulated genes. (B) EBNA3C and/or EBNA3A could regulate the expression of sequence-specific TFs and/or ncRNA and indirectly modulate the targeting of the chromatin remodelers to other EBNA3C and/or EBNA3A-regulated promoters.
Fig. 4.4. Gene bookmarking by EBNA3C and EBNA3A. EBNA3C and EBNA3A are represented as dark dots that maintain their association with the promoters of their target genes during mitosis and serve as a docking station for the chromatin remodelling enzymes re-establishing the mark in the daughter cells.

D) By regulating expression of the components of the epigenetic machinery (e.g. chromatin remodelling enzymes or microRNAs)

In cancer, components of epigenetic machinery such as chromatin remodelling enzymes are frequently deregulated skewing the expression of multiple genes. In this study I excluded the regulation of expression of EZH2 and H3K27 demethylases (JMJD3/KDM6B and UTX/KDM6A) by EBNA3C in LCL (Fig.3.1.16 and data not shown). Furthermore, the steady-state levels of EZH2, SUZ12, EED, RbAp48 and JMJD3 were assessed by western blotting protein extracts from an uninfected BL31 and BL31 infected with WT EBV-BAC or recombinant EBV-BAC viruses lacking EBNA3C and/or EBNA3A. None of the chromatin remodelers mentioned above was found to be consistently regulated in correlation with the EBV or EBNA3 status of the cells (Paschos et al., under revision). However, it is possible that EBNA3C and/or EBNA3A regulate the expression of some other component of epigenetic machinery (Fig. 4.5). In addition, EBNA3C and/or EBNA3A were shown to
transcriptionally regulate a subset of cellular microRNAs, which are as well considered one of the means of epigenetic regulation.

![Diagram showing transcriptional regulation of essential component(s) of epigenetic machinery by EBNA3C and/or EBNA3A. EBNA3C and/or EBNA3A could regulate the expression of a chromatin remodelling enzyme or some other component of the epigenetic machinery and thus impact on the regulation of multiple cellular genes.](image)

**Fig. 4.5. Transcriptional regulation of the essential component(s) of epigenetic machinery by EBNA3C and/or EBNA3A.** EBNA3C and/or EBNA3A could regulate the expression of a chromatin remodelling enzyme or some other component of the epigenetic machinery and thus impact on the regulation of multiple cellular genes.

E) By regulating signal transduction cascades that result in alterations of chromatin structure (e.g. TGFβ or/and Notch2 pathways)

Although the cellular epigenomes are able to respond dynamically to the outside stimuli, the mechanism by which target genes are regulated by environmental cues and signalling pathways is not yet well defined (Gehani et al., 2010). EBNA3C and EBNA3A are known to interfere with major cellular signalling pathways including the TGFβ pathway (Lydia
Eccersley, personal communication) and could mediate the communication of environmental signals towards the cellular epigenome.

In order to further address the mechanism of the role of EBNA3C and EBNA3A in chromatin remodelling in LCL, it would be informative to integrate genome-wide data including epigenetic marks, nucleosome turnover, promoter DNA methylation status and non-coding RNA expression with the gene expression and phenotype in the WT LCL and after the inactivation or mutation of EBNA3C and/or EBNA3A. With advancing developments of genome-wide technologies (e.g. ChIP-seq), it is now possible to adopt a dynamic approach, i.e. analyze data from several time-points as well as a single snapshot.

**EBNA3C and EBNA3A expression and their relevance during the initiation and progression of malignancy in vivo**

Due to the technical difficulties with detection of EBNA3C and EBNA3A *in vivo*, their expression has not been explored in a variety of tumours. In addition, the expression of EBNA3C and EBNA3A was assumed to be constantly associated with the expression of EBNA2, consistent with the latency III expression programme. However, it appears that in tumours *in vivo*, a more varied expression patterns of EBV latent proteins are present than the ‘classic’ latency expression programmes and the assumption of EBNA3C and EBNA3A co-expression with EBNA2 might not hold true.

EBNA3A expression has been detected in the majority of EBV-positive diffuse large B cell lymphomas (Nguyen-Van *et al.*, 2011) and expression of EBNA3s was found to be retained in small but significant subset (about 10%) of BL (Kelly *et al.*, 2006). Nevertheless to date, EBNA3C and EBNA3A have not been reported to be expressed in majority of EBV-associated lymphomas (Tsang and Munz, 2011). However, they have probably been expressed transiently in majority of the B cells containing EBV episome. It is possible that
transient expression of EBNA3C and EBNA3A is sufficient to prime the cell for long-term repression of $p16^{\text{INK4a}}$, BIM and/or tumour-suppressor microRNAs. The priming might mark the genes for subsequent modifications such as DNA methylation that would serve to preserve the EBNA3C and/or EBNA3A-mediated repression of their target genes in at least a subset of EBV-infected cells even after the expression of EBNA3C and EBNA3A is shut-off.

In the EBV-positive tumours associated with immunosuppression, EBNA3C and EBNA3A are frequently detectable in EBV-carrying cells (Heslop et al., 2005; Heslop et al., 2010) and might contribute to rapid cell cycle deregulation in a number of B cells in parallel.

**Therapeutic implications in cancer**

$p16^{\text{INK4a}}$ as a therapeutic target

At the level of the organism, $p16^{\text{INK4a}}$ regulation requires a fragile equilibrium, weighing the costs and benefits between the risk of cancer and accelerated aging. $p16^{\text{INK4a}}$ is not merely a biomarker of aging but causally contributes to the tissue loss in aging organisms by reduced self-renewal and senescence of tissue stem cells with age (Molofsky et al., 2006). Recently, it has been shown using a transgenic strategy for initiating selective apoptosis of senescent cells, that the removal of $p16^{\text{INK4a}}$-positive senescent cells in mice can prevent or delay age-associated diseases such as wrinkles, muscle wasting and cataracts (Baker et al., 2011). In general, it seems that therapeutically silencing $p16^{\text{INK4a}}$ would constitute a trade-off between increased cancer and reduced aging. However, it is not clear whether $p16^{\text{INK4a}}$ plays this dual role in all tissues or only some. It has been suggested that in some cellular compartments, such as melanocytes, $p16^{\text{INK4a}}$ exhibits a strong tumour suppressor effect but has very little impact on aging (Liu et al., 2011).
At this moment, the findings regarding p16$^{INK4A}$ relevance in aging and tumour-suppression in B lymphoid progenitors and B cell lineage are conflicting. The study by Liu et al. (2011) supports a view that p16$^{INK4A}$ has major tumour-suppressive effects and lesser role in aging in murine B cell progenitors. On the contrary, Signer et al. (2008) reported that p16$^{INK4A}$ and p14$^{ARF}$ accumulation in aged murine B lymphoid progenitors leads not only to an increase in their resistance to transformation but also to their reduced growth and survival. The authors believe that the age-related p16$^{INK4A}$ and p14$^{ARF}$ accumulation is actually the major underlying cause of severe growth defects in B lymphoid progenitors during aging not only in mice but in humans. These intriguing initial findings were obtained mainly in mice models and on the population of early B cell progenitors and so further research will be necessary to assess whether p16$^{INK4A}$ reactivation in lymphomas in humans would be an effective treatment.

Virally targeted restoration of WT p16$^{INK4A}$ expression into p16-deleted glioma cells inhibited angiogenesis induced by tumour cells \textit{in vivo} (Harada et al., 1999). In hepatocellular carcinoma, virally targeted p16$^{INK4A}$ restoration in cancer cells lead to their growth arrest and apoptosis (Hu et al., 2011). It therefore seems feasible to specifically restore p16$^{INK4A}$ expression only in the tumour tissue and thus avoid the potential negative effects of increased p16$^{INK4A}$ expression in the remaining tissues of the individual. Taken together, despite the dual role of p16$^{INK4A}$ in both promoting aging and suppressing tumorigenesis, p16$^{INK4A}$ should still be considered a potential therapeutic target in lymphoproliferations.

\textit{Epigenetic therapy}

DNA methyltransferase inhibitors and histone deacetylase inhibitors are currently approved for treatment of myelodysplastic syndrome and other haematological malignancies by the US Food and Drug Administration and have been in clinical use for several years (Kelly, de Carvalho and Jones, 2010). Current drugs are pan-inhibitors that block the entire family of
DNMTs and HDACs rather than a specific enzyme. The challenge of current epigenetic drug development is to produce specific epigenetic therapies. This includes the development of specific enzyme inhibitors such as PcG-specific inhibitors and therapies targeted to specific loci as opposed to genome-wide (Tuma, 2010; Crea et al., 2011).

**In summary**

EBV induces and maintains transformation of normal B cells *in vitro* through expression of only few viral genes. In this work I have studied the effects of inactivation of two EBV nuclear antigens - EBNA3C and EBNA3A - on regulation of the cell cycle in LCL, with the remainder of the EBV latent programme intact. The rationale behind the project was that both EBV cellular targets and the mechanisms the virus employs in their modulation can inform on the general mechanisms of B cell lymphomagenesis.

Several novel microRNA target genes are identified in this report, notably tumour suppressor cluster miR-143/145. Furthermore, I describe the mechanism by which EBNA3C and EBNA3A, in co-operation with a cellular co-repressor of transcription CtBP, specifically target and epigenetically re-programme the *p16^{INK4A}* locus, preventing exit from the cell cycle and/or differentiation. The absence of *p16^{INK4A}* abolishes the need for EBNA3C in the proliferation of established LCL, raising the questions regarding the relative functional relevance of the other EBNA3C-regulated proliferation-related genes in LCL and simultaneously offering a proliferation-independent system to explore EBNA3C function.
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