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INTERACTION OF THE GENOME MAINTENANCE PROTEINS OF ONCOGENEIC HERPESVIRUSES WITH CELLULAR CHROMATIN

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Stockholm 2013

Published by Karolinska Institutet

ISBN 978-91-7549-166-0

Printed by: US-AB
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“To my late father and my family”

ABSTRACT

The Epstein-Barr virus (EBV) nuclear antigen (EBNA)-1 is the only viral protein expressed in all virus-infected cells and EBV-associated malignancies. Similar to the genome maintenance proteins (GMPs) of other gamma-herpesviruses, EBNA1 binds to both viral and cellular DNA and controls the replication and transcription of the viral genome. EBNA1 expression affects cellular transcription but the mechanism and consequences are largely unknown. The work of this thesis aimed to investigate the interaction of EBNA1 with the host cell DNA and to understand how it may impact EBV oncogenesis. EBNA1 is a stable protein due to a Gly-Ala repeat (GAr) domain that acts as a portable inhibitor of proteasomal degradation. We found that, in the absence of the GAr, EBNA1 is rapidly degraded in the cytoplasm but remains long-lived in the nucleus. This correlates with anchoring to cellular chromatin via a bipartite Gly-Arg repeat (GRr) domain that resembles the AT-hook of High Mobility Group-A (HMGA) proteins. Grafting of the GRr to a soluble proteasomal substrate promotes detergent resistant binding to chromatin and inhibits degradation in spite of efficient ubiquitination. Thus the GAr and GRr may cooperatively regulate the stability and functions of EBNA1. This possibility was supported by experiments addressing the transcriptional effects of EBNA1. We found that the GRr is both necessary and sufficient for the capacity of EBNA1 to promote large-scale chromatin decondensation without recruitment of ATP-dependent remodelers. This correlates with rapid diffusion, measured by fluorescence recovery after photobleaching (FRAP), and with displacement of linker histone H1. Similar to architectural transcription factors, EBNA1 promotes a broad remodelling of transcription involving both up- and down-regulation of a large number of genes. The similarity is further substantiated by the capacity of EBNA1 to regulate the Twist promoter, a known target of HMGA2 architectural factors. Using a set of deletion mutants and GFP-fusion reporters, we found that the two GRr subdomains cooperatively determine the mobility of EBNA1, while mobility is increased by the interposed GAr in a length-dependent manner. The GMPs encoded by herpesviruses belonging to the genera Lymphocryptovirus (LCV) and Rhadinovirus (RHV) share a relatively conserved viral DNA binding domain but differ in their cellular-chromatin targeting module. We found that all GMPs promote chromatin decondensation. However, while the AT-hook containing GMPs of LCVs are highly mobile, the GMPs of RHVs are virtually immobile or show a significantly reduced mobility. Only the RHV GMPs recruit the bromo- and extra terminal domain (BET) proteins BRD2 and BRD4 to the site of chromatin remodelling. Thus, differences in the mode of interaction with cellular chromatin may underlie different strategies for host cell reprogramming during latency. Collectively the findings described in this thesis highlights previously unrecognized properties of the interaction of EBNA1 with cellular chromatin by which the viral protein may reset cellular transcription during infection and prime the infected cells for malignant transformation.

LIST OF PUBLICATIONS

This thesis is based on the following papers that will be referred in the text by their Roman numerals:

I. Giuseppe Coppotelli, **Nouman Mughal**, Diego Marescotti, and Maria G. Masucci. High Avidity Binding to DNA Protects Ubiquitylated Substrates from Proteasomal Degradation. The Journal Of Biological Chemistry (2011), 286, 19565–19575

II. Giuseppe Coppotelli, **Nouman Mughal**, Simone Callegari, Ramakrishna Sompallae, Laia Caja, Martijn S. Luijsterburg, Nico P. Dantuma, Aristidis Moustakas and Maria G. Masucci. The Epstein–Barr virus nuclear antigen-1 reprograms transcription by mimicry of high mobility group A proteins. Nucleic Acids Research (2013), 41, 2950–2962

III. Giuseppe Coppotelli*, **Nouman Mughal***, Maria G. Masucci. The Gly–Ala repeat modulates the interaction of Epstein–Barr virus nuclear antigen-1 with cellular chromatin. Biochemical and Biophysical Research Communications, (2013), 431, 706–711

IV. **Nouman Mughal*,** Giuseppe Coppotelli*, Simone Callegari, Stefano Gastaldello, Maria G. Masucci. Interaction Of Gamma-Herpesvirus Genome Maintenance Proteins With Cellular Chromatin. PloS ONE (2013) in press

* The authors contributed equally to the work

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LIST OF ABBREVIATIONS

DUBs	Deubiquitinating enzymes
DS	Dyad symmetry
E	Early genes
EBER	EBV encoded RNA
EBV	Epstein Barr virus
EBNA	Epstein Barr virus nuclear antigen
FLIP	Fluorescence loss in photobleaching
FR	Family of repeats
FRAP	Fluorescence recovery after photobleaching
GAr	Glycine Alanine repeat
GMP	Genome maintenance protein
GRr	Glycine Arginine repeat
HMG	High mobility group proteins
HSV	Herpes Simplex virus
HVP	Herpesvirus Papio
IE	Immediate early genes
IM	Infectious mononucleosis
KSHV	Kaposi's sarcoma associated herpesvirus
L	Late genes
LCV	Lymphocryptovirus
LANA	Latency associated nuclear antigen
LMP	EBV latent membrane protein
LR	Linking region
mnR1	Macaca nemestrina rhadinovirus 1
mnR2	Macaca nemestrina rhadinovirus 2
mu-LANA	Murine herpesvirus 68 Latency associated nuclear antigen
RHV	Rhadinovirus
sa-LANA	Herpesvirus Saimiri Latency associated nuclear antigen

1. INTRODUCTION

1.1 VIRUSES AND CANCER

Viruses that cause cancer in humans include Hepatitis B and C viruses (HBV, HCV liver cancer), Human Papillomaviruses (HPV, cervical and other anogenital cancers), Epstein-Barr virus (EBV, several types of B-cell lymphomas and carcinomas), Kaposi's sarcoma-associated herpesvirus (KSHV, B-cell lymphomas and Kaposi's sarcoma) and Human T-cell lymphotropic virus (HTLV, adult T-cell leukaemia). Human immunodeficiency virus (HIV) infection is an indirect cause of cancers that develop in AIDS patients as a result of immunosuppression.

1.2 HUMAN ONCOGENIC HERPESVIRUSES

Herpesviruses are highly spread in nature and most animal species are host of at least one but often several distinct herpesviruses. Members of this virus family share a common morphology and many significant biological properties. A typical herpes virion consists of a core containing a double-stranded linear viral DNA, an icosahedral capsid of 162 capsomers and approximately 125 nm in diameter, a tegument surrounding the capsid and an envelope as the outermost layer. Viral glycoproteins are embedded in the envelope. Common features of herpesviruses include their relative large genomes encoding enzymes required for viral nucleic acid metabolism and DNA replication, assembly of the virus particle in the nucleus of infected cells, and their the capacity to establish latent infections in the natural hosts (1).

1.2.1 Nomenclature and Classification

The genomes of herpesviruses vary in length between 125 to 250 kbp, mainly due to the presence and organization of repetitive sequence (Figure 1). The linear genomes terminate with repetitive sequences, the terminal repeats (TR), that vary in length between different virus isolates and are required for genome circularization (2-4). The TRs resemble, and in the case of HHV6 are identical, to the human telomere repeats (2). The guanine-cytosine (G-C) content of the DNA varies between 33% to 77% with local variations along the length of genome (5). The genomes also show variation in the distribution of some di-nucleotides, with an interesting deficit of CpG in some gamma-herpesviruses including EBV (6). Members of Herpesviridae family are classified into three subfamilies of alpha- beta- and gamma- viruses based on the characteristics of their productive and latent infections.

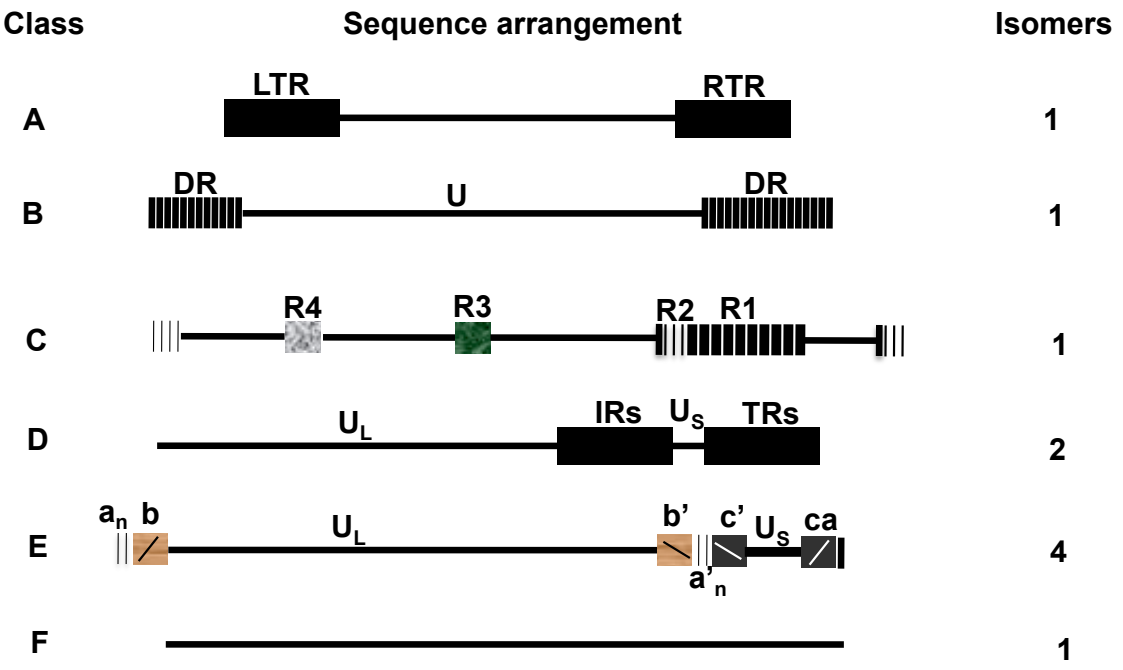

Figure 1
Schematic overview of the herpesviruses genome arrangements. Boxed parts represent repeated sequences.
LTR: left terminal repeat; RTR: right terminal repeat; DR: direct repeat; U: unique sequence; R1-R4: repeated sequences; U_L: unique long; U_S: unique short; IRs: internal repeat sequences flanking U_S; TRs: terminal repeat sequences flanking U_S; a, b, c and a′, b', c′: inverted repeat regions among group E genomes, primes indicate inverted orientation.

Alpha-herpesviruses are characterized by a relatively short replicative cycle, rapid spread in culture, efficient destruction of infected cells and capacity to establish latent infection primarily but not exclusively in sensory ganglia. Human viruses belonging to this family included Herpes simplex virus-1 and -2 (HSV-1/HHV1, HSV-2/HHV2) and Varicella zoster virus (VZV/HHV3).

Beta-herpesviruses are characterized by a more restricted host range, long replicative cycle and establishment of carrier state in vitro. They can establish latency in secretory glands, lymphoreticular cells, kidneys, and other tissues. Human viruses belonging to this family include cytomegalovirus (HCMV/HHV5) and roseolovirus (HHV-6 and HHV7).

Gamma-herpesviruses establish latent infections in lymphoid cells but also infect epithelial cells and fibroblasts where the infection is often productive. The family contains two genera: the lymphocryptoviruses and the rhadinovirususes. Human viruses belonging to this family include the lymphocryptovirus Epstein Barr virus (EBV/HHV4) and the rhadinovirus Kaposi's sarcoma associated herpes virus (KHSV/HHV8) that are associated with a broad variety of malignancies (1,7).

1.2.2 Life cycle

The life cycle of herpesviruses is characterized by their capacity to establish both latent and productive infections, which in many cases involves different host cell types (Figure 2).

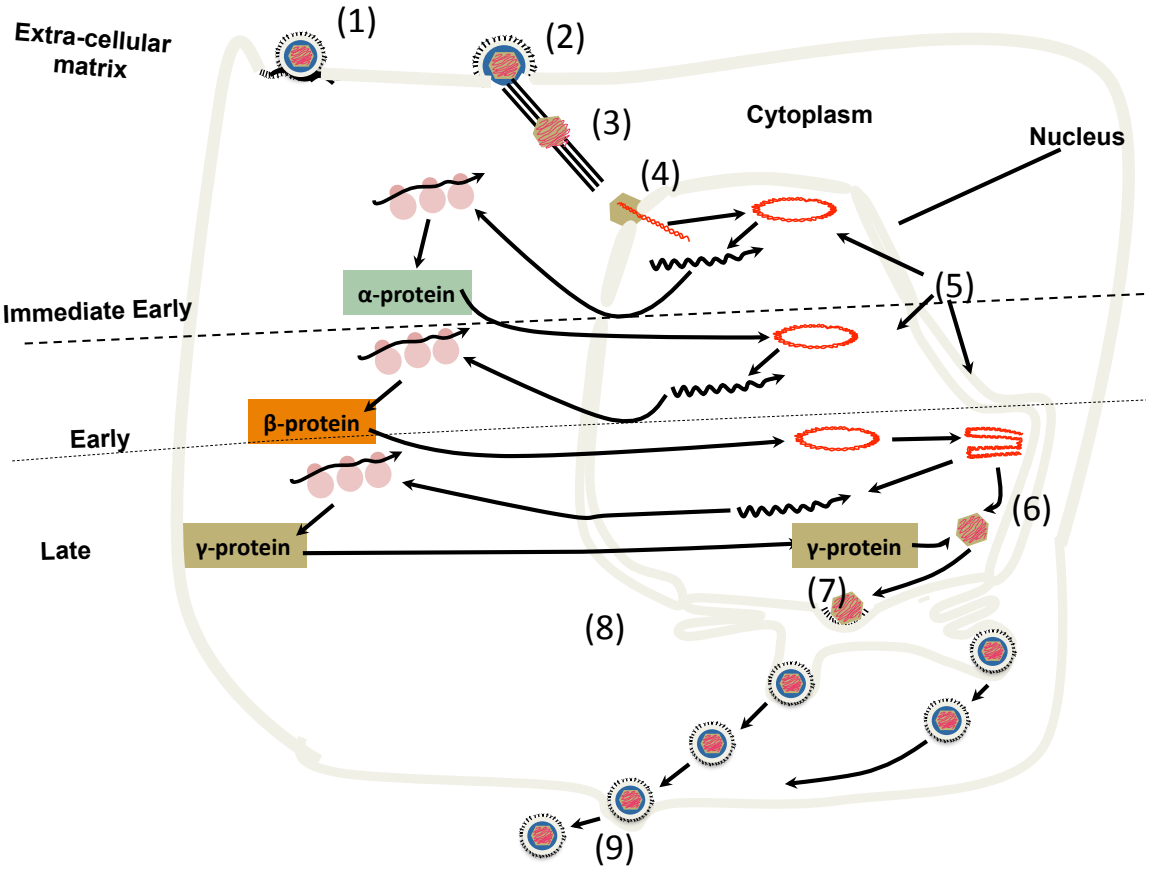

Figure 2
Replication cycle of the Herpes simplex virus.
After attachment (1) and penetration (2), capsids are transported to the nucleus (3) via interaction with microtubules (4), docking at the nuclear pore, where the viral genome is released into the nucleus (4) where transcription of viral genes and genome replication occur (5). Concatemeric replicated viral genomes are cleaved to unit-length during encapsidation into preformed capsids (6), which then leave the nucleus (7). Maturation occurs in the cytoplasm (8). After transport to the cell surface mature, enveloped virions are released from the cell (9).

The infection begins when the virus particle comes in contact with specific receptors, which mediates virus internalization via the viral glycoprotein gB, gH, and gL (8,9). Once inside the host cell, the capsid containing the viral genome is transferred to the nuclear pore where the viral genome is released inside the nucleus (10,11). In the nucleus the linear genome circularizes and transcription of the viral DNA begins with the help of cellular RNA polymerases. The viral genes, which are expressed during productive infection, can be categorised into three classes: immediate early genes (IE), early genes (E) and late genes (L) (12,13). The IE genes encode regulatory proteins and transactivators that initiate the transcription of E genes. The E gene products are enzymes needed to

increase the pool of nucleotides required for viral DNA synthesis, components of the viral polymerases and transactivators of the late genes. The L genes encode the structural components of the virus. Viral proteins are synthesized in the cytoplasm and are sent to the nucleus where the assembly of capsid proteins with genome-length viral DNA give rise to nucleocapsids. The nucleocapsids associate with segments of the nuclear membrane where they acquire a primary envelope. The virions are then transferred to the endoplasmic reticulum (ER) where they acquire a secondary and final envelope. Release occurs by exocytosis or, more frequently, following cell death (12). Expression of the genes associated with virus replication is restricted in certain cell types, which may result in the establishment of latent infections where only few and in some cases none of the viral gene products are detected. The molecular mechanisms that regulate the choice between latent and productive infections remain poorly understood although its is clear that cellular factors expressed in a cell type specific manner e.g. epithelial versus neuronal cells for alpha-herpesviruses, or cell activation/differentiation specific manner e.g. resting versus activated lymphocytes for gamma-herpesviruses, must be involved (14,15).

1.2.3 Latency

The establishment of latent infections depends on the balance between viral products and cellular factors. The virus contributes to this balance by keeping gene expression to a minimum level, which, together with the choice of specific cell types, protects the infected cells from the host immune response. Preserving the genome for entire life of healthy infected hosts is an important challenge for the virus. The three families of herpesviruses have adopted different strategies to solve this problem. Alpha-herpesviruses ensured episome persistence by establishing latency in non-dividing neurons. For example, HSV1 establishes latency in a subset of neurons in the trigeminal ganglia but replicates in other neurons found in these structures. In latently infected neurons, HSV1 expresses a single viral transcriptional unit (LAT). Current evidences suggest that the LAT products protect the neuron from programmed cell death (16). Beta-herpesviruses, such as HCMV, establish latency in cells of the myeloid lineage. The mechanisms involved in maintaining the viral genome in these cells are poorly understood. In various experimental models, semi-permissive cells such as monocytes, macrophages and lymphocytes were shown to express some IE and E genes without replication of the viral DNA (17). Gamma-herpesviruses have the unique capacity to establish latency in dividing cells, either in B or T lymphocytes. This poses a double challenge to the viruses. First, primary infection must result in the establishment of a pool of latently infected cells sufficiently large to allow life-long persistence. Second, specific strategies are required to avoid loss of the viral genome when the latent reservoir undergoes physiological rounds of cell proliferation. As discussed in more details in the following chapters, viruses of this family meet these challenges through the establishment of different types of latent infection that are adapted to distinct

differentiation stages of the host cells. A key feature of these strategies is the expression of viral proteins that promote the proliferation of the latently infected cells while ensuring the persistence of constant numbers of viral episomes. While essential for the establishment of life-long asymptomatic infections that allow spread of the virus to the vast majority of susceptible hosts, the capacity of gamma-herpesviruses to promote cell proliferation is also the main reason for their association with a broad repertoire of malignancies.

1.2.4 Epstein-Barr virus (EBV)

EBV was discovered in 1964 in a cell line derived from Burkitt's lymphoma, a childhood malignancy that is relatively common in central Africa (18). Efforts to establish a pathogenic relationship between infection and the development of the tumour based on the detection of specific antibodies soon led to two important discoveries: i. the virus infects over 95% of the adults worldwide and is probably the most common human pathogen; ii. primary infection usually occurs during childhood and is largely asymptomatic but, when delayed until adolescence or adulthood, it may cause the clinical symptoms of infectious mononucleosis (IM) (19,20)

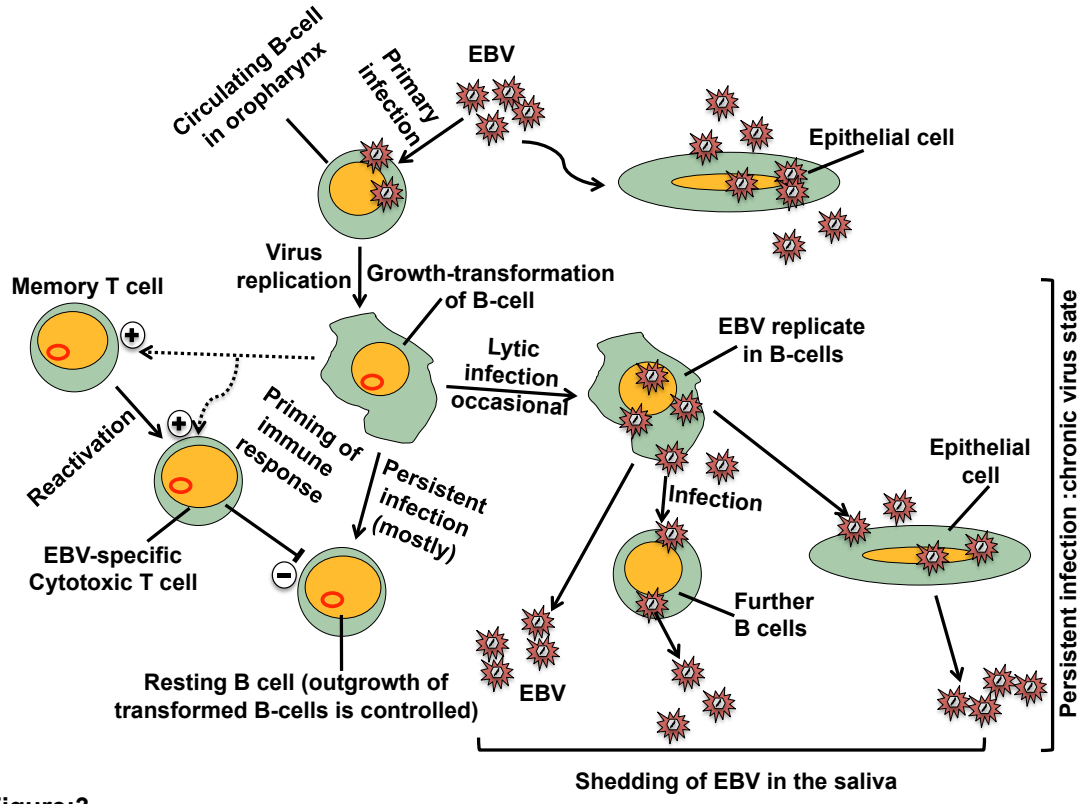

Figure:3
EBV life cycle

1.2.4.1 EBV life cycle

EBV infects lymphocytes and epithelial cell (Figure 3) (21). The B-cell tropism is

due to binding of surface glycoprotein gp350 to the CD21 receptor (22), while virus internalization is triggered by the interaction of gp42 with HLA class II. The infection of epithelial cells is initiated by binding of the BMRF-2 protein to β1 integrins (23-25) while binding of gH/gL to αvβ6/8 integrins triggers internalization (26). In B-lymphocytes the infection is mainly latent and promotes B-cell proliferation, which is required for the virus to gain access to the memory cell compartment where it persists for the entire life of the infected host. As discussed in more details in the next section, several viral proteins cooperate in the induction of B-cell proliferation. Switch from latent to productive infection rarely occurs in B-cells and the physiological triggers remains largely unknown although cross-linking of the B-cell receptor has long been suspected to play a key role (27-29). The switch has been extensively studied in cell cultures. Treatment with anti-Ig, TPA, sodium butyrate or calcium ionophores (28,30,31) activates transcription of the IE genes BZLF1 and BRLF1 (32-34). BZLF1 is a homologue of c-Jun and c-fos and it binds as a heterodimer to AP-1 consensus sites in viral and cellular promoters (35-40). BZLF1 and BRLF1 together activate the transcription of early genes. While the infection of B-lymphocytes is easily recapitulated *in vitro*, the infection of epithelial cells is technically more difficult and is often transient or progresses to lytic replication. This, together with the recently observed boosting effect of co-cultivation with infected B-lymphocytes (41), supports a scenario where primary infection occurs in B-lymphocytes circulating through the lymphoepithelial organs of the oropharynx. Rare B-cells entering the productive cycle may transfer the virus to epithelial cells where efficient virus replication occurs, with consequent shedding of large amounts of infectious virus. Many of the viral genes expressed in latently and productively infected B-lymphocytes are highly immunogenic, which promotes the activation of potent antigen-specific T-cell responses that control the infection by killing the infected cells. Few infected cells escape this immunological control by switching-off viral gene expression. Once effective cellular and humoral immunity is established, the virus persists in this cellular reservoir from which it periodically reactivates giving rise to episodes of virus production, epithelial cell infection and shedding of infectious viruses. At any given time the virus can be detected by sensitive PCR methods in the saliva of all healthy asymptomatic carriers.

1.2.4.2 Latency and B-cell transformation

Our understanding of EBV latency in B-cells is largely dependent on the capacity of the virus to immortalize these cells in vitro, leading to the establishment of transformed lymphoblastoid cell lines (LCLs). LCL cells express a restricted repertoire of viral genes whose products collectively contribute to B-cell transformation. These include six EBV encoded nuclear antigens (EBNAs), three latent membrane proteins (LMPs), two EBV non-coding RNAs (EBERs) and several microRNAs (Figure 4).

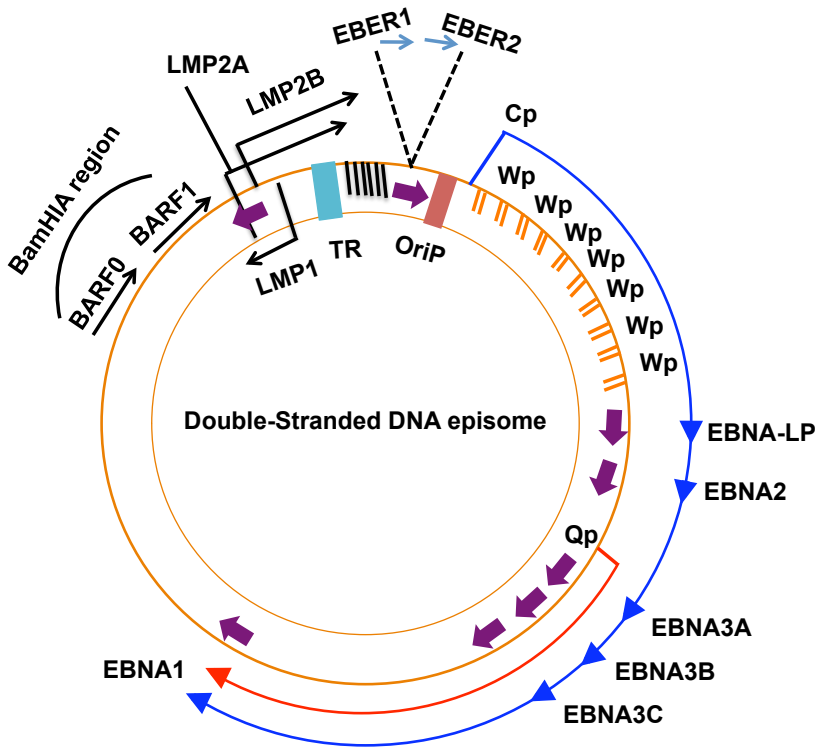

Figure: 4
Diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome.

EBNAs - All EBNAs are encoded by transcripts originating from the Cp or Wp promoters that are differentially spliced to generate the individual mRNAs (42-47). Short exons derived from the BamHW repeats give rise to **EBNA-LP, also known as EBNA5**. EBNA-LP is needed for the efficient outgrowth of LCLs (48) but its role in transformation is unclear. It interacts with pRB and p53 (49,50) and cooperates with EBNA2 in the regulation of viral and cellular genes (20). EBNA2 and EBNA-LP are the first viral protein to be expressed (51-53) and cooperatively promote the transition of B-cell from G0 to G1 (54). **EBNA2** is a transcription factor that regulates many viral and cellular genes, including the viral latent membrane proteins LMP1 and LMP2 and the cellular activation marker CD21 and CD23 (55,56). EBNA2 is a functional homologue of activated Notch and interact with the Notch target RBP-Jk, which is likely to explain its effects on cell differentiation, proliferation and apoptosis (57-59). The activity of EBNA2 is regulated by EBNA3 family proteins **EBNA-3A, 3B, -3C, also known as EBNA3, EBNA4 and EBNA6**. All three proteins compete with EBNA2 for binding to RBP-Jk. EBNA-3A and EBNA-3C are essential for B-cell transformation while EBNA-3B is dispensable (60). EBNA3C was shown to up regulate the expression of CD21 and LMP1 (61), to repress the Cp promoter by interacting with histone deacetylase-1 (62), to promote G1 to S transition by interacting with Rb (63) and to inhibit the mitotic checkpoint (64). **EBNA1** is the last exon of the polycistronic message but may also be transcribed from a dedicated Qp promoter (65). The functions of EBNA1 will be discussed in details in section 1.2.6.1

LMPs - Three EBV encoded membrane proteins are expressed in latently infected cells. **LMP1** is the main transforming protein of EBV (66) and promotes B-cell growth and differentiation by mimicking a constitutively active CD40 (67). The protein has three domains: an N-terminal cytoplasmic tail that anchors the protein in the plasma membrane, six hydrophobic transmembrane loops that are involved in self aggregation and activation of intracellular signalling, and a long C-terminal cytoplasmic region responsible for most of the signalling activity. LMP1 promotes the constitutive activation of the canonical and alternative NFkB signalling pathways, which regulates numerous genes that control apoptosis and cytokine production (68). In addition, LMP1 engages the MAP kinase pathway and activates the ERK, JNK, p38 and JAK/STAT pathways that control cell proliferation (69-73). Finally, through activation of the phosphatidylinositol 3-kinase (PI3-K) pathway, LMP1 promotes cell survival, actin polymerisation and cell motility (74). Two distinct forms of LMP2 are known: **LMP2A and LMP2B**, where LMP2A has an extra 119 amino acid cytoplasmic N-terminal domain (75). Neither of them is essential for B-cell transformation *in vitro* (76). LMP2A plays a central role in lymphocyte proliferation and differentiation by recruitment of the src family protein tyrosine kinases lyn and syk via ITAM (immunoreceptor tyrosine-based activation) motifs (77). LMP2A promotes the ubiquitination of lyn and syk, which blocks B-cell receptor signalling (78) and inhibits the reactivation of latent EBV in B-cells (79). The expression of LMP2A in transgenic mice disturbs normal B-cell development (80). LMP2A transforms epithelial cells through activation of the PI3-kinase/Akt pathway (81), which may be required for the long-term survival of persistently infected memory B-cells. LMP2B also modulate LMP2A activity (76,82).

EBER1 and EBER 2 - Two non-polyadenylated (non-coding) RNAs, EBER1 and EBER2 are highly abundant in latently infected cells (83). The EBERs are not required for B-cell transformation (84) but their expression was shown to enhance the malignant properties of EBV negative cells by promoting anchorage independent growth in soft agar, tumorigenicity in SCID mice and resistance to apoptosis (85). The EBERs induce the expression of IL-10 (86), which may be important for B-cell immortalization (87).

EBV micro-RNAs (miRNA) – EBV encodes several miRNAs in two clusters located in the BHRF1 exon and BART intron regions. The BHRF1 mRNA encodes for three precursors, miR-BHRF1-1, miR-BHRF1-2 and miR-BHRF1-3, that give rise to four mature miRNAs, while the BART intron encodes for twenty-two precursors, miR-BART1 to miR-BART22, and forty mature miRNAs. The BHRF1 miRNAs are expressed mostly in B-cells while the BART miRNAs are highly expressed in epithelial cells. EBV encoded miRNA targets many cellular and viral genes. The BART2-5p miRNA inhibits lytic replication by targeting the viral DNA polymerase BALF5 (88). BART1-5p, BART16-5p and BART17-5p have anti-apoptotic effects through targeting of LMP1 (89). BHRF1-3 and BART2-5p target the interferon-induced chemokine CXCL11 and the host stress-induced Natural Killer cell ligand MICB, which modulates the host response and avoids elimination by NK cells (90). BART5-5p targeting of PUMA

(p53 up-regulated modulator of apoptosis) promotes cell survival (91).

In addition to the latency program expressed in proliferating B-lymphocytes, more restricted forms of latency have been described in EBV infected resting B-cells and in B-lymphocytes that traffic through the germinal centers. As discussed in the next section similar forms of latency have also been observed in EBV-associate malignancies, confirming their relevance during different stage of the infection. Three distinct types of latent infections have been characterized depending on the pattern of viral gene expression (92) (Table 1).

Type of latency	Gene product	Example
I	EBNA1, EBER	Burkitt's lymphoma
II	EBNA1, LMP-1,-2A,-2B,EBER	Hodgkin's disease Nasopharyngeal carcinoma
III	EBNA1,-2,-3A,-3B, -3C,-LP, LMP-1,-2A,-2B,EBER	Infectious mononucleosis, Post-transplant lymphoproliferative disorder

Table: 1
Expression of latent viral proteins in different malignancies

In Latency I only EBNA1, the EBERs, and variable levels of BART miRNAs are expressed. LMP1, LMP2A and LMP2B are additionally expressed in Latency II while all the latent genes are detected in Latency III, which is characteristic of transformed B-cells in culture and is also found in circulating virus infected B-blasts during acute IM.

1.2.4.3 EBV associated malignancies

Although EBV establishes largely non-symptomatic infections in the vast majority of humans, it is also associated with a broad variety of malignancies of lymphoid and epithelial cells origin. Two mechanisms may contribute to the development of these tumors. Congenital and acquired severe immunosuppression may unleash the proliferative potential of virus-infected cells. In addition, genetic alterations induced by environment co-factors, such as chronic malaria infection or exposure to chemical carcinogens, may lead to the activation of cellular oncogenes that promote unrestricted cell growth. The contribution of the virus to the pathogenesis of the latter tumors is still debated. However, the role of the virus is strongly supported by continuous expression of one or more viral gene products in all the malignant cells.

Burkitt's Lymphoma (BL) is a B-cell cell malignancy categorized into three types: Endemic BL, Sporadic BL and HIV associated BL. EBV is detected in almost every case of Endemic BL while Sporadic BL and HIV associated BL

carry the virus in 15–20% and 30%–40% of the cases, respectively. EBV positive BLs express only EBNA-1 and the EBERs (latency I). The critical event in the BL oncogenesis is a chromosomal translocation that brings the *c-myc* oncogene on chromosome 8 under the transcriptional control of one of the immunoglobulin genes on chromosome 14, 2 or 22. Together with malaria or HIV infection, EBV may favor the translocation by inducing B-cell hyper-proliferation (reviewed in (93)).

Hodgkin's lymphoma (HL) is an unusual neoplasm since the malignant cells constitute only a minority of the tumor mass. Classical HL (cHL) is characterized by the presence of clonal, malignant multinucleated Hodgkin Reed Sternberg (HRS) cells in a background of inflammatory cells that includes lymphocytes, plasma cells, granulocytes, and histiocytes. HRS cells are derived from pre-apoptotic germinal center B-cells with rearranged immunoglobulin genes that often show crippling mutations, which would normally promote apoptosis. EBV infection is associated with approximately 40% of cHL cases, most frequently with the mixed-cellularity subtype (94). The EBV carrying HRS cells express Latency II, which includes EBNA1, LMP1, LMP2A, LMP2B the EBERs, BART miRNAs.

Nasopharyngeal Carcinoma (NPC) is an epithelial cell tumor which is virtually always associated with EBV (95). The tumor is particularly frequent in South East Asia, North Africa and in the Eskimo population of Alaska. This characteristic geographic distribution suggests the involvement of genetic and environmental co-factors. The malignant cells express Latency I or Latency II (96). LMP1, LMP-2A and LMP-2B are detected in 35% to 50% of cases (97). However, a higher frequency of LMP1 expression has been reported in pre-invasive lesions, suggesting that its expression may be necessary for the progression of early lesions (98).

Gastric Carcinoma (GC). About 10% of GC cases throughout the world are EBV positive. The malignant cells express Latency I, often together with LMP2A. The exact role of EBV in GC is still unclear especially when other factors like *H. pylori* infection are considered. EBV positive GC is characterized by non-random CpG island methylation in the promoters of cancer-associated genes such as PTEN. LMP2A may play an important role by up-regulating cellular DNMT1 through the phosphorylation of STAT3, causing CpG methylation of a tumor suppressor gene, such as PTEN (reviewed in(99)).

Post-Transplant Lymphoproliferative Disorders (PTLDs) and HIV/AIDS-associated Lymphomas arise in patients that are heavily immuno-suppressed after solid organ and allogeneic bone marrow transplant or HIV infection. Most cases of PTLD occurring early after transplantation are EBV positive. The tumors express latency III, which is consistent with failure to control virus induced B-cell proliferation. The strongest evidence for a direct role of the virus

in pathogenesis is the regression of the tumors upon discontinuation of the immunosuppressive therapy and their cure by infusion of EBV-specific CTLs (100). HIV/AIDS associated lymphomas include primary central nervous system (CNS) lymphomas, diffuse large B cell lymphomas (DLBCL), HL, BL, BL-like and primary effusion lymphomas. The EBV association varies depending on the subtype, being most frequent in CNS, DLBCL and HL, while 30-50% of HIV/AIDS associated BLs carry the virus (101-103).

1.2.5 Kaposi's sarcoma-associated herpesvirus (KSHV)

KSHV is a member of the gamma-herpesvirus family belonging to the genus rhadinovirus. The virus was isolated in 1995 from Kaposi Sarcoma, a rare tumor in the Mediterranean basin and central Africa that showed a dramatic increase at the surge of the HIV epidemics (104).

1.2.5.1 KSHV life cycle

KSHV is primarily sexually-transmitted and infects a broad range of host cell *in vivo*, which is confirmed by the presence of viral DNA and transcripts in circulating B-lymphocytes, the B-cells of primary effusion body-cavity lymphomas (PEL/BCBL), and multicentric Castleman's disease (MCD), endothelial cells and CD45+/CD68+monocytes in Kaposi's Sarcoma (KS), keratinocytes, and epithelial cells (105-109). The broad host cell range may be explained by the presence of multiple surface receptors, gB, gH, gL, gM, and gN, and unique glycoproteins (gpK8.1A, gpK8.1B, K1, K14, and K15) (110-114). Like all herpesviruses, the life cycle of KSHV includes latent and lytic replication phases (115). During the lytic cycle more than 80 IE, E and L genes are expressed (116-119). In immunocompetent individuals, KSHV persists as a circular episome in the nucleus of infected cells and gene expression is restricted to few latency genes (120). KSHV has poor transforming capacity *in vitro* and our knowledge of KSHV infection is manly based on the study of cell lines derived from KSHV positive malignancies. In latently infected PEL cells LANA1 encoded by ORF73 (121-126), viral cyclin D encoded by ORF72 (127-132), vFLIP encoded by ORF71 (133-135), Kaposin encoded by K12 (136-140) and viral miRNA (114,141-143) are expressed. A viral interferon regulatory factor is also consistently detected in all KSHV-induced tumors. Reactivation from latency is commonly observed in KS *in vivo*. The exact mechanism of this reactivation is not known but factors such as immune suppression, HIV infection, oxidative stress, and hypoxia seems to play a role. The mechanisms of KSHV reactivation have been extensively studied in PEL cells. Treatment with TPA promotes the expression of K-RTA (ORF50) that is both necessary and sufficient for KSHV reactivation (144-146). K-RTA induces the expression of a cascade of lytic genes, including vIL-6, PAN RNA, ORF59, ORF65, and K8.1, and the production of DNase-resistant encapsidated viral DNA (144-146). Expression is tightly controlled by various cellular and viral proteins, including, K-RTA itself

(145,147). LANA1 inhibits K-RTA expression by repressing basal RTA promoter activity as well as RTA-mediated auto-activation (147). The reactivation of KSHV seems to promote cell proliferation, angiogenesis, and local inflammation that are all required for tumor initiation and progression.

1.2.5.2 KSHV associated malignancies

Since its identification in KS lesions, KSHV has been strongly linked with multicentric Castleman's disease and primary effusion lymphoma while its association with diseases like marrow hypoplasia, haemophagocytic syndrome (HPS), multiple myeloma, sarcoidosis, angio-immunoblastic lymphoma and primary pulmonary hypertension is still debated.

Kaposi's sarcoma (KS) has been classified into four forms: classic, endemic, epidemic (AIDS-associated), and iatrogenic (115). KSHV DNA is present in 95% of all KS lesion regardless of the type. The KS tumours are comprised of spindle-shaped cells, slit-like endothelium lined vasculature and infiltrating blood cells (105). These spindles cells are the neoplastic component. The majority of the spindle cells express only four latent proteins: ORF73/Lana-1, ORF K12/kaposin, ORF K13/vFLIP and ORF72/v-cyclin, while in some cells the virus undergoes lytic replication (120,148,149). Thus, both latent and lytic viral products may contribute to the initiation and progression of KS (150).

Primary effusion body-cavity lymphoma (PEL/BCBL) is a rare, rapidly fatal lymphoma commonly found in HIV-infected patients (151). PEL cells usually express only four KSHV proteins, ORF73/Lana-1, ORF K12/kaposin, ORF K13/vFLIP, ORF72/v-cyclin, and lytic genes are detected in a small subset of cells. The viral interleukin 6 homologue (vIL-6) is found in 2-5% of cells while less than 1% expresses K8/kbZIP, K8.1, K9, K10, K11, ORF59/PF-8 and ORF65 (120,149).

Multicentric Castleman's disease (MCD) is a lymphoproliferative disorder of germinal center B-cells. KSHV genome is found in almost al cases of HIV-seropositive MCD cases and in approximately 50% of HIV seronegative cases (106,152). KSHV associated MCD contains large plasmablastic cells and is therefore called plasmablastic MCD (153,154). The pattern of KSHV gene expression is less restricted compared to PEL and KS. Many of the manifestations of MCD are thought to be mediated by IL-6 (155).

1.2.6 Genome maintenance proteins (GMPs)

In order to assure persistence of the viral episomes in latently infected proliferating cells the gamma-herpesviruses express genomic maintenance proteins (GMPs). These proteins perform two important tasks: i. they tether the

viral episome to cellular DNA and, ii. they regulate the replication of viral episomes.

1.2.6.1 EBV nuclear antigen (EBNA)-1

EBNA1 is the only viral protein expressed in all forms of latency, both in normal cells and EBV associated tumours. The EBNA1 encoded by B95-8 strain of EBV is 641 amino acids long and can be divided into several functional domains (Figure 5) including: nuclear localization signal (NLS, aa 379–386), a DNA binding and dimerization domains (DBD, aa 459–604), two Arginine-rich basic domains (GR, aa 33–83 and 328–382) that are involved in binding to cellular chromatin, and a Gly-Ala repeats domain (GAr) that inhibits processing by the proteasome and recognition by cytotoxic T cells (156,157).

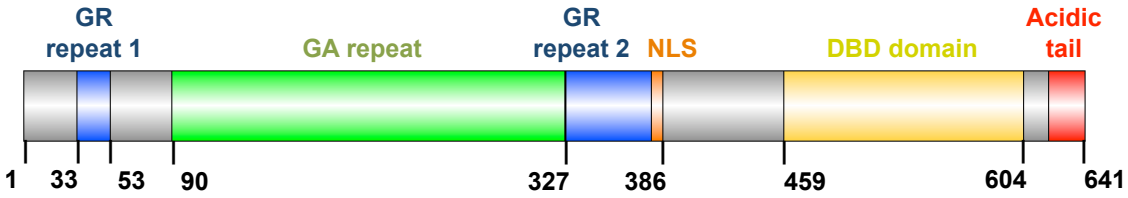

Figure 5:
Schematic representation of EBNA1 domains.

EBNA1 binds to the origin of latent viral DNA replication (*OriP*) via its DNA-binding and dimerization (DBD) domain. The *OriP* comprises of two functional elements: the dyad symmetry (DS) element and the family of repeats (FR), which contain 4 and 20 binding sites for EBNA1, respectively (158,159). EBNA1 binds as a dimer to palindromic recognition sites in the DS and FR and is constitutively bound through the cell cycle (158,160-164). The binding of EBNA1 to all 4 sites within DS element is sufficient for efficient replication of the episome (165,166). EBNA1 lacks any enzymatic activity (167) and acts by recruiting components of the cellular replication machinery (168-171). Another important function of EBNA1 is to ensure the proper segregation of the episomes in dividing B-cell. Binding to multiple recognition sites in the FR is crucial for the segregation function, as is the Gly-Arg-rich region (172). A cellular protein EBP2 is reported to facilitate the binding of EBNA1 to mitotic chromosome and may play an important role in plasmid segregation (172). However, it is not clear whether EBP2 enables the initial association of EBNA1 with the chromosomes or simply stabilizes the interaction. EBNA1 functions as a transcriptional activator by interacting with FR elements within *OriP* (173,174). This binding transactivates a promoter located in the BamHI-C region (Cp) that is approximately 3 kb distal to FR, and the LMP1 promoter (LMP1p) that is more than 10 kb away (175,176). In addition to the DBD, functions required for the activation of viral promoters have been mapped to the Gly-Arg-rich region (residues 325–376) and to the 61–89 N-terminal sequence. Several cellular proteins were shown to interact with these regions and may play a role in EBNA1 mediated transcriptional activation. For example, via the 61-83 region EBNA1 recruits Brd4, a cellular bromodomain protein that plays a role in cellular gene activation (177). Brd4 and EBNA1 colocalize at FR elements and Brd4

depletion was reported to abrogate EBNA1 mediated transcription (178). The cellular P32/TAP protein also interacts with EBNA1 in a region mapped between aa 325-376. A possible role of P32/TAP in EBNA1 mediated transcription is suggested by the ability of a P32/TAP C-terminal fragment to activate a reporter gene fused to the GAL4 DNA binding domain (179,180). EBNA1 also interact with the nucleosome assembly proteins NAP1 and TAF-I. This interaction is also mediated by the 325-376 region (181,182). NAPI and TAFI localize at FR elements with EBNA1 (181). NAP1 recruits p300 histone acetyltransferases at E2, a functional equivalent of EBNA1 in papillomavirus, and enhance its transcription (183). Depletion of NAP 1 and TAF-Iß diminishes the transcriptional activity of EBNA1 (181). EBNA1 also binds USP7 and the GMP synthetase complex that ubiquitylates Histone H2B (184). Depletion of USP7 in EBV infected cells has been shown to increase the levels of monoubiquitylated Histone H2B at the FR and decreased transcriptional activation (182). EBNA1 negatively control its own expression by binding to two recognition sites downstream of the Qp promoter (65). EBNA1 further interacts with cellular chromatin via two N-terminal linking regions LR1/GR1 (33–83) and LR2/GR2 (328–382,) that are spaced by a Gly–Ala repeat (GAr) (185).

Through the activation of cellular genes EBNA1 may provide functions necessary for cell survival and proliferation (186-189). Indeed, expression of EBNA1 increases tumorigenicity of EBV negative cell lines (190-193). However, although binding of EBNA1 to cellular promoters has been documented (194,195), only in few cases the regulation of cellular promoters was validated in reporter assays. This, together with the identification of a large number of candidate DNA binding sites across the human genome, both close and far apart from transcription start sites (196), suggests that the mechanism by which EBNA1 affects transcription may be different compared to conventional transcription factors.

1.2.6.2 KSHV Latency associated nuclear antigen (LANA)-1

LANA1 is transcribed as a polycistronic mRNA together with ORF72/viral-cyclin and ORFK13/vFLIP (197). LANA1 is divided into three domains: an N-terminal domain that binds to cellular DNA, a central highly hydrophilic domain, and a C-terminal domain that interacts with viral DNA (Figure 6).

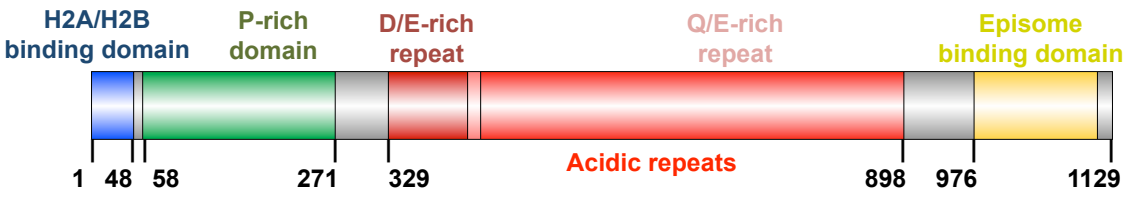

Figure 6:
Schematic representation of LANA1

The central domain of LANA1 is composed of multiple repeat elements predominantly containing the charged polar amino acids glutamine, glutamic acid, and asparagine while the N- and C-terminal domains are rich in basic amino acids. Binding of LANA1 to the viral episome occurs at two binding sites (LBS1/2) within each terminal repeat (TR) (198,199). Only the carboxyl-terminal domain of LANA1 is required for this site-specific DNA binding (200). LANA1 recruits host cellular factors that are required for viral genome replication, partitioning, and maintenance (201,202). It interacts with chromosomes by binding to nucleosomes in a region between histones H2A-H2B. The first 22 N-terminal residues of LANA1 are needed for this binding (203,204). The C-terminal domain of LANA1 is also implicated in chromosome binding via other chromosome-associated proteins such as MeCP2, Brd4, Brd2, DEK, HP-1 alpha and CENP-F (205-210). LANA1 works both as transcriptional activator and repressor. During latency, LANA1 regulates the transcription of the major latent promoter but how LANA1 mediate this effect is not known since its DNA binding sequence has not been identified. The regulation of RTA is an example of the function of LANA1 as a transcriptional repressor. RTA is the key transcriptional regulator that controls the switch from viral latency to lytic replication. Thus, by repressing RTA transcription, LANA1 keeps latent virus from reactivating (147,211). LANA1 also influences the transcription of various cellular genes by interacting with and altering the specificity of several cellular factors such as Daxx, CREB-binding protein, RING3, ATF4, and c-Jun (126,209,212-215). LANA1 has been shown to transform rat fibroblasts by cooperating with the cellular oncogene h-Ras (124) and up-regulates the human telomerase promoter by interacting with the Sp1 transcription factor (216). Activation of telomerase is a critical step in cellular transformation. LANA1 may also drive cell proliferation by binding and antagonizing various cellular tumor suppressor proteins. LANA1 sequesters glycogen synthase kinase-3 and Sel-10, which results in stabilization of their respective substrates c-Myc, β-catenin, and intracellular Notch (217-222). LANA1 binds and inactivate pRb and inhibits pRB- mediated cell cycle arrest (123,124). LANA1 interaction with p53 downregulates its transcriptional activity and prevents p53-mediated apotosis. Moreover, It was recently reported that LANA-1 promotes the degradation of p53 by recruiting it to the elongin B/C-cullin 5 ubiquitin ligases (223).

1.3 EPIGENETIC REPROGRAMMING IN ONCOGENESIS

Nucleosomes are the basic units of chromatin. In each nucleosome approximately 146 base pairs of DNA are tightly wrapped around an octamer of core histones (H3, H4, H2A and H2B) (224). The wrapping of DNA in nucleosome makes it inaccessible for different processes such as transcription, replication and DNA repair. The structure of chromatin is regulated by epigenetic processes. This may occur in four ways: i. methylation of DNA, ii. post-

translational modification of histones, iii. incorporation of histone variants, iv. nucleosome repositioning. These processes work together by influencing the dynamics of chromatin structure at localized level and their complex interplay marks the cellular genome in different cell types, developmental stages and disease states, including cancer (225-227). Many cancers are associated with global changes in DNA methylation patterns and histone modification profiles that result in deregulated gene expression (228). The altered epigenetic makeup may also play a role in cancer progression by silencing or deletion of genes such as tumour suppressor genes (229). In some cases epigenetic changes result in activation of oncogenes.

1.3.1 DNA structure and chromatin organization

Deoxyribonucleic acid (DNA) carries the information required for the development and proper functioning of living organisms (Figure 7). Each DNA molecule is a polymer of two-polynucleotide chains running around each other. Nucleotides are the basic unit of these polymers. Each nucleotide is composed of a deoxyribose sugar, phosphate group and a nitrogen base. Four types of nitrogen bases are present in DNA: Adenine (A), Cytosine(C), Guanine (G) and Thymine (T). In DNA it's the sequence of these four nitrogen-bases, which encode for information. The information encoded in the DNA sequence is first transcribed into RNA and then translated into proteins. A gene is a sequence of nucleotides, which encode for a protein or a polypeptide. The amount of DNA differs in different organisms. Humans DNA contains approximately 3 billion base pairs while the DNA of certain salamanders and plants can be up to 150 billions of base pairs. This huge amount of DNA is packaged in complex structures with the help of DNA binding proteins (230-236). The DNA binding proteins of eukaryotes can be divided into histones (nucleosomal histones and non-nucleosomal histones) and non-histones proteins. The complex of these proteins with DNA is called chromatin. Histones are small and charged proteins that organise and package the DNA into condensed chromatin fibers.

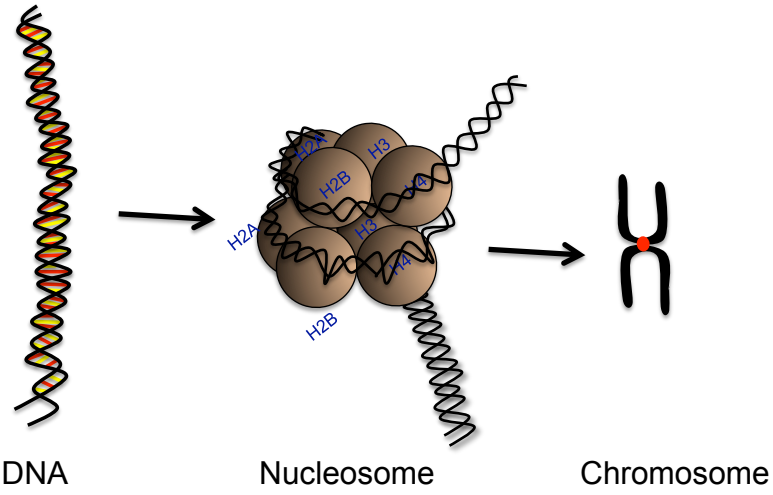

Figure 7:
Different level of chromatin organization.

Nucleosomal histones are responsible for the coiling of DNA into nucleosome while non-nucleosomal histones (linker histones) bind the nucleosomes at the entry and exit sites of DNA and ensure their locking into place. There are four kinds of nucleosomal histones: H2A, H2B, H3 and H4. Nucleosomal histones are very similar in structure and highly conserved among species. H3 and H4 are among the most conserved of all the proteins while the non-nucleosomal histones, H1 and H5, are less conserved and more than one isoform exists in a species. Some variant forms of histones also exist. They are similar in amino acid sequence and core structure to the respective class but have distinct features that distinguish them from the major histones. These variant forms are associated with specific functions of chromatin metabolism. For example, CENP-A, a variant of H3, is incorporated in nucleosomes only in the centromere region of the chromosome. H2A.Z, a variant of H2A, is associated with actively transcribed promoters and also prevents the spreading of constitutive heterochromatin. H2A.X replaces H2A at double-strand DNA breaks and marks the area for ongoing repair. Histone H3.3 is associated with region of active transcription. The N-terminal tails and globular domains of histones undergo posttranslational modification including acetylation, methylation, phosphorylation, citrullination, ubiquitination and sumoylation that play important roles in gene regulations, replication and DNA repair (237-240). The nucleosome is the basic unit of chromatin packaging in eukaryotes. In each nucleosome, a 146 base pair double stranded DNA is wound around a histone octamer. The linker histones sitting at the entry and exit of DNA increases the compaction by pulling the neighbouring nucleosome and organising them from “beads on string” to the 30 nm fiber. Higher amount of Histone H1 is associated with heterochromatin that is a more condensed form of chromatin. Extensive interactions exist between histone and DNA within the nucleosome. There are 142 hydrogen bonds between DNA and histones, of which half involve the amino acid backbone of histones and the phospho-diester bonds of DNA. Numerous hydrophobic interactions and salt bridge are also formed. The core histones are rich in basic amino acids such as Lys and Arg whose positive charge can neutralize the negative charge of the DNA. These extensive interactions make the nucleosome a very stable structure. In addition to its histone fold, each of the core histone has an N-terminal tail. Post-translational modification of the histone tails destabilizes these interactions making the DNA available for process like replication and transcription (reviewed in (241,242)). Several levels of chromatin organizations are present in eukaryotes. Interphasic chromatin can be distinguished into two main forms: a highly condense form termed heterochromatin and a less condense one called euchromatin. Heterochromatin can be further classified into facultative and constitutive heterochromatin. Approximately 10% of the genome is packed into constitutive heterochromatin during interphase. This is often composed of highly repetitive sequences, such as centromeres and telomeres, and is usually not transcribed. The position of facultative heterochromatin is not consistent in different cell types, being part of actively transcribed euchromatin in some cell types and silent heterochromatin in others. For example the β-globin gene is found in constitutive heterochromatin only in non-hematopoietic cells. Approximately 10% of the DNA is located in transcriptionally active euchromatin (243-247).

1.3.2 Chromatin-Remodeling

DNA that is tightly packed in heterochromatin is not accessible for critical cellular processes such as transcription, replication, recombination, and repair. This DNA is made accessible through the activity of two classes of enzymes: histone modifying enzymes and ATP-dependent nucleosome remodelers. Histone modifying enzymes target the N-terminal tails of histones, which alters the structure of chromatin and provides binding sites for regulatory proteins while chromatin remodeling complexes (CRCs) utilize the energy of ATP to disrupt nucleosome DNA contacts, move nucleosomes along DNA, and remove or exchange nucleosomes.

1.3.2.1 ATP-dependent chromatin remodelling complexes

ATP-dependent chromatin remodelling complexes are divided into four families: SWI/SNF, ISWI, CHD and INO80 (248,249). These families share the presence of an ATP-hydrolysing domain within their catalytic subunit (Figure 8) (250). Other binding sites present in the catalytic subunit recruit various attendant proteins at different times depending on the cellular context, which allows the remodelers to perform different tasks.

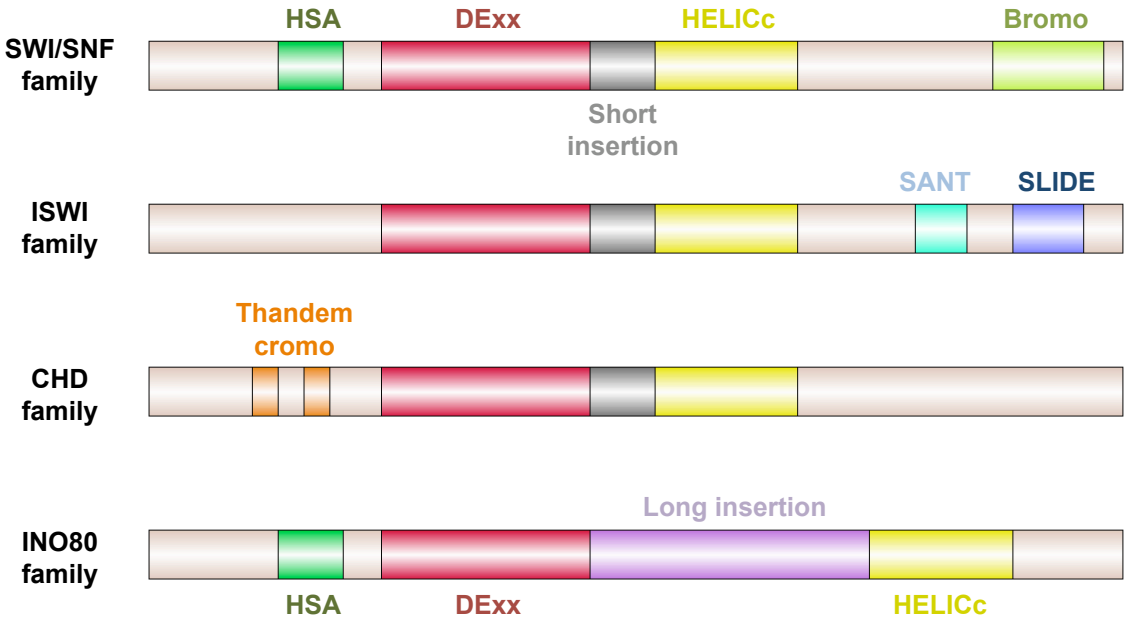

Figure 8:
Domains Graph (DOG v2.0) schematic representation of catalytic subunit of different remodelers family.
All families has a common ATPase subunit split in two domains, Dexx and HELICc ATPase. A short insertion is present within the ATPase domain of SWI/SNF, ISWI, and CHD families while long insertion in catalytic subunit of INO80. Each family is further defined by distinct combinations of flanking domains: Bromodomain and HSA (helicase-SANT) domain for SWI/SNF family, SANT-SLIDE module for ISWI family, tandem chromodomains for the CHD family, and HSA domain for the INO80 family.

The **SWI/SNF family remodelers** are composed of 8 to 14 subunits that form two types of complexes (yeast SWI/SNF and RSC; drosophila BAP/PBAP;

human BAF/PBAF) around one of two related catalytic subunits. The catalytic ATPase subunits comprise an HSA (helicase-SANT) domain, which can recruit actin-related proteins (ARP subunits), a post-HSA domain and a C-terminal bromo domain that plays a role in promoter targeting by interacting with acetylated histones. The ß-actin is part of the chromatin remodeling complexes of higher eukaryotes. These remodelers are mainly involved in activating gene expression with no role in chromatin assembly during replication (249,251-255).

The **ISWI family remodelers** are composed of 2 to 4 subunits. Most of the ISWI complexes are built around two related catalytic subunits. The C-terminal ATPase has a characteristic SANT domain and a SLIDE domain. SANT and SLIDE domain together form a module that recognises nucleosomes by interacting with nucleosomal and linker DNA and histone tails. Other proteins contributing to the formation of ISWI complexes contain domains for interaction with DNA (hCHRAC and dNURF301), plant homeodomains (PDH), and bromo domains (hBPTF, hACF1). The ACF and CHRAC complexes contribute to chromatin assembly and transcription by nucleosome spacing, while NURF promotes transcription by randomizing nucleosome spacing (248,249,255-257).

CHD family remodelers CHD1 consists of only one catalytic subunit and is the simplest member of this family while the NuRD complex, which is present in higher eukaryotes and assembles around the Mi-2 catalytic subunit, is composed of ten subunits. The N-terminus of the catalytic subunit contains two tandemly arranged chromo-domains that can recognise and methylate H3 tails. CHD remodelers promote transcription either by sliding or ejecting nucleosomes. Transcription repression by NuRD is mediated by the recruitment of other proteins such as (HDAC1/2) and methyl CpG-binding domain (MBD) proteins (249,254,255,258,259).

The **INO80 family remodelers** consist of more than ten subunits. A distinguishing feature of the INO80 catalytic subunit is the presence of a long insertion within two ATPases domain. This long insertion offers binding sites for ARPs and Rvb1/2 helicase related (AAA-ATPase) proteins. The ATPase subunit also binds to actin and ARP via its N-terminal HSA domain. INO80 are involved in numerous cellular processes including transcriptional activation and DNA repair. The chromatin remodelling activity of INO80 involves nucleosome mobilization, eviction of H2A.Z and removal or replacement non-acetylated H2A.Z (249,255,258,260). Similar to ISWI remodelers, INO80 promotes nucleosome mobility.

1.3.2.2 High mobility group proteins (HMGs)

HMG proteins are a class of non-histone chromosome binding proteins that are found in all eukaryotic organisms. They are among the most abundant and ubiquitously expressed proteins in the nucleus. All HMG proteins share similar biochemical and biophysical properties. They are accessory ‘architectural factors’ that work primarily by modulating the organization of nucleosome and

chromatin, and by recruiting other proteins for processes such as transcription, replication and DNA repair. In vertebrates, they have been divided into three structurally unrelated subgroups.

HMGA proteins are small proteins of about 100 amino acids. In mammals, the HMGA family consist of two subfamilies: HMGA1 and HMGA2 (225). There are three members in the HMGA1 sub-family: HMGA1a, HMGA1b and HMGA1c that are expressed from a single gene by alternative splicing (261). The HMGA2 sub-family has only one member encoded by a different gene. The HMGA family is characterized by the presence of three DNA binding domains known as AT-hooks. The AT-hook is a nine residues long domain of positively charged amino acids with a fully conserved Arg-Gly-Arg-Pro (R-G-R-P) motif (262,263). HMGA proteins bind preferentially to AT-rich sequences of B-form of DNA. The AT-hook acquires a disordered-to-ordered conformation, upon binding to DNA (264,265). A single AT-hook motif can bind to a stretch of four to six base pairs (266). Simultaneous binding of two or more AT-hooks on different DNA binding sites exponentially increases the avidity of proteins with DNA (267). HMGA1 also binds to non B-form DNA such as synthetic four-way junction structures, and to distorted or relaxed regions of DNA found on isolated nucleosome core particles (268-270). HMGA proteins have C-terminal acidic tails of unknown function. Some reports have suggested that it may have a role in protein-protein interaction and transcription enhancement (271,272). HMGA proteins also have protein-protein interacting domains, which allows this protein family to recruit/interact with different chromatin complexes at the chromatin thus influence the activation of different genes. The expression of HMGAs is tightly regulated by different cellular stimuli such as growth factors (273). They are highly expressed during embryogenesis while expression is almost undetectable in adult tissues (274-277). Very high levels of HMGAs are reported in virtually all cancers (278-288) where they are linked to malignancy and metastasis (289). Over expression of HMGA2 have been reported in malignant breast cancer, sarcomas, pancreas carcinoma, oral squamous cell carcinomas, and non-small cell lung cancer (290-294).

HMGB proteins regulate both nuclear and extra-nuclear processes (295-299). In mammalian HMG-box containing proteins are classified into two groups. The first group, including for example RNA polymerase I and the transcription factor UBF, contains two or more HMG-boxes while the second group includes highly diverse but less abundant proteins having mostly a single HMG-box (300-302). Four HMGBs (HMGB1-4) have been found in humans. Structurally HMGB1-3 consist of two DNA binding domains, HMG-box A and -B, and a long C-terminal acidic tail (302,303). HMGB4 lacks the C-terminal acidic tail (303,304). Both HMG box A and box B interact with the minor groove of B-type DNA structures and distort it in a sequence independent manner while the acidic tail interacts with the DNA binding surfaces of both HMG-boxes and seems to modulate the affinity of binding to DNA (305). In addition, HMGB protein also bind to already

distorted DNA structures (305). Posttranslational modification of HMGBs plays an important role in fine-tuning their interaction with DNA/chromatin and in determining their relocation from the nucleus to the cytoplasm and subsequent secretion (300,302,306). HMGB proteins participate in multiple nuclear processes such as transcription, replication, V(D)J recombination and DNA repair and promote gene transcription through several mechanisms (297,299). One mechanism is mediated by the ability of the HMGB proteins to bind to nucleosomes and recruit chromatin remodeling proteins (e.g., ACF/CHRAC) that induce nucleosome sliding, thus exposing previously blocked regions of DNA (307,308). A second mechanism employs the so called 'hit-and-run' mode of action in which HMGB proteins facilitate the stable binding of transcription factors to their DNA recognition sites (299). The capacity of HMGBs to repress transcription is associated with their ability to form a stable HMG-1/TBP/TATA complex that inhibits the assembly of the pre-initiation complex on promoters (309,310).

HMGN proteins are very small and highly charged proteins that specifically interact with the nucleosome core. HMGNs are the only non-histone proteins that bind inside the nucleosome between the gyres of DNA and the octamer core with the tails of histone H3 and H2B being involved in the interaction (311). The HMGN family includes five members in vertebrates: HMGN1, HMGN2, HMGN3a, HMGN3b and HMGN4 (311). The HMGNs can be divided into 3 distinct functional domains: a bipartite nuclear localization signal (NLS), a nucleosomal binding domain (NBD) and an acidic C-terminal regulatory domain (RD) (311). The NBD anchors HMGNs to the core of nucleosome while the RD domain influences chromatin unfolding and histone posttranslational modifications (312,313). The expression of HMGNs is tightly linked to cellular differentiation (314,315). Two possible mechanisms have been proposed to explain the chromatin de-compaction activity of HMGNs: out-competition of H1 for chromatin binding sites and regulation of the posttranslational modifications at histone proteins tails (316-320).

1.3.3 VIRUSES AND CHROMATIN REMODELING

Viruses are obligatory parasites. Successful infection relies on their ability to manipulate cellular processes and exploit its resources. Nuclear chromatin offers a formidable challenge to viral gene expression and genome replication due to its complex and dynamic nature. Viruses have evolved different mechanism to modulate chromatin or chromatin related processes to their own advantage (321). Due to the important role of HATs (histone acetyltransferases) in gene regulation and DNA replication viruses often target these proteins. Several viral proteins were shown to interact with and recruit cellular HATs (322). The HIV Tat protein interacts with pCAF, CBP, p300 and GCN5 (323,324) to regulate viral gene expression, virus replication and pathogenesis. In few cases, the

interaction of viral proteins with HATs inhibits their activity, with consequences such as block of apoptosis or cell cycle arrest. For example HPV-E6 binds with p300 to block p300-mediated p53 acetylation and activation (325). HDACs (histone deacetylases) are another group of cellular factor that are targeted by many viruses due to their ability to repress viral transcription and replication. Interaction with viral protein often inhibits HDAC activity and promotes viral transcription and replication. For example that IE1 protein of MCMV has been reported to form a complex with HDAC2, PML and Daxx, and inhibit the deacetylation activity of HDAC2, promoting virus replication (326). Histone H1 inhibits transcription by promoting chromatin condensation and the formation of higher order chromatin structure (327). It also promotes the condensation of viral DNA, which inhibits transcription and replication. Some viral proteins, such as adenovirus-2 (Ad2) capsid-hexon protein, interact with Histone H1 to promote genome de-compaction (328). The cellular TAF-Ia protein is a histone chaperon that promotes nucleosome deposition at viral genomes, which keeps HSV from reactivating. The VP22 protein of HSV binds to TAF-Ia and inhibits this suppression (329). The cellular DAXX protein is also targeted due its ability to promote the assembly of viral DNA into repressed heterochromatin. Many viral proteins, such as CMV IE1, HSV ICP0 and HPV L2, target DAXX (326,330,331). The IE1 of CMV, pp71, plays a role in CMV reactivation by degrading DAXX via an uncommon, proteasome-dependent but ubiquitin-independent mechanism (332). Some viral proteins target ATP-dependent chromatin remodeling complexes due to their ability to alter nucleosome positioning and histone interactions. For example the HIV integrase and EBV EBNA2 target the INI1/SNF5 protein of SWI/SNF complexes. In HIV this interaction is needed for proper integration of HIV genome while for EBNA2 it plays a role in the activation of repressed viral and cellular genes (333,334).

2. AIMS OF THIS THESIS

EBNA1 is the only viral protein expressed in all EBV infected cells and EBV carrying malignancies. This protein has been extensively studied with the hope that a better understanding of its functions would provide new insights about the mechanisms by which the virus establishes latent infections and promotes malignant transformation. Previous studies have often overlooked an important property of EBNA1, namely its capacity to interact with cellular chromatin throughout the cell cycle and independently of its well-known viral episome anchoring function. In the work described in this thesis, my colleagues and I have addressed this aspect of the biology of EBNA1 with the following specific aims:

I. To characterize the chromatin-binding module of EBNA1 and understand how it may influence its turnover and functions

II. To understand how the interaction of EBNA1 with cellular chromatin may impact its ability to regulate cellular gene expression

III. To compare EBNA1 and its functional homologues encoded by other gamma-herpesviruses with the ultimate goal to identify shared properties that may help to understand their role in oncogenesis

3. METHODS

Fluorescence Recover After Photo-bleaching (FRAP)

In order to investigate the nuclear dynamics of our protein of interest, we made use of Fluorescence Recovery After Photobleaching (FRAP) (335). The FRAP technique is extensively used to study qualitatively or quantitatively the mobility and (transient) immobilization of molecules in living cells (336). The protein of interest is fused with GFP or its derivatives and expressed in a suitable cell line. In a typical FRAP experiment, we bleached once a small-defined region (strip or circle) within a larger volume (nucleus) using high intensity laser and scanned the same area for a certain amount of time (336). Bleaching will result in lost of GFP fluorescence within the defined region, a process referred to as photobleaching. If the protein of interest is mobile, it will diffuse into the bleached area until the signal becomes equal to the surroundings. If the protein is immobile it will not diffuse in the bleached area, resulting in incomplete recovery of the florescence signal. FRAP analysis will tell us three essential component of mobility: diffusion coefficient, immobile fraction, and the time spent in the immobile state. Assuming elementary binding kinetics, the size of the immobile fraction and the duration of immobilization are determined by the on- and off-rates of the investigated protein to and from immobile complexes.

Fluorescence Loss In Photobleaching (FLIP)

FLIP is a common variant of FRAP. In a typical FLIP experiment, we bleached a defined region at regular intervals using a high intensity laser and monitored lost of fluorescence in a region or structure distant from the bleached region (337). FRAP and FLIP can be combined (FLIP-FRAP): two regions at two poles of nucleus are monitored at the same time after beaching only one of them. FLIP or FLIP-FRAP experiments are useful to measure the resident time of proteins within sub-nuclear structure, such as heterochromatin foci, telomere, speckles, repair foci etc (338-341).

Nucleosome array conformation analysis

In order to investigate the chromatin remodelling capacity of the proteins of interest, we made use of an in vivo targeting system that allows tethering of protein of interest to chromatin. The proteins were fused to the Escherichia coli lactose repressor protein (LacR) and the chimeric proteins were expressed in cell lines that contain multiple copies of the bacterial lactose operator (LacO) inserted in a region of heterochromatin. The LacR protein binds with very high affinity of the LacO sequence that appears as an intensely fluorescent dot in cells expressing the chimeric Lac repressor (LacR) fused to GFP or its

derivatives (342,343). Targeting of chromatin remodelers to the array by fusion to LacR is accompanied by large-scale chromatin de-condensation that can be quantified as increased size of the fluorescent area relative to the size of the nucleus.

Micrococcal Nuclease digestion assay

In order to investigate the chromatin-remodelling capacity of our protein of interest, we also used a Micrococcal Nuclease digestion assay. The assay is based on two properties of Micrococcal nuclease: i. The ability to work as an endonuclease that cleaves within the nucleosome linker region. ii. the capacity to reduce the size of the chromatin fragment by exonucleolytic digestion proceeding bi-directionally from the initial cleavage point (344,345). Exonucleolytic digestion results in the histone octamers sliding towards each other, which subsequently exposes additional nucleotides to digestion and results in a gradual decrease in the size of the oligonucleosome fragment. The presence of architectural transcription factors on nucleosome, such as HMG proteins, affects the kinetics of micrococcal nuclease digestion in several ways. The ability of HMG proteins to induce chromatin de-compaction will facilitate access to the linker DNA and increase the rate of the initial endonucleolytic attack. While the ability of HMGs to prevent octamer sliding by stabilizing the histone octamer, will protect several bases at the end of the chromatin particle, and decrease the rate of the exonucleolytic digestion. In this way, digestion of HMG containing chromatin will proceed with faster kinetics and produce a cleaner nucleosome ladder with fewer smears in between the oligonucleosomal fragments (346).

4. RESULTS

The results of four published papers included in this thesis will be briefly summarized.

4.1 PAPER I

As discussed in the introduction, EBNA1 is the only EBV protein constantly expressed during latency due to its critical involvement in episome maintenance (158,174,200). Its constant expression makes EBNA1 an ideal target for immune responses but earlier studies have shown that this viral antigen is poorly recognized by rejection responses mediated by CD8 positive MHC class I restricted CTLs (347). This was initially attributed to the GAr domain that acts as a portable signal that inhibits proteasomal degradation (348,349). However, later studies have shown that the inhibitory effect of the GAr can be bypassed since GAr deleted EBNA1 remains a very long-lived protein (182,350). This observation led us to investigate the probable contribution of other protein domains to EBNA1 stability.

Since EBNA1 is a nuclear protein, we first investigated whether the nuclear localization influences protein stability. To this end, we generated deletion mutants lacking the GAr either alone (EBNA1dGA) or together with the nuclear localization signal (EBNA1dGA/NLS). In the absence of the NLS, EBNA1dGA was rapidly degraded in the cytoplasm of transfected HeLa cells suggesting that stability is only achieved in the nucleus. EBNA1 interacts with cellular chromatin via two linking regions (LR1 and LR2) that are located at each side of the GAr. These DNA binding domains contain multiple repeats of Arg-Gly-Arg polypeptide and are therefore identified in our work as GR1 and GR2 repeats. Deletion of the repeats promoted rapid proteasomal degradation of the EBNA1dGA/GR mutant, suggesting that binding to chromatin may be responsible for the nuclear stability of EBNA1.

An artificial model was then constructed in order to explore in more detail the mode of action of the GRr. Different combination of the GR1 and GR2 were fused to the variable kappa chain (VK) of a conserved Ig gene that is often expressed in B-cell malignancies and is considered as a possible candidate for tumor specific immunotherapy (351-354). The VK polypeptide is efficiently expressed in the cytoplasm of transfected cells and is an excellent target for proteasomal degradation. Grafting of the GR1 and GR2 domains resulted in nuclear accumulation of the VK chimera and prevented proteasomal degradation. Thus, the inhibitory activity of the GR1/GR2 domain appears to be associated with chromatin binding and can be transferred to other proteasomal substrates. This led us to ask whether protein stabilization is a unique feature of this domain or a general property of DNA binding. To this end, VK was fused to

the DNA binding domain of Histone H1, the AT-hook (AT1/2/3) of HMGA1a, the DNA-targeting module of histone lysine N-methyl-transferase SUV4-20h2 (SUV-DBD) or the A/B box of HMGB-1 (A/B box). Analysis of the turnover of these proteins in short cycloheximide (CHX) assay showed that only DNA binding through H1 or the AT-hook of HMGA1 stabilized VK to the same extent as the GR1/GR2 of EBNA1.

In order to investigate whether the effects of different DNA-binding domains might be explained by the avidity of binding, their nuclear localization was investigated after treatment with a mild detergent such a Triton X100. Only the DNA binding domain of H1, the AT-hook of HMGA1a and the GR1/GR2 domain conferred resistance to detergent extraction suggesting that the avidity of DNA binding plays a key role in protein stabilization. This was further confirmed by the analysis of VK chimeras fused to different subdomains of the GR and HMGA AT-hook chromatin targeting modules. Only the intact GR1/2 of EBNA1 and the AT1/2/3 of HMGA1 were able to mediate detergent resistant binding to DNA and protected VK from proteasomal degradation.

Ubiquitination of the substrate is a key requirement for proteasomal degradation (355,356). We asked therefore whether binding to chromatin might affect this step of the degradation process. Analysis of protein ubiquitination in cell lysates prepared in the presence of chemical inhibitors of the deubiquitinating enzymes (DUBs) and proteasomes revealed no appreciable difference. Thus high affinity interaction with DNA appears to play a key role in regulating protein turnover.

4.2 PAPER II

EBNA1 promotes extensive changes in the expression of cellular genes (357-363). Attempts to ascribe this transcriptional effect to the binding of EBNA1 to cellular promoters via its viral DNA binding domain have yielded inconclusive results (364). Although binding was demonstrated in vitro, in most of the cases this did not correlate with the capacity of EBNA1 to efficiently transactivate the promoters in conventional reporter assays. This, together with the observation that EBNA1 establishes a strong interaction with cellular chromatin via its AT-hook like domain (185), and supported by the identification of numerous EBNA1 binding sites on cellular chromatin by ChIP assays (194,196,365), suggests that the viral protein may not act as a conventional transcription factor.

In order to explore the possibility that the effect of EBNA1 on transcription may be associated with changes in chromatin organization, we first investigated its possible accumulation in discrete regions of cellular chromatin. Fluorescence analysis in transfected NIH3T3 cells and U2OS cells co-transfected with the heterochromatin binding protein HP1ß revealed in both cases a clear exclusion of EBNA1 from heterochromatic regions. This was further confirmed by

immunofluorescence analysis that showed partial colocalization of EBNA1 with markers of euchromatin and facultative heterochromatin, such as H3K9me2, but exclusion for heterochromatin regions marked by H3K9me3. The possibility that EBNA1 may regulate the abundance of heterochromatin was then investigated by counting the number of bright heterochromatin foci in NIH3T3 cells stained with DAPI and U2OS cells transfected with GFP-HP1ß. A significant decrease in the number of foci was detected in both cell types suggesting that EBNA1 may promote chromatin decompaction. This possibility was directly addressed in micrococcal nuclease (MNase) digestion assay performed with DNA from the EBV-negative B-lymphoma line BJAB and its EBNA1-transfected subline BJAB-EBNA1. The analysis revealed a faster MNase digestion in EBNA1 positive cells and production of shorter nucleosome fragments, further supporting the possibility that EBNA1 may induce chromatin decompaction.

In order to assess the impact of EBNA1 expression on chromatin organization in living cells, we took advantage of the A03-1 reporter cell line that carries multiple copies of a 256-repeats array of the Lac operon (LacO) integrated in heterochromatin region that appears as an intensely fluorescent dot in cells expressing a chimeric Lac repressor (LacR) fused to GFP or its derivatives. The expression of the mCherry-LacR-EBNA1 fusion protein had a striking effect on the organization of the heterochromatic region, indicated by a highly significant 4-fold increase in the size of the fluorescent area. An even stronger effect was observed in the NIH2/4 reporter cell line that carries a shorter LacO array. The degree of array decondensation was comparable with that induced by a chimeric mCherry-LacR fused to the herpes simplex virus transactivator VP16, a prototype inducer of large-scale chromatin unfolding in mammalian cells. We then moved to investigate, which domain of EBNA1 is responsible for this effect. The size of the LacO array was monitored in A03-1 cells expressing mChery-LacR fused to EBNA1, EBNA1-DBD, EBNA1dGA, EBNA1dN/dGA, EBNA1-GR2-/DBD, or the juxtaposed GR1/GR2 domains alone. Fusion of mChery-LacR to the GR1/GR2 domain was necessary and sufficient for chromatin decompaction suggesting that the interaction of EBNA1 with cellular chromatin through this domain plays a key role in the chromatin remodeling effect.

Potent viral transactivators, such as VP16, recruit ATP-dependent chromatin remodelers that weaken the interaction of DNA with histones and promote nucleosome sliding. Alternatively, HMG proteins may promote nucleosome sliding by displacing linker histones. To investigate which mechanism may be responsible for the EBNA1 effect, A03-1 cell transfected with mCherry-Lac fused to EBNA1, VP16 or HMGA1 were co-transfected with GFP or YFP tagged enzymatic subunits of ATP-dependent chromatin remodeling complexes. As expected, all the ATPases were recruited by VP16 whereas recruitment was not observed in cells expressing EBNA1 or HMGA1 thus

confirming the involvement of different mechanisms. Moreover effect of VP16 was efficiently inhibited by depletion of ATP while the treatment had no effect on the activity of EBNA1.

The capacity of HMGA proteins to displace histone H1 is dependent on their rapid mobility on DNA. We tested therefore whether EBNA1 is a mobile protein and, if so, whether it shares with HMGA the capacity to displace linker histones. This analysis revealed that EBNA1 and HMGA1 share a similar mobility and are both capable to decrease the recovery time of Histone H1 in FRAP assays. The mobility of EBNA1 was dependent on the GR1/GR2 domains and fusion of the domains to GFP was sufficient to promote chromatin retention. The functional similarity of EBNA1 with HMGA proteins was further substantiated by comparison of the transcription profile of cells expressing EBNA1, HMGA1 and other chromatin-binding proteins that lack canonical transcription factor activity. This analysis revealed that EBNA1 expression is associated with low but significant up- and down-regulation of a large number of genes. Furthermore, similar to HMGA2, EBNA1 was shown to induce robust activation of the Twist promoter that regulates a variety of cellular events, including Epithelial-to-Mesenchymal Transition (EMT) during embryogenesis and in invasive carcinomas.

4.3 PAPER III

Having established the role of chromatin binding through the GR domain for the capacity of EBNA1 to promote chromatin remodelling, we sought to identify cellular factors and structural features of EBNA1 that may influence this interaction.

Fluorescence loss in photobleaching (FLIP) and Fluorescence recovery after photobleaching (FRAP) assays were used to compare the mobility of GFP-tagged EBNA1, HMGA1a and histone H1. The mobility of GFP-EBNA1 was close to that of HMGA1a in both FLIP and FRAP assays and was not influenced by the presence of viral episomes. We also tested whether the mobility of EBNA1 may change in mitosis when a more stable interaction with cellular chromatin could be required to anchor the episomes to the migrating chromosomes. To this end, the FRAP recovery curves of GFP-EBNA1 were compared in transfected U2OS cells during interphase or in cells arrested in mitosis by treatment with colcemide. There was a significant difference in the kinetics of fluorescence recovery in interphase and mitotic cells and suggesting that at least a proportion of EBNA1 is immobile during mitosis.

GA-repeats of variable length separate the chromatin-targeting modules encoded in the GR1 and GR2 domains of EBNA1. In order to investigate whether this structural organization influences the interaction of EBNA1 with cellular chromatin, the mobility of GFP-EBNA1 was compared with that of

deletion mutants lacking either the GAr alone (GFP-EBNA1-dGA) or the entire N-terminal domain (GFP-DBD). As expected, deletion of the N-terminus abolished the ability of GFP-DBD to interact with cellular chromatin resulting in half-time fluorescence loss in FLIP and half-time fluorescence recovery in FRAP comparable to those of the soluble GFP-NLS. In addition, the mobility of EBNA1 was significantly impaired by deletion of the GAr suggesting that repeat may play an important role in modulating the chromatin remodeling function of EBNA1.

4.4 PAPER IV

All gamma-herpesviruses express EBNA1-like protein during latency. Like EBNA1, the EBNA1-homologues also ensure episome maintenance by tethering the episome to host chromosome and regulate episome replication and proper segregation into daughter cells. We therefore asked whether the GMPs share the capacity of EBNA1 to promote chromatin decompaction and, if so, whether they also share the same mechanism of action based on displacement of H1. For this study we selected the GMPs of seven gamma-herpesviruses. EBNA1 and baEBNA1 are encoded by viruses of the genera lymphocryptovirus (LCV) while LANA1, mnR1LANA, mnR2LANA, muLANA and saLANA are the GMPs of rhadinoviruses (RHV). Bioinformatics analysis showed that all GMPs share a relatively conserved C-terminal episome-binding domain while the N-terminal chromatin-binding domain is only conserved among members of the same genera. The N-terminus of EBNA1 and baEBNA1 contains multiple Arg-Gly-Arg repeats that resemble the AT-hook of HMGA proteins, whereas a basic N-terminal domain is likely to be involved in the interaction of RHV GMPs with nucleosomes or nucleosome binding proteins.

We first investigated the nuclear localization of GMPs in transfected NIH3T3 cell line. This cell line was chosen due to the easy visualization of heterochromatic regions in the nuclei of murine cells stained with DAPI. Like EBNA1, the GMPs showed an exclusive nuclear localization and exhibited a diffuse fluorescence with no apparent association with distinct nuclear sub-compartments. Since the two GMPs families differ in their chromatin-binding domain, we asked whether this might influence their avidity to interact with chromatin. To this end, the fluorescence of GFP-fused GMPs transfected in U2OS cell was measure after extraction with Triton X100. Similar levels of fluorescence were measured after extraction, indicating that the GMPs interact with chromatin with similar avidity.

In order to investigate the chromatin-remodelling capacity of the GMPs, A03-1 cells were transfected with mCherry-LacR reporters fused to each of the GMPs. In spite of individual variations in the magnitude of the effect, targeting of the GMPs to the LacO region was in all cases accompanied by a highly significant increase in the average size of the array. Interestingly, the effect was more pronounced in cells expressing the GMPs encoded by the human tumor viruses EBV and KSHV, EBNA1 and LANA1. We then investigated whether the

chromatin remodelling function correlates with the mobility of the proteins on cellular chromatin. The LCV encoded EBNA1 and baEBNA1, that shares 56% amino acid sequence homology with EBNA1 and a highly conserved AT-hook-like chromatin-targeting module, were equally mobile. In contrast, a poorer recovery was observed in cells expressing the RHV GMPs. Only 25% to 35% of the initial fluorescence was recovered in cells expressing LANA1 and mnR2-LANA, suggesting a very slow overall mobility and possibly the presence of a large immobile fraction. The two proteins belong to different RHV subfamilies but share a highly conserved domain structure, and more than 45% sequence identity in the N-terminal domain that mediates the interaction of LANA1 with cellular chromatin via binding to histone H2A and H2B. The remaining RHV GMPs showed intermediate recoveries. The N-terminal domain of mnR1-LANA is very similar to that of LANA1 and mnR2-LANA, suggesting that they could have similar interacting partners, whereas the N-terminal domains of saLANA and muLANA share the overall prevalence of basic amino acid residues but no sequence similarity with the corresponding regions of other RHV GMPs.

EBNA1 promotes chromatin remodelling with a slow kinetics and without recruitments of ATP-dependent remodelling complexes. We therefore tested whether these properties are shared by other GMPs. To this end, we compared mCherry-LacR-tagged GMPs for their ability to recruit a panel of GFP- or YFP-tagged ATPase subunits of known ATP-dependent remodelling complexes, including the BRG1 subunit of SWI/SNF complexes, the SNF2H subunit of ISWI complexes and the CHD4 subunit of NuRD complexes, histone acetyltransferases, such as GCN5, pCAF and p300, and two bromo and extra terminal domain (BET) proteins, BRD2 and BRD4, that bind to acetylated histones and are often hijacked by viruses to promote transcription. All the tested proteins were recruited to the site of chromatin decondensation in cells expressing mCherry-LacR-VP16, whereas, in accordance with our previous findings, none of the proteins was recruited by EBNA1 and a similar behaviour was observed with baEBNA1. In line with the presence of high affinity binding sites for BRD2 and BRD4 in the C-terminal domain of LANA1, the two BET proteins were recruited to the site of chromatin remodeling in cells expressing mCherry-LacR-LANA1, and a similar recruitment was observed with all the RHV encoded GMPs. Occasional recruitment of other components of ATP-dependent chromatin complexes and HATs was occasionally observed with the RHV GMPs. Thus, in some of the cells LANA1 recruited BRG1 and p300, and mnR1-LANA recruited pCAF, p300 and GCN5.

5. DISCUSSION

Pathogenic viruses and intracellular bacteria have evolved elaborate strategies for manipulating the host cell environment, often resorting to the production of multifunctional proteins that hijack or mimic the activity of cellular regulators. A common property of DNA tumor viruses is the establishment of non-productive infections characterized by the expression of a restricted repertoire of latency-associated viral genes. Remodelling of the infected cells by the products of these genes is an enabling feature of viral oncogenesis but, in spite of intensive research, their mechanisms of action are still poorly understood. The work described in this thesis has highlighted key features of the interaction of the genome maintenance protein of the human oncogenic herpesvirus EBV, EBNA1, with cellular chromatin, pointing towards novel mechanism by which the virus may reshape the host-cell and promote malignant transformation.

Proteasomal degradation is a complex process whose molecular details and regulation are only partially understood. We have shown that the GRr, the bipartite chromatin-anchoring module of EBNA1, acts a portable signal that inhibits proteasomal degradation. The GRr resembles the AT-hook of HMGA proteins that bind in a sequence-independent manner to AT-rich stretches of DNA via three conserved Pro-Arg-Gly-Arg-Pro motifs. The structural and functional similarity is confirmed by the finding that the AT-hook of HMGA1 can substitute for the GRr in all the functions of EBNA1 that require interaction with cellular DNA, including partitioning of the viral episomes in proliferating cells (366). NMR studies indicate that the AT-hook undergoes a structural transition upon DNA binding, assuming a crescent-shaped conformation that fits deep into the narrow minor groove (262). Hydrophobic interactions of the Arginine side chains with Adenines place the domain in a fixed orientation towards DNA while the cooperative action of three properly spaced AT-hooks is required for high avidity binding. The EBNA1 GRr lacks the Proline residues and tripartite organization of the canonical AT-hook but contains long stretches of Arg-Gly-Arg repeats, which is likely to enhance binding avidity by mediating contact with multiple AT pairs.

Histone H1, the AT-hook containing HMGA proteins and A/B-box containing HMGB protein bind to partially overlapping regions in the minor groove of DNA but the interaction is clearly different. Based on their rate of diffusion, the AT-hook and A/B-box containing proteins are highly mobile while histone H1 has a slower diffusion rate, indicating a more stable interaction with DNA. We found that tethering to DNA via the GRr or the AT-hook did not interfere with the efficiency of ubiquitylation and the majority if not all of the ubiquitylated substrates were resistant to detergent extraction suggesting that they remain bound to DNA. One interesting possibility is that stable interaction with DNA may

hamper the capacity of the proteasome to pull the ubiquitylated substrate away from the complex.

The finding that EBNA1 is protected from proteasomal degradation by two stabilization signals, the GAr and GRr, both acting downstream of ubiquitylation is intriguing. The resistance of EBNA1 to proteolysis is likely to play a pivotal role in the establishment of life-long persistent infections in healthy EBV carriers by allowing the maintenance of sustained levels of the protein in a latent reservoir of non-proliferating memory B-lymphocytes where transcription of the viral genes is tightly downregulated. It remains to be seen how the two domains, that are clearly capable of providing independent stabilization signals upon grafting to unrelated proteasomal substrates, cooperate in regulating the stability of EBNA1 in different cellular compartments.

We have found that, through the GRr, EBNA1 promotes a widespread remodelling of chromatin organization and a broad rearrangement of transcription, featuring both up- and down-regulation of a large number of genes. Dynamic changes in chromatin structure play a key role in DNA transcription, replication, recombination and repair. The access of transcription factors to gene regulatory elements is controlled by the local chromatin architecture, which contributes to their tissue-specific action. The effect of EBNA1 is reminiscent of that of “architectural” or “pioneer” transcription factors that confer competence for gene expression by opening the chromatin for binding by remodelers, transcription factors and co-repressors, and play critical roles in cell programming and in the responsiveness to environmental cues.

EBNA1 induces chromatin decompaction with slow kinetics and independent of the recruitment of ATP-dependent remodelers, sharing these two properties with HMGA1a. The HMG “architectural factors” modulate nucleosome and chromatin structures by weakening the chromatin binding of linker histones. By rapid diffusion through the nucleus, HMGs may gain access to temporarily vacated nucleosomal sites, counteracting thereby the ability of linker histones to stabilize higher order chromatin structures. Similar to HMGAs, EBNA1 enhances the mobility of histone H1, which correlates with global chromatin decompaction, increased accessibility to micrococcal nuclease digestion and reduced length of the nucleosome repeat. The importance of H1 displacement for these effects is supported by the induction of a similar phenotype in mouse embryonic stem cells upon diminished occupancy of the linker region following knockdown of multiple H1 variants (367). HMGA proteins are physiologically expressed at high levels during embryogenesis and have important roles in development (274-277). Their oncogenic potential is highlighted by the ectopic expression in a broad spectrum of human malignancies, and is confirmed by the induction of different types of neoplasia in transgenic mouse models (279-284,289,291,368). The transforming ability of HMGs is attributed to their capacity to cooperate with oncogenes in the regulation of genes that control cell proliferation and apoptosis.

Although EBNA1 is regularly expressed in all EBV associated malignancies, its role in oncogenesis is debated and conflicting data have been reported on the capacity to serve as an oncogene in transgenic mouse models (369-373). Our findings provide a possible explanation for these discrepancies by highlighting a scenario where the chromatin remodelling function of EBNA1, although insufficient to promote malignancy in the absence of co-factors, may sensitize the infected cells to the activity of cellular or viral oncogenes. Indeed, EBNA1 was shown to synergize with *c-Myc* in a mouse model of lymphomagenesis (371).

We have found that EBNA1 is less mobile on mitotic chromosomes compared to interphase chromatin. The possibility that post-translational modifications may regulate the interaction of EBNA1 with DNA or with various DNA-binding proteins in a cell-cycle dependent manner remains an interesting focus for future research. We have found that deletion of the GAr significantly reduces the mobility of EBNA1 and causes the appearance of a slow or possibly immobile fraction. It is noteworthy that a four-fold decrease of diffusion rate could have important functional consequences by decreasing the capacity of EBNA1 to compete for temporarily vacant linker histone binding sites, hampering thereby the chromatin remodelling function. Using a set of chimeric proteins containing GFP fused to different combination of the structural elements present in EBNA1, we have found that both the GR1 and GR2 domains are required for efficient tethering to chromatin while the intervening GAr regulates the diffusion property in a length dependent manner. The requirement for both the GR1 and GR2 is in agreement with functional data demonstrating that both domains are required for the episome maintenance function of EBNA1, and is also in line with our finding that both domains are required for resistance to detergent extraction and proteasomal degradation. Thus, the peculiar architecture of the chromatin-targeting module of EBNA1 may be a critical determinant for its activity. The effect of the GAr is particularly interesting since it illustrates a previously unrecognized mechanism by which this protein domain could regulate the function of EBNA1. Although dispensable for the capacity of EBV to immortalize B-lymphocytes *in vitro*, the GAr is present in all virus isolates, and is conserved the EBNA1 homologues encoded by lymphocryptoviruses. The length of the repeat is also likely to be important *in vivo* since, although the size varies in different isolates, shortest documented repeats are at least 50-60 amino acids long. Based on our findings, this may be the minimal length required to significantly affect the diffusion properties of EBNA1.

The GMPs of gamma-herpesviruses have been extensively studies with the hope that a better understanding of their properties may lead to the design of novel antiviral therapeutics capable of halting the risk of malignant transformation. While comparing the chromatin interaction properties of GMPs encoded by viruses belonging to the LCV and RHV genera, we have found that the GMPs share the capacity to establish detergent-resistant interactions with

cellular DNA and to promote chromatin decompaction, which could play an important role in the regulation of latent infection and cooperation with viral or cellular oncogenes. However, although the effect on chromatin organization appears to be similar, the interaction of the GMPs with cellular chromatin is profoundly different. While the GMPs encoded by the EBV and HVP (LCVs) are highly mobile on chromatin and promote chromatin decompaction without recruitment of ATP-dependent remodelling complexes, five RHV encoded GMPs were significantly less mobile and their capacity to promote chromatin decompaction correlated with regular recruitment of the BET proteins Brd2 and Brd4.

The consistent behaviour of LCV and RHV GMPs suggests that their different chromatin-targeting modules could be a key determinant of mobility. Most importantly, while the LCV GMPs may directly bind to cellular DNA via the AT-hook, the targeting of RHV GMPs is achieved via interaction with DNA-binding proteins that may dictate the mobility of the complex. Interestingly, the N-terminal domains of all RHV GMPs also contain several relatively well-conserved Thr and Ser residues that are phosphorylated in LANA1 by the CK1, PIM1, GSK-3 and RSK3 kinases (374). Short-term treatment of transfected cells with RSK inhibitors reduced the interaction of LANA1 with histone H2B and promoted protein degradation, suggesting a possible strategy for interfering with the binding of RHV GMPs to cellular chromatin (374).

The capacity of all RHV GMPs to recruit Brd2 and Brd4 to the site of chromatin remodelling is in agreement with the presence of multiple conserved high-avidity binding sites in the C-terminus of the molecules (375). Brd2, Brd4 and related bromodomain proteins provide a scaffold for the recruitment of E2F transcription factors, histone deacetylases, histone H4-specific acetyltransferase and protein complexes involved in chromatin remodeling, including SWI/SNF and elements of the Mediator complex (376). Future studies should focus on dissecting the role of Brd2 and Brd4 in the chromatin remodelling induced by RHVs.

6. ACKNOWLEDGEMENTS

I would like to thank all the current and former member of our lab who supported me in accomplishing this thesis. In particular, I would like to thank:

My supervisor, Prof. Maria G. Masucci, for the patient guidance, encouragement and advice, she has provided throughout my time as her student.

I wish to thank Teresa Frisan (co-supervisor) and Javier for their support and encouragement through all these years.

I especially like to thank Giuseppe Coppotelli (mentor) for teaching me all the techniques (from western blot to FRAP). It was a long and eventful collaboration. But I had lot of fun.

I am thankful to Stefano for graciously providing me with his technical assistance whenever I asked for.

I would also like to thank Jamie and Marcela for all their support and for boosting my enthusiasm from time to time.

A special thanks to Linda Thörn and Matti Nikkola for all their support and administrative assistance.

Best thanks to present and past members of the group:

Helena, Ricardo, Laura, Kristina, Rama, Siamak, Omid, Lina, Eugenie, Diego, Sebastian, Sandra, Harsha.

Specially like to thank my friends: Khalid, Rukun, Seema, Roberta, Kelly, Daniela, Simone (AH), Mia, Ximena, Bin Zhao, Valeria and Xinsong Chen (AH) for all the fun that we shared during these years. Without your support and encouragement, I would never have made it so far.

I would also like to thank Mom, Naheed, Zaman, Nazia, Salman, Kamran, Sami and Sana for their love and support through all these years.

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