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ROLE OF LATENT EBV GENES IN THE INDUCTION OF GENOMIC INSTABILITY IN BURKITT'S LYMPHOMA

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ABSTRACT

Epidemiological and molecular evidence link Epstein-Barr virus (EBV) infection to a variety of lymphoid and epithelial malignancies but the contribution of the virus to tumorigenesis is unclear. Genomic instability, defined by the establishment of a "mutator phenotype" and characterized by the occurrence of non-clonal chromosomal aberrations, excessive DNA damage and defects in DNA repair, is the hallmark of malignant transformation. In the studies described in this thesis, we have explored the possibility that EBV may promote malignant transformation by inducing genomic instability. As a first step in this analysis, the presence of non-clonal chromosomal aberrations, including dicentric chromosomes, chromosome fragments, gaps, rings, satellite associations and double minutes, was investigated in EBV positive and negative Burkitt's lymphoma (BL) cell lines, *in vitro* EBV converted BLs and EBV genome-loss variants of EBV positive tumors. EBV carriage was associated with a significant increase in abnormal metaphase plates with prevalence of dicentric chromosomes, fragments and gaps. Increased phosphorylation of H2AX and lengthening of telomeres were detected in EBV positive cell lines suggesting DNA damage and telomere dysfunction as possible molecular mechanisms. Analysis of EBV gene expression revealed an increase of abnormal metaphases in cells expressing EBV latency I and further increase in latency III, suggesting that more than one viral protein may be responsible for this phenotype.

EBNA-1 is always expressed in EBV carrying proliferating cells. We investigated therefore the occurrence of genomic instability in cells expressing stable or inducible EBNA-1. Chromosomal aberrations, increased DNA damage and activation of the DNA Damage Response (DDR) as detected by phosphorylation of the DNA damage sensing kinase ATM and its downstream target histone H2AX, were observed in EBNA-1 expressing cells. These signs of genomic instability were associated with a significant increase of endogenous reactive oxygen species (ROS). Bioinformatic analysis of EBV regulated genes identified four genes within the ROS metabolic pathway as possible targets of EBV transcriptional regulation. The catalytic subunit of the ROS producing NADPH Oxidase 2 (Nox2) was shown to be selectively upregulated in EBNA-1 expressing cells. The involvement of Nox2 in the production of ROS and induction of genomic instability was confirmed by functional inactivation using chemicals and RNAi.

The possibility that more than one latency associated EBV product may be involved in the induction of genomic instability was addressed by investigating the occurrence of chromosomal aberrations increased DNA damage and DDR activation in a panel of transfected sub-lines of the B-lymphoma line BJAB carrying individual latency genes. In addition to EBNA-1, expression of EBNA-3C and LMP-1 was associated with these phenotypic markers of genomic instability. Each of these viral proteins appears to promote genomic instability through a different mechanism. Only EBNA-1 directly induced DNA damage via ROS, while expression of LMP-1 was associated with inhibition of DNA repair via downregulation of ATM, which resulted in failure to phosphorylate Chk2 and consequent inactivation of the G2 checkpoint. EBNA-3C expression induced a high degree of aneuploidy that was associated with inactivation of the mitotic spindle checkpoint and transcriptional downregulation of BubR1.

Collectively these results indicate that multiple cellular functions involved in the maintenance of genome integrity are independently targeted by EBV, pointing to the induction of genomic instability as critical event in viral oncogenesis.

LIST OF PUBLICATIONS

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

I Kamranvar SA*, **Gruhne B***, Szeles A, Masucci MG. Epstein-Barr virus promotes genomic instability in Burkitt's lymphoma. Oncogene. 2007, 26,5115-5123.
* These authors contributed equally to this work.

II **Gruhne B**, Sompallae R, Marescotti D, Kamranvar SA, Gastaldello S, Masucci MG. The Epstein-Barr virus nuclear antigen-1 promotes genomic instability via the induction of reactive oxygen species. Proc. Natl. Acad. Sci. 2009, 106. 2313 – 2318.

III **Gruhne B**, Masucci MG. Three Epstein-Barr virus latency proteins independently promote genomic instability by inducing DNA damage, inhibiting DNA repair and inactivating cell cycle checkpoints. Submitted.

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LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia, mutated
ATR	Ataxia telangiectasia and Rad3 related
BER	Base excision repair
BL	Burkitt's lymphoma
CIN	Chromosomal instability
CTAR	C-terminal activation region
DAPI	4,6-diamino-2-phenylindole
DDR	DNA damage response
DNA-Pk	DNA dependent protein kinase
DSB	Double-strand break
EBER	EBV encoded RNA
EBNA	EBV latent antigen
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor
HR	Homologous recombination
hTERT	Human telomerase reverse transcriptase
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
miRNA	Micro RNA
MMR	Mismatch repair
MRN	Mre11-Rad50-Nbs-1 complex
NER	Nucleotide excision repair
NHEJ	Non-homologous endjoining
NPC	Nasopharyngeal carcinoma
PTLD	Post-transplant lymphoproliferative disease
pRb	Retinoblastoma protein
ROS	Reactive oxygen species
SSB	Single-strand break

1 INTRODUCTION

1.1 EPSTEIN-BARR VIRUS (EBV)

EBV is a gamma herpes virus that infects the majority of the world's population. Following primary infection the host remains a lifelong carrier of the virus. Primary infection occurs in childhood and presents either no symptoms or minor symptoms of a cold. However if the primary infection is delayed until adolescence or adulthood, it may cause a severe disease called infectious mononucleosis that is accompanied by high fever, glandular swelling and sore throat. EBV infects preferably B-cells and epithelial cells. The EBV genome is a linear, double-stranded, 172-kb long DNA molecule. EBV is associated with a number of malignancies of lymphoid and epithelial cell origin such as Burkitt's Lymphoma (BL), nasopharyngeal carcinoma (NPC) and Hodgkin's Disease (HD) [1]. The contribution of the virus to tumorigenesis remains unknown, however epidemiologic evidence supports the notion that EBV at least in part is responsible for tumor formation.

1.1.1 The Virus Life Cycle

The life cycle of the virus can be divided into four separate steps: primary infection, transformation, latency and reactivation of the productive life cycle (Figure 1).

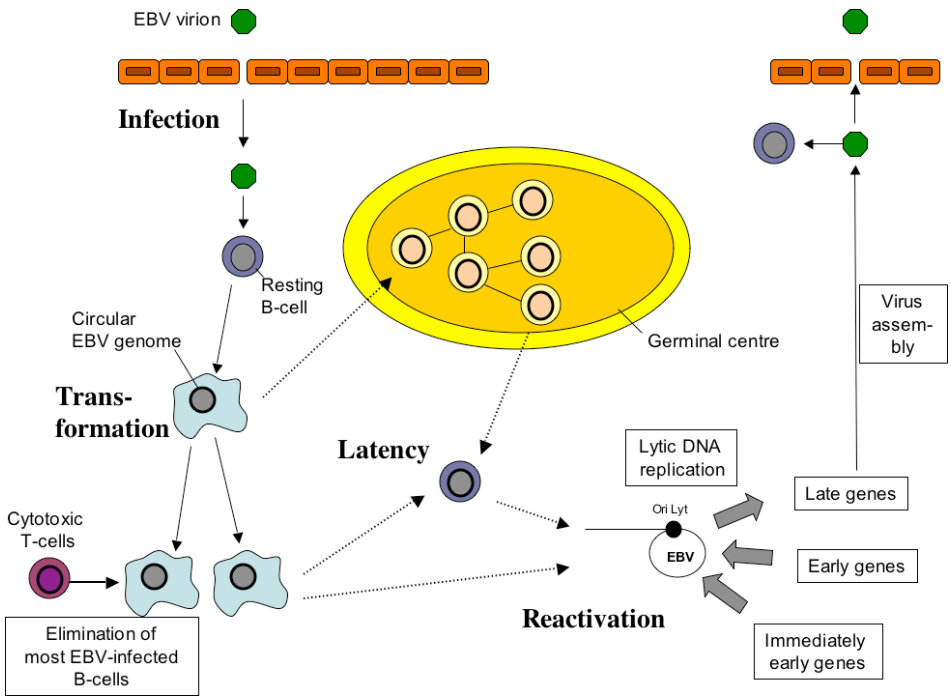

Figure 1: The life cycle of EBV infection in B-cells.

EBV infects mainly B-cells in a resting state and activates these B-cells to enter into the cell cycle, maintain continuous proliferation and prevent cells from undergoing apoptosis. This state is called growth transformation. Downregulation of all latent viral products leads to a latent state, in which affected cells are not recognized by the immune system. The virus replicates occasionally in most asymptomatic carriers of EBV and reactivates the productive lytic cycle. The newly produced infectious particles infect other B-cells or are shed into the saliva. EBV uses the normal B-cell biology to establish infection, persistence and replication [2].

Infection

The transmission of the virus from host to host occurs via saliva. The first sites of infection are oropharyngeal epithelial cells and B-cells. The infection of B-cells is mediated through the interaction of the viral envelope glycoprotein gp350 with the host CD21 receptor [3]. The CD21 receptor is a receptor for the C3d complement component CR2. The C3d/EBV receptor is not restricted to B-cells, in fact identical or related molecules have been found in T-cells and epithelial cells [4,5]. However EBV infection has been also described in C3d negative cells, suggesting a C3d-independent mode of infection. The viral envelope fuses with the host membrane involving three other glycoproteins namely gp85, gp25, gp42 [6]. One of these glycoproteins, gp42, binds furthermore to the MHC class II molecule and enhances the infection of B-cells [7]. Once the virus envelope has fused to the host membrane, the uncoated virus is transported to the nucleus. The immediate circularization of the viral DNA in the nucleus serves as a signal for activation of the W promoter. The first latent proteins to be expressed are the EBV latent antigens (EBNA)-2 and -5 and later all other nuclear antigens are expressed [8]. The promoter for the latent membrane proteins (LMP) is activated by the expression of EBNA-2 and EBNA-3C and drives the transcription of the LMPs [9]. Cytotoxic T-lymphocytes respond to almost all latent proteins leading to an immediate decrease in infected B-cells. Several antibodies against the EBV viral capsid antigen, the early antigen D and R are produced and released by plasma cells.

Transformation

The expression of the latent proteins transform the B-cells into continuously growing B-cells *in vitro* as well as *in vivo* [10]. EBV *in vitro* transformed resting B-cells grow continuously in lymphoblastoid cell lines (LCLs). The B-cells undergo blast transformation characterized by an increase in nuclear size, cytoplasmic volume and expression of certain cellular genes, such as CD23. Around 36h after infection, the DNA synthesis is initiated and the cell division takes place. During transformation, all latent proteins, including six EBV nuclear antigens (EBNA-1, -2, -3A, -3B, -3C, and -LP) and three latent membrane proteins (LMP-1, LMP-2A, LMP-2B), are expressed designated as the “growth program” or latency III expression pattern ([11], see Table 1). Additionally two untranslated RNAs, EBV encoded RNA (EBER)-1 and -2, and some micro RNAs (miRNAs) are detected during activation of the growth program. Five latently expressed proteins have been shown to be essential for EBV transformation namely LMP-1, EBNA-2, -3A, -3C and -LP [12-16]. EBNA-1 has a promoting effect for immortalization by enhancing the transformation [17] and by driving the expression of EBV’s transforming genes [18]. In LCLs B-cell activation markers such as CD32, CD30, CD39 and CD70 and cellular adhesion molecules like LFA1, LFA3 and ICAM1 are highly upregulated

simulating the state of antigen or mitogen activated B-cells. These observations suggest that EBV-induced immortalization uses the same cellular pathways that drive the physiological B-cell proliferation. Expression of all latent viral proteins are believed to enable autonomous B-cell proliferation and thereby expand the initial pool of infected B-cells allowing the virus to access the long-lived memory B-cell compartment.

Latency

Following growth transformation and expansion of the B-cell pool, the virus protein expression is more restricted. The latency II program is characterized by the expression of EBNA-1, LMP-1, LMP-2 and the EBERs RNA [19]. The expression of the latent membrane proteins activates the latently infected B-cell blasts to progress to the germinal centre and differentiate into latently infected memory B-cells [20]. This latency type is additionally to the germinal centre expressed in NPC and HD. Later the virus enters the state of latent persistence and shuts down the expression of all viral proteins [21]. The latently infected memory B-cells circulate in the peripheral blood without being detected by the immune system. The silent state of the virus is called latency 0. Only when the memory B-cells divide, they express one latent viral protein, EBNA-1 [21]. During this state, latency I, only one viral cis element, OriP, and one viral protein, EBNA-1, is required for viral maintenance [22,23]. OriP consists of two clusters of EBNA-1 binding sequences; the family of repeats and the dyad symmetry. EBNA-1 tethers the family of repeat region to the chromosomes and thereby permits the retention of replicated DNA in daughter cells. The latency I expression pattern is found in BL.

Table 1: Expression of latent viral proteins in different malignancies.

Latency	Protein expression	Malignancies
I	EBNA-1	Burkitt's lymphoma
II	EBNA-1, LMP-1, -2	Nasopharyngeal carcinoma
III	EBNA-1, -2, -3A, -3B, -3C, -LP, LMP-1, -2	Lymphoproliferative disease

Reactivation of the Productive Life Cycle

Reactivation of the productive life cycle has been observed in BL cell lines upon cross-linking the B-cell receptor, a surface immunoglobulin, with anti-IgG antibody resembling the binding of the B-cell receptor with the antigen [24]. Some BL cell lines may be reactivated by treatment with transforming growth factor [25]. It has been assumed that this reactivation occurs also *in vivo*. Indeed several studies describe reactivation upon different kinds of physiological and psychological stress *in vivo* [26-28]. The viral gene expression of the lytic phase follows a sequential order, first the immediately early genes, the early genes and then the late genes are expressed. The expression of immediately early genes, such as BZLF1 and BRLF1 are induced directly by signalling through B-cell receptor activation [29]. The expression of the transcription factor BZLF1 is sufficient to start the lytic cascade. BZLF-1, showing sequence similarity to c-fos, drives the expression of several viral early gene promoters, cellular promoters and the lytic origin of replication, oriLyt. It activates its own expression further by binding to its own promoter and activating the adjacent gene BRLF-1. Both transcription factors together, BZLF-1 and BRLF-1, activate most of the early genes in the next phase of the lytic cycle. Early genes execute the viral replication. In the EBV genome six genes are

essential for oriLyt-dependent replication, namely BALF-5 [30], BALF-2 [31], BMRF-1, BBLF-1, BSLF-1 and BBLF-2/3. Balf-5 encodes for the catalytic subunit, while BMRF-1 encodes for the accessory subunit of the DNA polymerase. Balf-2 encodes a single-stranded DNA binding protein. The function of BBLF-4, BSLF-1 and BBLF2/3 has not been described yet, but they are suspected to be helicase, primase and helicase–primase associated protein, respectively. Following DNA replication, the late genes, mostly structural genes, are expressed and assembled into virions. Consequently the virions, packaged with viral DNA, are released as infectious particles, which in turn infect adjacent B-cells or are shed into the saliva.

1.1.2 EBV Latent Proteins

EBNA-1

EBNA-1 is the only protein expressed in all EBV-associated malignancies. Its main function is the replication and maintenance of the episomal EBV genome, which is achieved by the binding of EBNA-1 to the plasmid origin of viral replication, oriP and to the host chromosomes during mitosis [32]. EBNA-1 also acts as transcriptional transactivator activating the C promoter. EBNA-1 has a glycine-glycine-alanin (gly-ala) repeat separating amino- and carboxy-terminal domains from each other. The repeat varies in size in different EBV isolates and inhibits antigen processing via the ubiquitin proteasome pathway [33]. EBNA-1 is therefore protected from endogenous presentation through the MHC class I pathway [34].

EBNA-1 is dispensable for growth transformation *per se*. However its absence attenuates the growth transformation efficiency [17], most likely due to the essential role of EBNA-1 in maintaining the EBV genome in the cells. Indeed the few immortalized B-cells that contain EBNA-1 deleted EBV, had the EBV genome integrated into the host DNA [17]. EBNA-1 has been shown to enhance cell growth and proliferation [35] as well as protect the cells from the induction of apoptosis [36,37]. This phenotype might be triggered by the competition of EBNA-1 and p53 to the same binding pocket of USP7/HAUSP [38]. USP7/HAUSP plays an important role in regulating cell proliferation and apoptosis through p53 and Mdm2 interactions. The binding of p53 to USP7/HAUSP leads to the stabilization of p53. EBNA-1 binds with a 10-fold higher affinity to USP7/HAUSP and thereby promotes the destabilization of p53. Cells expressing mutant EBNA-1, deficient in binding to USP7/HAUSP, undergo apoptosis by p53 stabilization.

The oncogenic potential of EBNA-1 was studied in transgenic mice. Wilson et al. backcrossed two transgenic mouse lines onto C57BL/6 genetic background expressing EBNA-1 under the control of an immunoglobulin heavy chain intron enhancer. These mice develop spontaneously B-cell lymphomas [39]. However Kang et al. described three EBNA-1 expressing transgenic mouse lines in a FVB background that did not induce B-lymphoma [40] and neither if backcrossed into the C57BL/6 background [41]. In another mouse model, EBNA-1 expressing Hodgkin's Disease cells were non-tumorigenic in SCID mice but demonstrated enhanced lymphoma development in non-obese diabetic SCID mice [42]. It seems that EBNA-1 is not an oncogene *per se*, but needs cofactors or characteristic environment to induce B-lymphomas in transgenic mice.

EBNA-2

EBNA-2 is the first protein expressed together with EBNA-LP after infection of primary B-cells [8]. EBNA-2 and EBNA-LP are both responsible for the transition of the

infected cells from G0 to G1 [43]. EBNA-2 transcriptionally activates the expression of all other viral proteins expressed in LCLs by transactivating the C promoter [44] and the LMP promoter [45] as well as some cellular genes such as CD21 and CD23 [46]. EBNA-2 interacts with the Notch target RBP-Jk suggesting that it acts as a viral functional homologue of an activated Notch receptor [47]. EBNA-2 is essential for growth transformation and it is believed that the RBP-Jk interaction is necessary for an efficient growth transformation. Notch signalling may affect a number of cellular processes, including the differentiation, proliferation and apoptosis. The mimicking of Notch by EBNA-2 might promote tumorigenesis in EBV associated malignancies.

The EBNA-3 Family

The three members of the EBNA-3 family, EBNA-3A, -3B and -3C (alternatively called EBNA-3, -4 and -6) are arranged in a tandem in the EBV genome and share a similar exon/intron structure. The common characteristic of the three proteins is the interaction with the cellular protein RBP-Jk [48]. EBNA-3A and EBNA-3C are essential for B-cell transformation, whereas EBNA-3B is dispensable. EBNA-3C is believed to play a major role in cell cycle regulation. It downregulates the retinoblastoma protein (pRb) and thereby enhances the progression from G1 to S phase [49]. Furthermore EBNA-3C expressing cells are able to overcome the spindle checkpoint after nocodazole treatment and progress into M phase [50].

EBNA-LP

EBNA-LP and EBNA-2 are the first genes to be expressed after EBV infection of B-lymphocytes. EBNA-LP is required for efficient B-cell transformation and subsequent LCL outgrowth [51]. It has been shown to interact with pRb [52,53]. However this interaction does not affect the E2F activity or the cell cycle progression [54].

LMP-1

LMP-1 was the first EBV latent protein shown to have oncogenic potential by developing B-cell lymphoma in transgenic mice [55]. The latent membrane protein mimics the CD40 receptor and functions as a constitutive active tumor necrosis factor receptor by activating a number of cellular signalling pathways (Figure 2) [56]. The activation of intracellular signalling requires aggregation and oligomerization of LMP-1. Only the C-terminal cytoplasmic region, containing the C-terminal activation regions (CTAR)-1, -2 and -3, are required for signal activity. The N-terminal cytoplasmic tail, that orients the protein in the membrane, and the hydrophobic transmembrane loop, involved in self-aggregation, do not possess signalling activity. LMP-1 activates the NFκB pathway either through CTAR-1 or alternatively through CTAR-2 and subsequent stimulation of downstream targets like TRAF-1 and -3 or TRADD and TRAF-2 respectively [57,58]. Once NFκB is activated, it regulates the transcription of a large number of genes including stress response genes, antiapoptotic genes and genes involved in differentiation and proliferation. LMP-1 regulates the epidermal growth factor receptor (EGFR) in a NFκB-dependent manner. The activation of EGFR and the downstream mitogen activated protein kinase (MAPK) pathway leads to proliferation of cells. LMP-1 induced expression and activation of EGFR [59] result in the transactivation of the key regulators of the cell cycle including cyclin D1, cyclin E and E2F1. Another effect of the constitutive activation of NFκB by LMP-1 is the expression and activation of the human telomerase reverse

transcriptase (hTERT) [60], a catalytic subunit of the human telomerase. Expression of hTERT is upregulated in many malignant tumors and cancer cell lines and its activation is thought to be a prerequisite of immortalization since it elongates telomeres and hinders cells to progress into telomere length-controlled senescence. The expression and activation of hTERT by LMP-1 may lead to enhanced oncogenic potential.

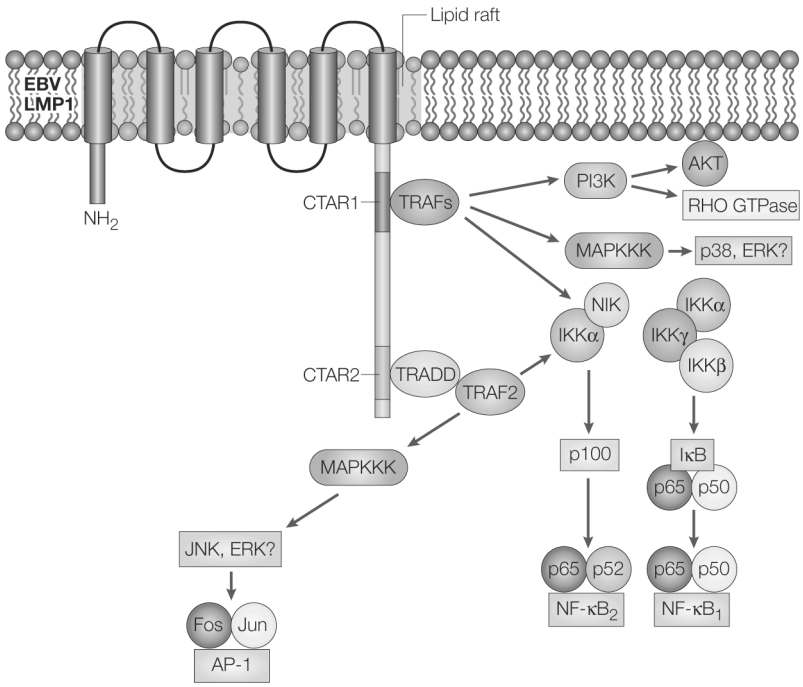

Figure 2: Different signalling pathways activated by LMP-1 [61].

Additionally LMP-1 has been shown to increase expression of the apoptosis inhibitor survivin in an NFκB and AP-1 dependent manner in NPC cells [62]. Other than survivin, LMP-1 upregulates the anti-apoptotic protein FLIP in B-lymphoma cells through NFκB signalling [63]. The upregulation of survivin and FLIP may lead to a protection against apoptosis in LMP-1 expressing cells. AP-1 is a transcription factor that activates a wide range of cell events including cell transformation, proliferation, differentiation and apoptosis. LMP-1 activates AP-1, which in turn regulates the expression of the cell cycle regulator p16 that may lead to aberrant cell proliferation and tumor development. The mentioned cross-talk between NFκB and AP-1 plays an important role in NPC tumorigenesis. Just recently it has been shown that NFκB signalling together with AP-1 and LMP-1 are required for cell transformation. In that study LMP-1 positive cells showed colony formation in soft agar only if NFκB and AP-1 were active. Furthermore the ability to metastasize was enhanced in LMP-1 positive NPC cells as shown in a trans-well migration assay [64].

LMP-2

There are two known variants of LMP-2, LMP-2A and LMP-2B, which differ in the first exon [65]. LMP-2A is not essential for transformation [66] and interferes with the B-cell receptor by mimicking a B-cell antigen through dominant negative effects of protein tyrosine kinases that are normally associated with the receptor [67]. LMP-2A is thereby thought to maintain the B-cells in a latent stage, since activation of the B-cell receptor is needed to activate the lytic promoting gene BZLF-1. When LMP-2A is expressed in transgenic mice, normal B-cell development is impaired. This suggests that LMP-2A induces proliferation and survival of B-cells [68]. The survival signal is provided by an active phosphatidylinositol 3-kinase (PI3K)/Akt pathway [69] and an active Ras pathway [70].

EBER-1 and EBER-2

The non-polyadenylated RNAs are the most abundant viral transcripts and therefore used as target molecules for detection of EBV in infected cells. Both EBERs are transcribed in equal rates, but the steady-state level of EBER-1 is 10 fold higher than EBER-2. The EBERs are considered as oncogenes, since tumorigenicity was shown in EBER expressing transgenic mice [71]. Furthermore EBER expression was accompanied by clonability in soft agar and resistance to apoptosis. EBERs are not essential for transformation, since recombinant EBV with deleted EBER genes are able to transform B-lymphocytes [72].

Micro-RNAs (miRNAs)

The miRNAs are 21-24nt long, non-coding RNAs that can post-transcriptionally downregulate the expression of mRNAs containing the complementary target sequence. A recent study describes 17 miRNAs in EBV infected cells [73]. The expression of the different miRNAs depends on cell type and viral gene expression. The miRNAs are organized into two clusters; one is located in the introns of the viral BART genes containing 14 miRNAs. The second cluster is located adjacent to the BHRF-1 and contains three miRNAs. The miRNAs of the BART cluster are highly expressed in latently infected epithelial cells, while almost absent in B-cells. In contrast the miRNAs of the BHRF-1 cluster are mostly prevalent in B-cells and expressed in low levels in epithelial cells. Each of the miRNAs appears to have very specific functions in different cell types.

MiRNAs are able to regulate latent gene expression and act antiapoptotic. Lo et al. showed that the BART cluster miRNAs suppress the expression of LMP-1 and modulate the downstream LMP activation of NFκB [74]. In that study LMP-1 expression was downregulated and NFκB was inactivated upon miRNAs expression. LMP-1 induces cell growth and reduces apoptosis, however overexpression of the latent membrane protein results in growth inhibition and sensitization to apoptosis. The negative regulation of LMP-1 by the miRNAs of the BART cluster may favour EBV associated cancer development by balancing growth advantages and disadvantages of LMP-1. Another study reveals the antiapoptotic function of miRNAs. The p53 upregulated modulator of apoptosis (PUMA) is downregulated by the miRNA-BART5 miRNA [75]. Depletion of miRNA-BART5 or expression of PUMA led to apoptosis upon treatment with proapoptotic agents in NPC cells.

1.1.3 EBV Associated Malignancies

Burkitt's Lymphoma

Burkitt's lymphoma (BL) is a B-cell lymphoma first described by the English surgeon Denis Burkitt in children of equatorial Africa [76]. Burkitt himself suggested that a vector-borne virus was responsible for that type of tumor [77] and Epstein, Achong and Barr identified herpesvirus-like particles by electron microscopy in a BL derived cell line [78]. Later BL was classified into three different forms, the endemic, sporadic and HIV associated form. The endemic form (eBL) is associated with EBV (over 95% of eBL cases are EBV positive) and is predominant in the African equatorial belt and other parts of the world where malaria is hyperendemic [79-82] (Figure 3).The incidence is 5-10/100,000 children between 6 and 8 years old. The sporadic form of Burkitt's lymphoma (sBL) occurs worldwide with an incidence of 0.1/100,000 humans of all ages. The HIV associated form occurs worldwide with a variable incidence. The tumor appears very early in the AIDS disease long before the manifestation of the full-blown clinical picture of AIDS. In all three forms of Burkitt's lymphoma the majority of patients are male with a 3:1 male-to-female ratio [83]. The consistent feature of BL is the activated c-myc gene resulting from the reciprocal translocation between the c-myc gene and one of the three immunoglobulin loci, namely IgH, Igκ or Igλ. The c-myc protein is the key regulator of proliferation, differentiation, apoptosis and cell growth control [84]. It drives cells through G1-phase and into S phase entry and is even able (if ectopically expressed) to drive quiescent cells into S phase without mitogens. Additionally c-myc activates protein synthesis and energy metabolism, reduces cell adhesion, stimulates angiogenesis, causes metastasis, and genomic instability [84-90]. Furthermore c-myc is able to regulate miRNAs that may to lead to tumorigenesis [91,92].

Nasopharyngeal Carcinoma (NPC)

NPC is a malignant neoplasm of the epithelial cells in the pharynx with a very high incidence in Southeast Asia, southern China and the arctic regions as well as a very low incidence in Europe. Genetic, environmental and virological factors enhance the incidence of NPC and may explain this uneven geographical and ethnic distribution of NPC. Virtually all undifferentiated cases of NPC are EBV positive and express the latency II phenotype [93].

Hodgkin's Lymphoma (HD)

HD is characterized by the presence of large atypical tumor cells, also called Reed-Sternberg cells. These cells represent less than 1% of the cells leaving all surrounding cells non-malignant. In Western Europe and North America 30-50% of HD patients are EBV positive. The tumor shows latency II expression pattern.

Gastric carcinomas

The pathogenic role of EBV in gastric carcinoma is still unclear, however EBV was detected in 5-25% of gastric adenocarcinomas [94-96]. EBV may enter the gastric epithelium without the use of a receptor. The latency pattern in gastric adenocarcinoma is different than the other EBV associated malignancies. BARF-1 transcripts, a homologue to the human colony-stimulating factor 1 receptor and the intracellular adhesion molecule 1, are expressed while LMP-1 is absent [97,98].

1.1.4 EBV Associated Lymphomas in Immunocompromised Individuals

Post-transplant Lymphoproliferative Disease (PTLD)

PTLDs are a group of B-cell lymphomas occurring in immunosuppressed patients following organ transplantation. The diseases are characterized by uncontrolled proliferation of infected B-lymphocytes. Most PTLDs result from reactivation of the virus due to transplantation or infection from the donor.

X-linked Lymphoproliferative Disorders

This group of disorders is characterized by fatal or full-blown infectious mononucleosis, B-cell lymphoma and dysgammaglobulinemia. EBV is believed to trigger this immune dysfunction leading to hepatic necrosis, bone marrow failure, uncontrolled cytotoxic T-cell response and cell death [99]. Defects on the long arm of the X chromosome in the gene SH2D1A/SAP are probably responsible for the decreased ability to control immune responses to viruses such as EBV [100].

AIDS-related Lymphoproliferative Disorders

These disorders include diffuse large cell lymphomas, immunoblastic lymphomas, Burkitt's lymphomas with an EBV positivity ranging from 30% to 90% [101]. The heterogeneous group of lymphomas arise in the presence of HIV-associated immunosuppression.

1.2 EBV AND TUMORIGENESIS

1.2.1 The Role of EBV in Tumorigenesis

EBV is considered a human tumor virus, however the molecular mechanism for tumorigenesis remains unclear. Epidemiological evidence link EBV to BL. Indeed almost all endemic BL cases carry the EBV episome. A study in Uganda showed that the virus titer in children that develop BL is particularly high [102]. In children with a low virus titer the incidence of BL is much lower. Furthermore EBV has a strong association to NPC. All undifferentiated cases of NPC and most differentiated cases in China and Southeast Asia, have been shown to carry the EBV genome. Epidemiologic studies in HD patients show that the risk to develop the disease tripled if the patient had mononucleosis earlier in life. Furthermore HD patients displayed higher EBV titers to EBV antigens than control subjects indicating that the patients were infected with the virus and the reactivation was initiated [103]. Obviously EBV is not the only factor inducing these malignancies, since a very high percentage of adults are infected with the virus, but only a very small subset develops the diseases. Environmental or genetic cofactors contribute to the risk of developing the mentioned diseases (Figure 3).

The oncogenic potential of the virus however remains a highly discussed issue. EBV is able to immortalize human B-lymphocytes *in vitro* by establishing continuous growing lymphoblastoid cell lines (LCLs). Freshly established LCLs are not tumorigenic, however after extensive culturing these cells reveal high levels of aneuploidy and tumorigenicity in mice [104]. Recent studies distinguish transformed LCLs from immortal LCLs by defining immortal LCLs as cell lines that have passed 180 population doublings (PD) [105]. The immortal LCLs demonstrate aneuploidy, genomic instability and high telomerase activity in contrast to transformed LCLs that do not reach 180 PD and do not feature numerical and structural chromosome aberrations as well as increased telomerase

activity [104,106,107]. This finding suggests that other factors such as cellular and environmental factors in cooperation with the EBV latent proteins play a key role in transformation. In immunocompromised patients, viral products drive the proliferation of cells in B-lymphoma. However, upon reconstitution of EBV specific immune responses, the highly aggressive tumors regress [108]. It seems that only in the absence of a functioning immune system, the virus is able to reveal its oncogenic potential.

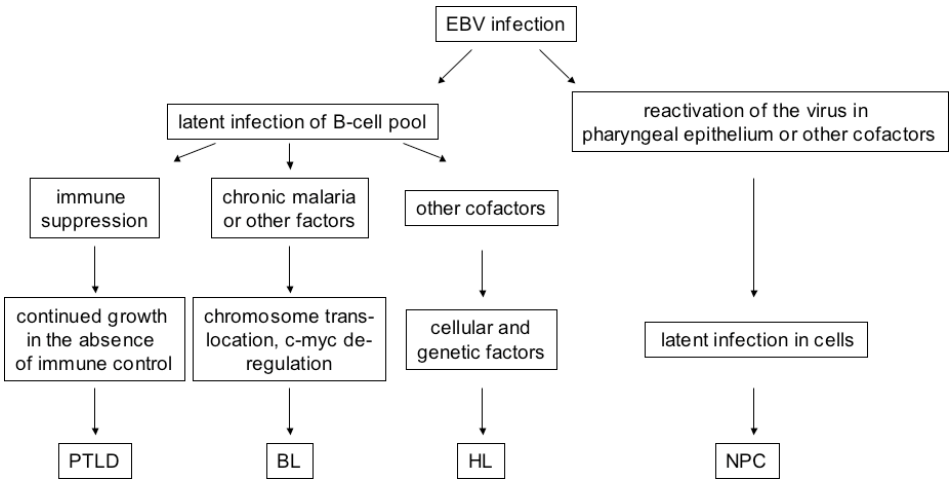

Figure 3: Events and cofactors during development of EBV associated malignancies.

Tumors like BL or HD arise in EBV positive and EBV negative variants suggesting that the virus is rather a promoting than initiating factor and other unknown factors are sufficient for the development of BL and HD in the absence of EBV. Though, several viral products have been shown to affect various aspects of malignant growth such as induction of proliferation, inhibition of apoptosis, deregulation of the cell cycle and induction of genomic instability. As stated before, LMP-1 and EBERs possess oncogenic potential by developing B-cell lymphomas in transgenic mice. LMP-1 activates a number of signalling pathways involved in cell proliferation and apoptosis. LMP-2A may induce proliferation and survival of B-cells. EBNA-1, LMP-1 and some miRNAs act antiapoptotic. EBNA-2 mimics the Notch signalling pathway affecting proliferation and apoptosis. EBNA-3C seems to be involved in cell cycle deregulation.

1.2.2 General Characteristics of Cancer Cells

The development of cancer is a complex multi-step mechanism that usually occurs over many decades. Many types of cancers in the human population occur in an age-dependent incidence rate suggesting several rate-limiting, stochastic events [109]. Intermediate steps in cancer formation have been observed in pathological samples in a number of organ sites indicating a process evolved from normalcy via a series of premalignant states into invasive cancers [110]. Furthermore transgenic mouse studies have described multiple rate-limiting steps in tumorigenesis [111]. During tumor

progression the cells have to manifest different alterations that dictate malignant cell growth. The expression of these alterations requires certain capabilities early and later in tumor development. Early acquired capabilities are self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis and limitless replicative potential. Late acquired capabilities are described as sustained angiogenesis, tissue invasion and metastasis. The order in which these early capabilities are acquired is variable across the spectrum of cancer and cancer subtypes. Moreover, in some tumors several early capabilities may arise simultaneously and thereby decrease the number of mutational steps required for tumorigenesis.

Self Sufficiency in Growth Signals

Mitogenic growth signals are required for the progress from a quiescent state to an active proliferating state in cells. The growth signals are transmitted into the cell by transmembrane receptors that bind to distinct classes of signalling molecules. In tumor cells, proto-oncogenes are mutated to oncogenes that mimic normal growth signalling. Tumor cells show a reduced dependence on exogenous growth stimulation indicating that these cells generate endogenous growth signals. The autonomy from exogenous growth signals is achieved by alteration of extracellular growth signals, transcellular transducers of those signals and intracellular circuits that translate those signals into action. The signals may influence cell behaviour leading from quiescence in normal tissue to entrance into the active cell cycle.

Insensitivity to Anti-growth Signals

Multiple antiproliferative signals control the proliferation during tissue homeostasis or cellular quiescence. At the molecular levels, the antiproliferative signals are transmitted by the pRb. The retinoblastoma protein is a tumor-suppressor protein and prevents excessive cell growth by regulating the cell cycle. It inhibits the progression from G1 to S phase if DNA is damage. The pRb protein is dysfunctional or absent in many human tumors, mainly by mutation of the gene or by functional inactivation of the protein. Disruption of the pRb pathway allows cell proliferation and renders the cell insensitive to anti-growth signals.

Evading Apoptosis

Apoptosis, programmed cell death, regulates the expansion of cell populations. Almost all types of cancers demonstrate resistance to apoptosis. Once the apoptotic program is started, the cellular membranes are disrupted, cytoplasmic and nuclear skeleton are broken down, the cytosol is extruded, the chromosomes are degraded and the nucleus is fragmented within 30-120 minutes. Finally the remaining cell corpse in engulfed by nearby cells. The well-studied tumor suppressor p53 elicits apoptosis. This tumor suppressor is mutated in almost 50% of all human cancers. The protein p53 arrests cell growth upon DNA damage and further induces apoptosis if the damage is not repairable. Elimination of p53 function is sufficient to inactivate the whole apoptotic machinery in many types of cancer cells [112]. In other tumor types the parts and subunits of the apoptotic machinery are inactivated or absent [113].

Limitless Replicative Potential

Deregulation of the replication program is expected to enable the generation of vast cell populations. However, it seems that the acquired replicative potential is not sufficient

to ensure expansive tumor growth. Every cell has a finite replicative potential [114], after a certain number of population doublings the cell enters a state of senescence and crisis. The number of doublings depends on the length of the telomeres. Telomeres are the regions at the end of the chromosomes protecting these from destruction. Normal telomere shortening in every cell division limits the cells to a fixed number of divisions and thereby constricts the lifespan of a cell. In almost 90% of human cancers the telomeres are elongated by the reactivated telomerase followed by replicative immortality. Telomerase is suppressed in most human cell types [115]. The remaining 10% of human cancers not expressing high levels of telomerase, elongate telomeres with a mechanism called "alternative lengthening of telomeres" [116].

Sustained Angiogenesis, Tissue Invasion and Metastasis

The oxygen and nutrition supply by the vasculature is crucial for cell function and survival. Solid tumors cannot grow greater than 2mm without acquiring access to the circulatory system [117]. Tumors are able to release angiogenic signals, attract and stimulate endothelial cells. Endothelial cells then construct capillaries connecting the tumor mass with the existing vasculature system of the host. The capillaries provide the required nutritients and oxygen for the tumor to grow further. During development of most human tumors, pioneer cells move out from the tumor tissue and invade adjacent tissues or travel to distant sites founding new colonies. These capabilities are mostly acquired later during tumor progression.

Genome Alterations as a Trigger

The mentioned capabilities are acquired mostly through changes in the genome of the cancer cells. However, random mutations of specific genes are very rare events and unlikely to happen within a human life span. The cancer incidence in the human population is rather frequent, suggesting that the genomes of tumors cells require increased mutability in order to progress to tumor formation [118]. The increased mutability may be directly caused by increased genome instability. The genome of mammalian cells, although the DNA is a very stable molecule itself, meets constantly destabilizing factors like normal replication and cell division but also intracellular and extracellular environmental stresses such as reactive oxygen species, chemical agents or background radiation. The cells have developed a number of defence mechanisms against these insults to maintain the genomic integrity, ensure fidelity of DNA replication, repair the damaged DNA and control the progression of the cell cycle. Indeed, several human tumors show a loss of genes responsible for the maintenance of the genome. Numerous genes ensure the stability of the genome such as genes involved in sensing and repairing damaged DNA, genes assuring correct chromosomal segregation during mitosis, gene encoding for proteins controlling cell cycle checkpoints and proteins involved in induction of apoptosis upon DNA damage. Genomic instability in these cells is associated with increased rate of mutagenesis, loss of cell cycle control, alteration in DNA repair processes and may manifest in altered gene expression and cellular changes.

Genomic instability is the precondition of increased mutability, which leads to acquisition of capabilities needed for the cell to develop a malignant phenotype.

As important as genetic alterations are epigenetic alterations. Epigenetic alterations are changes in phenotype or gene expression by mechanisms other than changes in the DNA sequence. The changes remain in the cell through cell divisions. The most studied epigenetic alteration is the methylation at CpG dinucleotide sites. Most CpG dinucleotide sites are located at proximal promoter regions. In normal cells these promoter regions are unmethylated, while in almost all tumor types, the hypermethylation of these regions leads to inappropriate transcriptional silencing of genes [119]. The loss of the gene function may provide an advantage for neoplastic cells. Hypermethylation of promoters from genes involved in genome integrity may lead to genomic instability.

1.3 GENOMIC INSTABILITY

The term genomic instability combines a group of events capable of causing unscheduled genomic alterations, either of a temporary or permanent nature. Genomic instability may be caused by endogenous or exogenous insults or accumulated due to impaired DNA damage response. Endogenous insults, that damage the DNA regularly and unavoidably, include deaminations, depurinations, DNA polymerase errors and reactive oxygen species (ROS). Further damage can be inflicted by exogenous insults from UV and gamma radiation. Once the DNA is damaged the cell possesses several mechanisms to sense the damage and then to halt the cell proliferation in order to repair the damage. If the damage cannot be repaired, the cell will undergo apoptosis. Thus genomic instability may be accumulated due to altered DNA damage response, deregulated cell cycle, the inefficiency of the cell to repair the damage or due to the inability to die by apoptosis upon unrepairable damage.

1.3.1 Types of Genomic Instability

Genomic instability includes instability at genome and chromosome level. The instability at genome level may lead to instability at chromosome level and vice versa. Instability at genome level is characterized as subtle sequence changes and gene amplifications. Subtle sequence changes include base substitutions, deletions and inversions of a few nucleotides. Missens mutation, a point mutation with a single-nucleotide change, occurs for example in the K-ras gene in 80% of pancreatic cancers [120]. Gene amplification can be detected by cytogenetic methods as double minutes or heterogeneously stained regions. Multiple copies of amplicons, including 0.5-10 megabases of DNA, may contain growth-promoting genes and thereby enhance tumorigenicity. Chromosomal instability (CIN) includes numerical and structural chromosome aberrations. Numerical chromosome aberrations, known as aneuploidy, describe losses or gains of whole chromosomes. Almost all human tumor types, including EBV associated malignancies, feature aneuploidy [121]. Once a chromosome is lost or gained, all the genes located on that chromosome will be expressed half or double, respectively. Normal cells counteract losses or gains with either reduplication of the remaining chromosome or by dosage-compensation as a result of numerous feedback loops that regulate most cellular pathways.

Structural chromosome aberrations comprise clonal and non-clonal chromosome aberrations. Clonal chromosome aberrations such as translocations, inversions, deletions, duplications of long stretches of chromosomes are propagated to the progeny, while non-clonal chromosome aberrations such as dicentric chromosomes, chromosome fragments,

gaps, satellite associations and ring chromosomes cannot proceed into the next cell cycle following metaphase and are newly induced in each cell cycle (Figure 4).

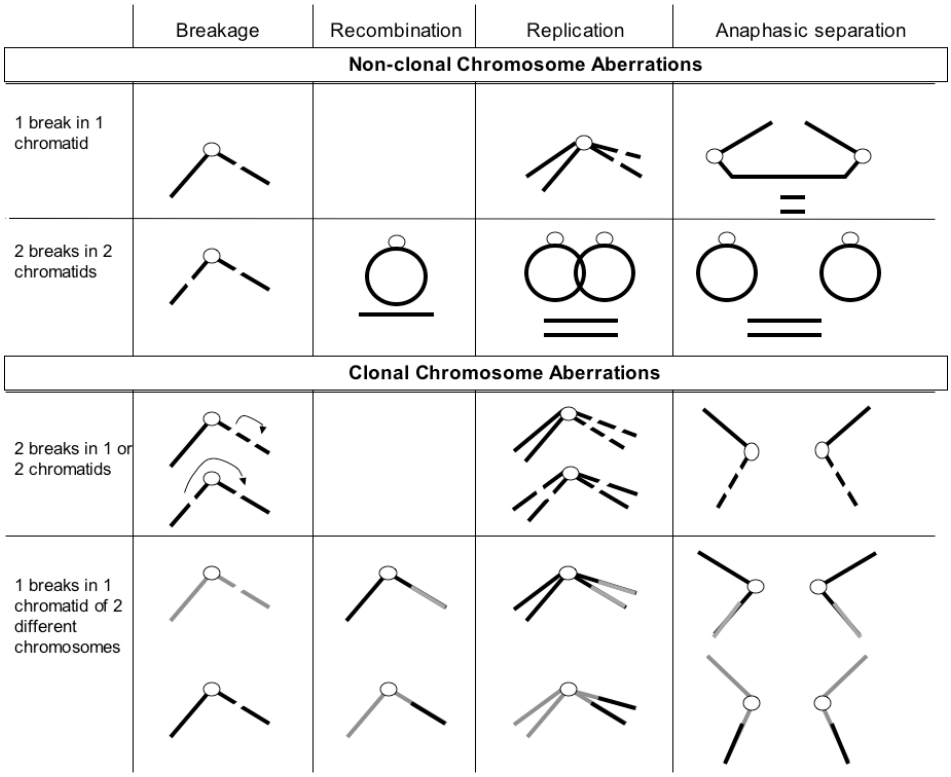

Figure 4: Origin of non-clonal and clonal structural chromosome aberrations and their evolution during the cell cycle.

Clonal chromosome aberrations have been described in EBV infected B-cell derived tumors [122]. These chromosome aberrations can be detected by cytogenetic methods. At the molecular level, translocations or inversions give rise to fusions between two different genes. The fused transcript may provide the cells with tumorigenic properties. Further gaining (duplications) or losing (deletions) parts of chromosomes may lead to inactivation of tumor-suppressor genes. If both alleles of tumor suppressor genes are mutationally inactivated, the cell acquires a growth advantage [123]. The loss of heterozygosity (LOH) occurs if one allele is lost due to mitotic recombination, chromosome loss or other structural changes and may be one mechanism explaining how tumor-suppressor genes are inactivated. Other than inactivation of tumor-suppressor genes, CIN may provide growth advantages to cancer cells by extensive changes in gene expression. However, despite these mechanisms, gross chromosomal changes are likely to influence the expression of genes, leading to increased cell proliferation or decreased cell death. The transformation of a LOH cell into a metastatic tumor is a very slow process driven by

sequential mutations in oncogenes and tumor suppressor genes leading to growth advantages and clonal expansion.

Non-clonal chromosome aberrations may be generated by DNA strand breaks or telomeric dysfunction. DNA breaks are dangerous lesions that can lead to mutations, neoplastic transformation or cell death. Mammalian cells possess potent and efficient mechanisms to repair DNA damage. However, when these repair mechanisms fail, DNA breaks may lead to tumor formation or progression. Proper telomere function prevents uncontrolled cell growth and therefore defines the proliferation potential of a cell. In cells that have lost proliferation control, length changes in telomeres are the main source of genomic instability allowing the cells to become immortal or to acquire tumor phenotypes. Telomere length independent instability, due to modifications of the telomere nucleoprotein complex, was shown to induce telomere uncapping and chromosome fusions [124]. Dicentric chromosomes are generated as chromosome fusions due to eroded telomeres or DNA double-strand breaks (DSBs) (Figure 4). Chromosome fragments indicate excised fragments from DSBs. Chromatid gaps are induced by DSBs at one chromatid. Ring chromosomes are formed if chromatids of the same chromosome bind to each other or if breaks at each chromatid occur. Satellite association emerge upon fusion of the satellite regions of chromosomes.

1.3.2 Sources of Genomic Instability

Endogenous Sources of Genomic Instability

Water attacks DNA leading to DNA damage by hydrolysis of the glycosylic bond, resulting in the formation of mutagenic abasic sites [125]. Non-repaired lesions lead to the incorporation of deoxyadenosine opposite the abasic site [126]. Depurinations, resulting in changes in the nucleotide sequence, occur with a much lower frequency than hydrolytic deaminations [127] (Figure 5).

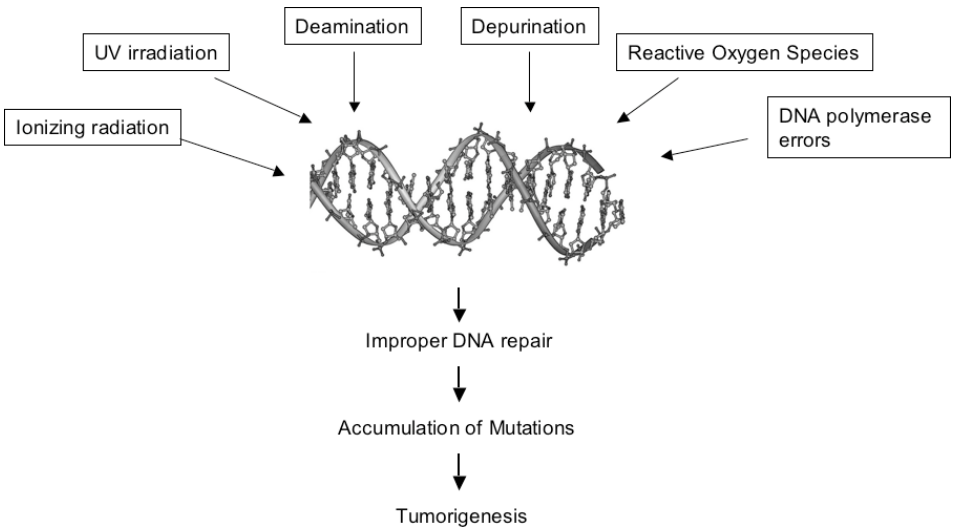

Figure 5: Sources of unavoidable DNA damage under physiological conditions (adapted from Ref [128]).

DNA polymerases work very efficiently with a very low error rate. However, misincorporations do exist and misincorporated nucleotides are excised by a exonucleolytic proofreading system during replication [129]. Mutations in DNA polymerases may lead to enhanced error rates.

Reactive oxygen species (ROS), including superoxides, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Figure 6), are a major endogenous source of DNA damage. In cells with aerobic metabolism, cytochrome oxidase catalyzes the metabolism of oxygen to water. Thereby some additional oxygen molecules are metabolized that possess enough energy to damage nearby macromolecules like DNA. ROS are generated by a number of normal cellular processes, such as respiration, cell injury, inflammation and phagocytosis [130]. The estimates of a daily exposure to ROS revealed 10^{12} molecules of oxygen with 200,000 damaged bases in each human cell per day. To cope with this exposure, cells have evolved multiple defence mechanisms including repair enzymes, detoxifying enzymes and ROS scavengers. Nevertheless, some ROS are able to escape these protective machineries. ROS generate single-strand breaks (SSB), DSBs, abasic sites and several nucleotide modifications [131]. If nucleotides are attacked by ROS, alterations of nucleotides such as 8-hydroxyguanine, 5-hydroxycytidine, thymidine and cytosine glycols are induced. The most common nucleotide modification is the 8-hydroxyguanine, also called 8-oxoguanine [132].

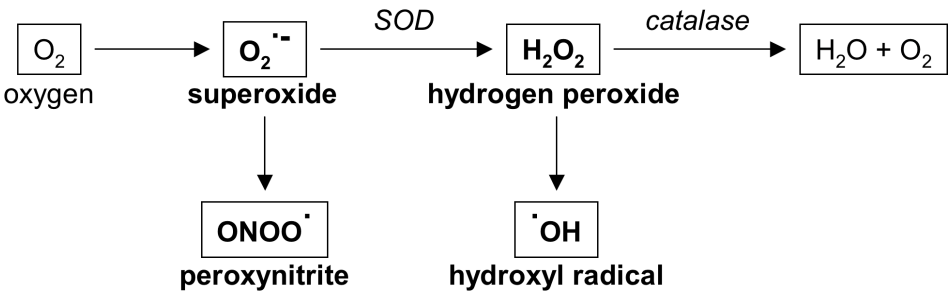

Figure 6: Formation of different kinds of ROS (in bold) and neutralization of ROS by the enzymes (in italic) catalase and superoxide dismutase (SOD).

Exogenous Sources of Genomic Instability

Ionizing radiation can be regarded as subatomic particles or electromagnetic waves energetic enough to detach electrons from atoms. Radiation produces a variety of lesions to DNA including rupture, alteration of bases, destruction of sugars, crosslinks and formation of dimers leading eventually to genomic instability. A correlation between the dose of radiation and the number of DSBs has been observed. SSBs are mostly caused by radiation-induced hydroxyl radicals. The genomic instability, induced by ionizing radiation, is transmitted over many generations to the progeny of surviving cells [133]. UV radiation has significant effects on the DNA of all living organisms. UV radiation is absorbed by cellular DNA and may severely damage the DNA. The two most common lesions caused by UV-B radiation are thymine and pyrimidine dimers. Both lesions distort the DNA helix leading to SSBs, which eventually contribute to the formation of DSBs after replication [134]. In addition to direct DNA damage, the short wavelength photons can be absorbed by chromophoric molecules leading to the formation of singlet oxygen or

free radicals which, in turn, destroy membranes, DNA and other cellular components [135].

1.3.3 DNA Damage Response (DDR)

Sensing DNA Damage

Different machineries depending on the type of damage sense the DNA damage. DSBs are sensed by the Mre11-Rad50-Nbs1 (MRN) complex. This complex is recruited to broken DNA molecules and activates the kinase ataxia telangiectasia, mutated (ATM) at the DSB [136]. Inactivated ATM exists as dimer that dissociates and autophosphorylates upon activation at DSBs [137]. ATM phosphorylates Nbs1 leading to activation of the MRN complex, which in turn enhances ATM kinase activity. Three other proteins join the double-strand sensing complex, namely BRCA1 and the mismatch proteins MSH2/6 and MLH2 [138]. ATM in conjunction with the MRN complex can activate the alternative kinase ataxia telangiectasia and Rad3 related (ATR) at the site of DSBs. ATR is recruited to the DNA damage together with ATRIP in a replication protein A (RPA) dependent manner [139]. ATR is also able to sense SSBs. Single-strand binding protein complex RPA binds and recruits ATR together with its regulatory subunit ATRIP to the DNA lesion [140].

Mediator proteins act directly downstream of ATM and ATR kinases (Figure 7). These proteins operate as substrates or recruit additional substrates. At the site of damage, the histone H2AX is phosphorylated on Ser139 by ATM or ATR [141] followed by recruitment of Mdc1, which in turn amplifies the phosphorylation of H2AX [142]. Several other mediators, including 53BP1, are recruited almost immediately after the damage to the lesions leading to the generation of damage-induced foci. Approximately 30 minutes after the damage, additional DDR proteins, such as Rad51 and Rad52, are recruited to the foci. ATM and ATR control downstream response by regulating the serine/threonine kinases Chk1 and Chk2. These kinases are structurally unrelated but share overlapping substrate specificity. Chk2 is mainly phosphorylated by ATM, leading to a p53-dependent G1 arrest and p53-independent G2 arrest. Both ATM and Chk2 phosphorylate p53 synergistically to ensure p53 activation only when the pathway is fully active. Chk1 is highly phosphorylated in response to hydroxyurea and UV radiation and moderately phosphorylated upon gamma irradiation. The kinases Chk2 and Chk1 target several effector proteins that function in cell cycle, DNA repair and cell death machineries. Since the ATM-Chk2 and ATR-Chk1 modules share many substrates among checkpoint effectors proteins [143], a crosstalk between the two pathways is possible. Both modules trigger cell cycle arrests in G1 or G2 before entry into S phase or mitosis respectively, if the DNA was exposed to damage. Cell cycle checkpoints are control mechanisms present in each cell cycle phase to ensure the fidelity of cell division. These checkpoints verify whether all processes have been accurately completed in each phase before progression into the next phase. Upon DNA damage the DDR pathways activate cell cycle checkpoints to allow the cell to repair the damage before progressing into the next cell cycle phase.

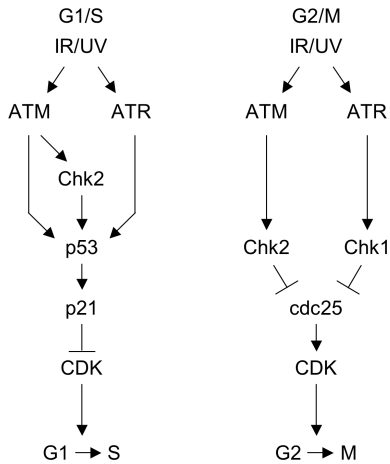

Figure 7: Scheme of ATM/ATR modules.

Regulation of Cell Cycle Checkpoints

Dividing cells have evolved several control mechanisms to ensure the fidelity of cell division. These control mechanisms are cell cycle checkpoints that verify whether the processes at each pahse of the cell cycle have been accurately completed before progression into the next phase (Figure 8). In G1 phase the retinoblastoma protein pRb binds to E2F-transcription factors. Cdk4/6 and Cdk2 phosphorylate pRb, E2F transcription factors are released and the transcription of S phase promoting genes is activated. If DNA damage happens in G1 phase, ATM/ATR and Chk1/Chk2 are activated and phosphorylate MDM2, which normally binds to p53 and ensures rapid turnover of p53 [144]. The phosphorylation of MDM2 leads to the stabilization and activation of p53, which in turn upregulates p21. The cyclin-dependent kinase inhibitor p21 silences cyclin E/Cdk2 kinase and thereby causes a G1 arrest leading to the inability to initiate DNA synthesis and to a preservation of the already active pRb/E2F pathway in its growth-suppressing mode. The G1 checkpoint control is most commonly deregulated in human cancer [145,146]. An alternative pathway is able to block the cells in late G1 upon DNA damage. Irradiation-activated ATM and Chk2 lead to the phosphorylation and destruction of Cdc25a. Subsequently DNA synthesis is inhibited [147].

Upon DNA damage in S phase, the DNA replication origins are inhibited while ongoing DNA replication forks are not affected [148]. This intra-S phase checkpoint is triggered by two different pathways. An ATM-independent pathway involves the inhibition of Cdk2 activity after genotoxic insults, followed by the blockage of cdc45 onto chromatin. Cdc45 is required to recruit DNA polymerase alpha into the re-replication complex. Inhibiting Cdk2 activity prevents thereby initiation of new origin firing [149,150]. The second pathway involves the activation of ATM by genotoxic insults. It has been shown that an ATM-dependent phosphorylation of Nbs1 is required for the S phase checkpoint [151]. To fully block the cells in S phase, Nbs1 needs to build a complex together with Mre11, Rad50 and BRCA1 [152].

The G2 (or G2/M) checkpoint prevents cells from initiating mitosis when the cell experience genotoxic insults in G2 phase. Upon DNA damage and activation of

ATM/ATR, the kinases Chk2/Chk1 phosphorylate and translocate Cdc25c to the cytoplasm leading to a dephosphorylation of Cdc2 [153]. Phosphorylated Cdc2 usually forms a complex with cyclin B1 that triggers progression from G2 phase [154]. Tumors with mutant p53 (such as Burkitt's lymphoma) selectively accumulate in G2 after DNA damage, which indicates that p53-independent mechanisms are sufficient to sustain the G2 checkpoint.

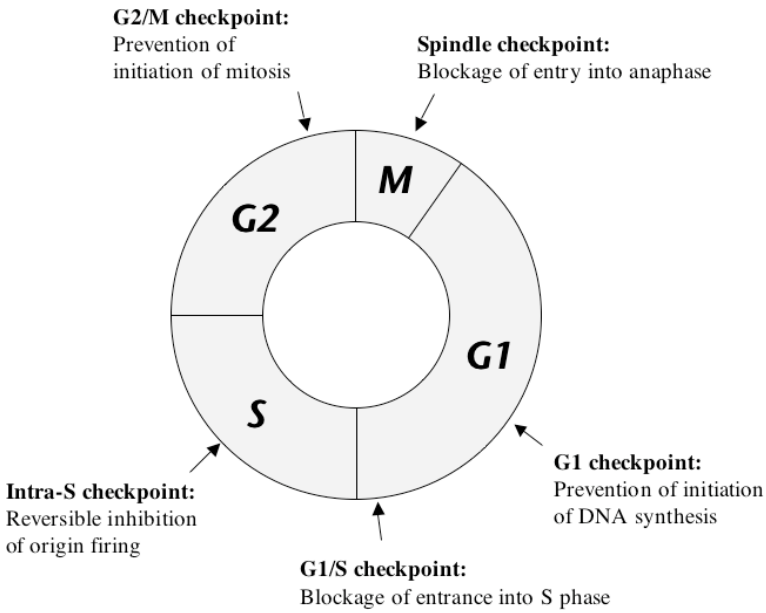

Figure 8: Cell cycle checkpoints.

Finally, spindle checkpoint blocks entry of cells into anaphase until all chromosomes are properly attached to the mitotic spindle. The kinetochore on each sister chromatid must be correctly attached to the opposite spindle pole to ensure that each daughter cell receives one copy of the chromosomes. The spindle checkpoint is activated by unattached or not properly attached kinetochors and missing tension between the pole and the kinetochore. Once all kinetochors are properly attached to the poles, Aurora B proteins localize to the kinetochore recruiting a whole complex of proteins, namely Bub1, BubR1, Bub3, CENP-E and Mad2 [155]. Mad2, BubR1 and Bub3 form the mitotic checkpoint complex. This complex binds to and inhibits the function of cdc20 leading to an inactivation of the anaphase-promoting complex (APC/C). Once all kinetochors are properly attached, the mitotic checkpoint complex dissociates from cdc20 and activates the APC/C. The APC/C, comprising an E3 ubiquitin ligase complex, joins polyubiquitin chains to the securin protein and targets it for degradation [156]. Securin inhibits the protease activity of separase. Once securin is degraded, separase cleaves the Scc1 subunit of cohesin [157] and thereby resolves the linkage between the sister chromatids.

1.3.4 DNA Repair Pathways

Efficient DNA repair mechanisms are essential to all living organisms, since DNA damage is predominant in all cells. Only a proper DNA repair involving removing and replacing damaged bases and nucleotides protects the cells against accumulation of multiple lesions, genome degradation and loss of vital genetic information. DNA lesions include SSBs, DSBs, inter- and intra-strand cross links and modified bases. For each of these lesions one or more DNA repair pathways are active within a cell (Figure 9). Three pathways overlap in function, the mismatch repair (MMR), base excision repair (BER) and the nucleotide excision repair (NER) which operate to repair aberrant bases or nucleotides from one strand of the double-helix, using the other strand as template for the new DNA synthesis. Three other pathways repair specifically DSBs, namely non-homologous end joining (NHEJ), homologous recombination repair (HRR) and microhomology-mediated end joining (MMEJ).

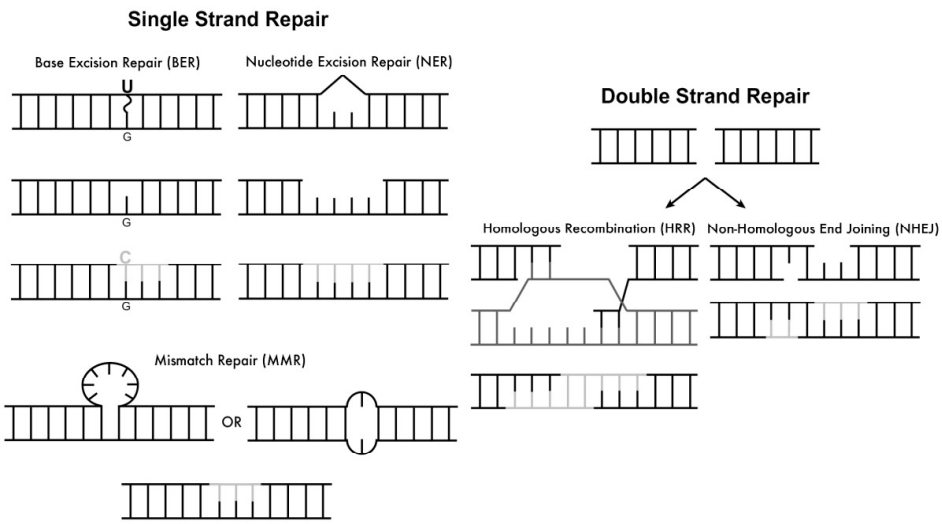

Figure 9: Comparison of the different DNA repair pathways [158]; the grey DNA stretches indicate newly synthesised DNA.

Base Excision Repair

BER involves only a small number of proteins and is considered to be the least complex DNA repair pathway. A damaged base is recognized and removed by a DNA glycosylase through the cleavage of the bond between the base and the deoxyribose. A non-specific apurinic/apyrimidinic endonuclease eliminates the remaining deoxyribose creating a gap. The gap is filled by DNA polymerases.

Nucleotide Excision Repair

Bulky lesions such as inter- and intra-strand crosslinks are removed by NER. After DNA damage recognition, the DNA is unwound and incisions are made on either side of

the aberrant base where a total of 15 to 30 nucleotides are removed. DNA polymerases fill the gap and DNA ligases seal it. For completion of the process, five multi-protein complexes, nucleotide excision repair factors 1 to 4 and the replication protein A complex are recruited.

Mismatch Repair

Single unpaired nucleotides, insertion and deletion loops are recognized by the mismatch repair machinery. The exonuclease Exo1 excises the mismatched base, a DNA polymerase fills the gap and DNA ligase seals the gap.

Homologous Recombination Repair

The repair of DSBs by HRR corresponds to an exchange of identical sequences between the DNA molecule carrying the DSB and the intact DNA molecule. HRR is considered very faithful if the DNA template used for repair is identical to the sequence at the break. In case the DNA template is not identical, the repair of DSB may lead to local mutations, deletions or gene rearrangements. HRR involves several proteins including sensing proteins, DNA nucleases, helicases, topoisomerases, polymerases and ligases. HRR is divided into three steps: 1) DNA damage sensing by Mre11, Rad50 and Nbs1 initiates resection of the 5'-ended DNA strand at the break ends. 2) Rad51 replaces RPA at the 3' single-strand DNA and invades into the homologous DNA duplex followed by a strand exchange. The extended strand than dissociates and anneals with the processed end of the non-invading strand on the opposite site of the strand. The remaining gaps are filled by DNA polymerases and DNA ligases. The last step involves a strand resolution of the recombination intermediates.

Non-homologous End Joining

NHEJ acts predominantly in G1 and early S phase. This pathway ligates the two broken chromosome ends together. NHEJ is considered as most effective DSB repair pathway, since it has the ability to ligate any kind of DSB ends without requirement of a homologous sequence. NHEJ has for a long time been considered as error-prone mechanism generating small insertions and deletions, but later it was observed that these lesions are derived from the ligation of blunt DNA ends [159]. If fully compatible DNA ends are ligated, the error rate of NHEJ is very small. Ku complexes, comprising of Ku70 and Ku80 bind to the DSB ends shortly after DSB formation. Ku70 and Ku80 form a heterodimeric complex essential to NHEJ. The DNA-dependent protein kinase (DNA-PK) with its catalytic subunit DNA-PKcs is recruited to the Ku complex. DNA-PK bridges the ends of the broken DNA and allows DNA ligase 4 and its cofactor XRCC4 to bind to the DNA [160]. The ligation may be prevented if terminal nucleotides are damaged or modified and mismatch correction or gap filling is required. In that case the polynucleotide kinase (PNK) is recruited through its interaction with XRCC4 to the DNA to correct the modification [161]. After pairing of partial complementary overhangs or nuclease processing, DNA polymerases are mobilized to the DSB to fill some single-stranded gaps.

Microhomology-mediated End Joining

MMEJ is a Ku-independent end-joining repair mechanism in the absence of the core NHEJ factors. MMEJ requires extensive resection of DSB ends and therefore implies

much larger sequence deletions [162]. It can be assumed that this pathway is used, whenever HRR and NHEJ are not available.

1.4 EBV AND GENOMIC INSTABILITY

1.4.1 Chromosomal Instability in EBV Associated Malignancies

Established lymphoblastoid cell lines do not show the BL typical translocation of the c-myc gene but rather random karyotypic abnormalities. Aneuploidy and karyotype changes have been described as a common feature after immortalization of B-cell with EBV [104,163,164]. The aneuploidy most likely originates from disrupture of the mitotic spindle apparatus, which then leads to chromosomal imbalances allowing for further mutations in critical genes.

Karyotypic abnormalities were frequently described in BL and other EBV associated malignancies. The second most frequent cytogenetic lesion, beside the c-myc translocation, is chromosome one abnormality. A recurrent change in endemic and AIDS-related BL is the partial duplication of the long arm of chromosome one, involving variable bands but consistently including 1q23 [165,166]. Furthermore copy number gains were described in EBV positive gastric carcinomas and lung lymphepithelioma-like carcinomas [167]. Additionally to karyotype changes and aneuploidy, strong telomerase activity and micronuclei formation have been observed in EBV associated malignancies [168]. Integration of the EBV genome into the host genome leads to achromatic gaps causing fragile sites prone to break and eventually enhance chromosome instability [169].

Latent and lytic EBV proteins have been described as associated with genomic instability. LMP-1, annotated as oncogene, has been shown to negatively influence the tumor suppressor gene RASSF1A, disrupt microtubule structures and thereby induce chromosomal aberrations in human epithelial cells [170]. A very recent study described increased appearance of micronuclei and disrupture of the spindle checkpoint in EBV associated B-lymphomas [171].

1.4.2 Deregulated DNA Response

Sensing the DNA damage

It does not seem that EBV impairs the DNA sensing machinery, however the virus uses the machinery for its own purposes. The single-strand binding protein RPA for example, interacts with EBNA-1 both in solution and when EBNA-1 is bound to the EBV origin. This interaction might be advantageous for the virus to activate the latent origin of replication by recruiting host factors to stabilize the EBV-chromosome complex [172].

Checkpoint Responses

EBV products have been shown to alter several major contributors of the cell cycle checkpoint control. EBNA-3C for example has been shown to mediate the degradation of pRb through a cellular ubiquitin ligase [49]. Degraded pRb is released from E2F-transcription factors and the transition from G1 to S phase is ensured. EBNA-3C expression in cells may lead to an enhanced G1/S transition. Another latent protein, LMP-1, also promotes G1/S transition by overexpression of cyclin D1 and subsequent phosphorylation of pRb [173].

Additionally to the deregulation of the G1 checkpoint, EBV products inhibit the G2 checkpoint. One study shows that EBV negative BL cell lines are arrested in G2 after

treatment with the DSB inducing agents cisplatin and etoposide, while cells infected with EBV fail to arrest in G2 and either undergo apoptosis or proliferate [174,175]. These results allow the hypothesis, that once DNA damage occurs in EBV positive cells the checkpoint machinery may not activate the G2 checkpoint to arrest the cells and repair the damage. Instead the cells overcome G2 phase and enter M phase accumulating all acquired DNA damage during the following generations. Expression of LMP-1 using tetracycline regulated vectors showed an accumulation of cells in G2/M phase [176]. Although no mechanism for this phenomenon has been described yet, LMP-1 may control the proliferation of EBV-infected cells by regulating the progress through G2/M phase.

EBV latent antigens have been shown to deregulate the spindle checkpoint. Cells treated with the spindle poison nocodazole enter mitosis but cannot form metaphase spindles because microtubules cannot polymerize. EBV infected cells overcome the spindle checkpoint after nocodazole treatment [177] and this phenomenon was attributed to the expression of EBNA-3C [178].

DNA Repair

LMP-1 induces the accumulation of micronuclei and represses DNA repair [179]. Further, it was shown that the repression of DNA repair occurs in a p53-dependent manner [180]. The DNA repair is repressed by a LMP-1 mediated phosphorylation and translocation of FOXO3 [181]. The transcription factor FOXO3 is a downstream target of the PI3K/Akt pathway and is active in its unphosphorylated form in the nucleus. Once FOXO3 is translocated to the cytoplasm it loses its activity.

2 AIMS

The aim of the work presented in this thesis was to investigate the ability of the Epstein-Barr virus to induce genomic instability.

The specific aims were to:

- Investigate the presence of chromosomal instability and DNA damage response in EBV positive Burkitt's lymphoma cell lines compared to EBV negative Burkitt's lymphoma cell lines.
- Examine which latent viral protein(s) are responsible for the induction of genomic instability.
- Elucidate the mechanism by which the different latent viral proteins induce genomic instability.

3 METHODOLOGICAL CONSIDERATIONS

This section describes the methodological considerations of crucial experiments. The reason for the choice of some methods and the discard of others is highlighted. A detailed description is not given here, as this is presented in the appended papers.

3.1 DETECTION OF DNA DAMAGE

The first aim was to investigate chromosomal instability in EBV positive cell lines. Chromosomal instability has been demonstrated in EBV associated malignancies using cytogenetic methods [121,122]. Methods like spectral karyotyping (SKY) or comparative genome hybridization (CGH) visualize aneuploidy, clonal chromosome aberrations, gene amplification and losses of DNA sequences. The SKY techniques utilize fluorescently labelled probes for each chromosome, showing single chromosomes in a characteristic colour. Using SKY techniques, every chromosome is marked with different mixtures of fluorochromes, thus exposing deletions, translocations and inversions. CGH is based on co-hybridization of differentially labelled tumor and normal DNA to human metaphase chromosomes allowing the detection of DNA sequence copy number changes throughout the genome. These methods are limited in sample size; usually only 20 to 30 metaphase plates can be processed. Several publications use the micronucleus assay or the multinucleus assay to describe chromosomal instability in EBV associated malignancies [168,179]. Interphase nuclei are stained with 4,6-diamidino-2-phenylindole (DAPI) and broken or detached chromosomes are visualized by dots outside the nuclei or several connected nuclei. In our experience these methods are both qualitatively and quantitatively inferior to our methodology. The results varied substantially depending on the experimentator and were not consistently reproducible. Therefore these methods were not employed.

A simple reproducible method was necessary to efficiency test numerous metaphase plates for the induction of non-clonal chromosome aberrations such as dicentric chromosomes, chromosome fragments, chromatid gaps, double minutes, satellite associations as well as chromosome rings. These aberrations are easily recognizable in a conventional metaphase spreads. Therefore cells were blocked in metaphase, dropped on glass slides, fixed with appropriate fixing solution and DNA was stained with DAPI. With this method it is possible to process relatively fast 100 metaphase plates and analyse them later using imaging software (Paper I, II and III). The observed structural chromosome aberrations are not propagated, instead these aberrations are newly induced in each cell cycle suggesting that DNA damage is produced continuously in the cells. A standard method to examine the presence of DNA damage, or more specific DNA strand breaks, is to stain for the histone H2AX. That histone is phosphorylated (pH2AX) at the site of the break (Paper I, II and III). The more intense the signal, the more DNA strand breaks are present in the cell. An alternative method to demonstrate the DNA strand breaks, the comet assay, detects DNA fragmentation. The cells are placed into a layer of agarose and the DNA is unwound. If the cell carries DNA strand breaks, the DNA pieces are transported by electric force resulting in a comet-like shape (Paper II and III). The DNA is thereafter stained with DAPI. The size of the comets are analysed using specific software. The size of the comets is thereby directly proportional to the DNA damage in the cell.

3.2 MEASUREMENT OF REACTIVE OXYGEN SPECIES

Once the DNA damage was documented, the mechanism of induction was investigated. First endogenous insults leading to the induction of DNA damage were tested. A standard method for detection of reactive oxygen species (ROS) utilizes a colourless probe that turns fluorescent upon oxidation of endogenous ROS [182]. 2,7-dichlorodihydrofluorescein diacetate (DCFDA) is a lipid-soluble probe that can detect the intracellular ROS formation of various types, including hydroxyl, peroxyl, alkoxyl, and nitroxyl free radicals, as well as peroxynitrite. Subsequently the next more challenging question was, how these ROS were induced. To answer that question, a bioinformatical approach was applied. Cellular gene expression data were extracted from a reverse engineering study of the cellular network including gene expression profiles from 336 human B-cell phenotypes [183]. Transcriptome data from a subset of 24 samples, comprising EBV positive and EBV negative samples, were extracted. Genes involved in ROS metabolism were selected using the Gene Ontology database categorising 134 genes capable of generating or removing ROS. The expression profile of these 134 genes were investigated in 24 samples. Tested gene levels using PCR techniques and protein levels using western blotting techniques confirmed the obtained data [184,185].

3.3 DETECTION OF DNA DAMAGE CAPACITY

DNA damage was not induced by endogenous insults in all cells suggesting that other mechanisms may contribute to the accumulation of DNA damage. One possible reason might be impaired DNA repair capacity. Unavoidable unrepaired DNA damage accumulated in the cell, might lead to the observed DNA damage. Several methods were considered to assess DNA repair capacity: 1) tests based on DNA damage induced by chemicals or physical agents; 2) indirect tests of DNA repair such as unscheduled DNA synthesis; 3) tests based on more direct measures of repair kinetics; 4) test to measure genetic variation associated with DNA repair [186]. In the first category the cells are treated with DNA damage inducing agents, placed back in cell culture condition and are allowed to repair the induced damage. Samples are taken at indicated time points. The remaining DNA damage at these time points are compared to the DNA damage at time zero. The second category measures DNA repair synthesis after excision and removal of a stretch of DNA following induction of DNA damage. The test is based on incorporation of tritium labelled thymidine into the DNA. The third category is the most direct test to measure DNA repair capacity. Using this method irradiated luciferase plasmids are transfected into the cells and the capacity of the cells to repair the plasmids is measured through the amount of luciferase luminescence restored after a certain incubation time. Finally the fourth category to measure DNA repair capacity is to determine microsatellite polymorphisms.

In the present study the first category was used. The cells were treated with gamma radiation or etoposide, sampled at the indicated times and the remaining DNA damage was determined by staining for phosphorylated H2AX (Paper I) or measuring comet size (Paper III). An additional method to show DNA repair capacity was used, since different cells may show a different sensitivity to the DNA damaging agents, independent from the DNA repair capacity. The second category was discarded as being regarded too indirect. The fourth category was deemed unsuitable as it measures genetic variations in single

chromosomes but general effects are of interest here. The most direct test was used. Luciferase-encoding plasmids are treated with gamma or UV radiation and transfected into the cells. After 24h the cells are harvested and the luciferase luminescence signal is measured. The signals from irradiated plasmids are related to non-irradiated plasmids; low ratios suggested decreased DNA repair capacity (Paper III).

3.4 CHARACTERIZATION OF THE ACTIVITY OF CELL CYCLE CHECKPOINTS

DNA damage accumulation may occur due to defective DNA damage response. Normal cells react to DNA damage with cell cycle arrest to permit DNA repair at the sites of DNA damage. Not all stable transfected cell lines showed induced genomic instability or deficiency in DNA repair. Therefore the activity of cell cycle checkpoints was assessed. Since our model, BL cell lines, possess mutated p53 and therefore the G1 checkpoint is inactivated, this specific checkpoint was ignored. The G2 checkpoint and the spindle checkpoint were activated upon gamma irradiation and nocodazole treatment respectively. Different cell cycle phases were detected with DNA staining using propidium iodide. Fluorescence activated cell sorter (FACS) profiles reveal in a normal cell cycle distribution two peaks. One peak showing the DNA content at 1n, characterizing the cells in G1, and a second peak with a DNA content of 2n, characterizing cells in G2 or M. Using this method, cells in G2 and in M phase cannot be distinguished. Since the G1 checkpoint in these cells is inactivated and only a small number of cells reside in G1 and S phase, almost all parental cells are prevalent in G2 phase after gamma irradiation. Nocodazole treatment hinders the polymerization of microtubules leading to activation of the spindle checkpoint and arrest in M phase. Indeed parental cells were found mainly in G2/M phase. In some cells two peaks, one in the G1 fraction and one in the G2/M fraction, were present after treatment suggesting that either some cells have progressed to G1 from M phase or these cells G1 phase never progressed to S and G2 phase. If the cells have progressed from M to G1, cell division must have taken place at least once, while in the other scenario the cells did not divide. The probe, carboxyfluorescein diacetate succinyl ester (CFSE), enters the cells and is converted by intracellular esterases to a membrane impermeable, fluorescent molecule that is not transferred to the adjacent cell. During each round of cell division, the relative fluorescence intensity of the dye is reduced by 50%. Using this technique it is possible to detect, whether the cells divided after treatment with gamma radiation and nocodazole (Paper III).

4 RESULTS AND DISCUSSION

4.1 EBV INDUCES CHROMOSOME ABERRATIONS IN BL CELL LINES

Epidemiological and molecular studies have suggested a role of EBV in the pathogenesis of a number of EBV associated malignancies of lymphoid and epithelial cell origin, but the mechanism by which EBV contributes to tumorigenesis remains largely unknown. Genetic alterations such as aneuploidy and clonal chromosome aberrations have been described in EBV associated malignancies. Genomic instability is a common feature of cancer cells and is believed to represent an early step in the progress of tumorigenesis.

Through a systematic approach, genomic instability was measured by counting abnormal metaphases containing non-clonal chromosome aberrations such as dicentric chromosomes, chromosome fragments, chromatid gaps, double minutes, satellite association and chromosome rings. Around 3500 metaphase spreads of EBV positive (EBV+) and EBV negative (EBV–) cell lines were investigated (Paper I). Significantly more abnormal metaphase plates in EBV+ cell lines compared to EBV– cell lines were observed. This effect was reproduced in EBV+ and EBV– cells within the same genetic background through *in vitro* infection of negative Burkitt's lymphoma cell lines. Loss of the viral genome in Akata (–) and Oma cl.4 from the original cell line containing the viral genome (Akata (+) and Oma cl.6) led to reversion of the phenotype with less abnormal metaphase plates. Reintroduction of the viral genome into the Akata (–) cell line caused a restoration of the phenotype observed by increased abnormal metaphase plates. These results suggest that EBV plays a causal role in the induction of chromosomal instability in BL cells. The induction of chromosomal aberrations by EBV has been reproduced recently in a non-tumor background. Lacoste et al. observed increased levels of dicentric chromosomes and fragments in fresh B-cells four weeks after infection with EBV (personal communication).

Dicentric chromosomes, chromosome fragments and chromatid gaps were most frequent in the metaphases, whereas double minutes, satellite associations and rings were rare events suggesting a prevalence of chromosome breaks, telomere dysfunction or defects in DNA repair in these cells (Paper I). Telomere dysfunction was investigated by measuring telomere length using fluorescent in situ hybridization (FISH). The number of telomere signals at the end of chromosomes and at the fusion sites of dicentric chromosomes was tested. EBV+ cells showed more frequent abnormal numbers of telomeres (more or less than four telomeres) indicating that EBV induces DNA strand breaks or inappropriate repair at telomere sites. The numbers of telomere signals in the fusion site of dicentric chromosomes were counted to elucidate the mechanism of the development of dicentric chromosomes. Dicentric chromosomes with less than four telomere signals at the fusion site may be generated by non-homologous end joining of DSBs [187] or by the fusion of eroded or broken telomeres [188].

Broken chromosome ends are sticky and eventually bind to other broken chromosomes or intact chromosomes generating dicentric chromosomes without telomere signals or two telomere signals respectively. Joining of eroded telomeres may lead to four telomere signals at the fusion site. While in EBV– BL cells, almost all dicentric chromosomes demonstrated four telomere signals at the fusion site, EBV+ BL cell lines

had an even distribution of none, two or four signals at the fusion site of dicentric chromosomes suggesting different mechanisms to be responsible for the formation of dicentric chromosomes in EBV+ cells and EBV– cells (Paper I). Shortening of telomeres has been shown to induce the formation of dicentric chromosomes. Surprisingly our study showed a significant increase in telomere length in EBV+ cell lines compared to EBV– cell lines. The contribution of this abnormality to the observed phenotype is not clear.

The occurrence of dicentric chromosomes and chromosome fragments may result from DNA breakage. Consequently, the DNA strand breaks in BL cells were investigated. Fixed cells were stained for pH2AX. The phosphorylation of this variant of H2AX occurs in chromosome regions adjacent to DNA breaks. The detection of high levels of pH2AX in EBV+ BL cell lines indicates the presence of DNA strand breaks. The phosphorylation of H2AX was reverted upon viral genome loss in the Akata (–) cell line and restored upon re-introduction of EBV in the Akata (JSWT) cell line pointing to EBV as a cause of the damage. The response to damage is intact in these cells, since all cell lines demonstrate a phosphorylation of H2AX after treatment with the DNA damaging agent etoposide (Paper I).

The random chromosome aberrations described in BL cells have been attributed to the over-expression of c-myc and its capacity to induce genomic instability [189-191]. The DNA damage in the absence of c-myc was tested using the P493-6 cell line. This cell line carries a recombinant EBV with an estrogen-driven EBNA-2 and tetracycline-inducible c-myc gene. Culturing of this cell line with estrogen and tetracycline induces a latency III gene expression program (LCL expression pattern), whereas withdrawal of these compounds leads to latency I like expression pattern (BL expression pattern). Cells cultured in LCL condition demonstrated phosphorylated H2AX, however upon c-myc overexpression in BL-line condition, the phosphorylation is increased suggesting that expression of latent proteins without c-myc deregulation is sufficient to induce DNA damage. However, the DNA damage response is enhanced upon overexpression of c-myc.

4.2 EBNA-1 PROMOTES GENOMIC INSTABILITY VIA THE INDUCTION OF ROS

The tested EBV+ cell lines were categorized in latency I and latency III expressing cell lines. A significant increase in abnormal metaphase plates was observed in latency I expressing cells compared to EBV– cell lines. However, an even further increase was detected in cell lines expressing latency III suggesting that more than one protein is involved in the induction of the phenotype (Paper I). EBNA-1 is the only protein expressed in latency I cell lines and may be responsible for the induction of DNA damage. Therefore cells expressing EBNA-1 constitutive or by withdrawal of tetracycline in the tet-off regulated EBNA-1 cell line, were tested for the induction of abnormal metaphase plates. In stable transfected cell lines significant more abnormal metaphase plates were observed compared to the EBNA-1 negative parental cell line. A similar increase in the number of abnormal metaphase plates was observed after one week of EBNA-1 expression in the tet-off regulated EBNA-1 cell line. An alternative way to examine DSBs and DNA fragmentation was used by measuring comet length in the neutral comet assay. The comet length is directly proportional to the amount of DSBs. EBNA-1 expressing cells showed an increase in comet length compared to the parental cell line. Addition or removal of tetracycline in the tet-off regulated EBNA-1 cell line confirmed that the

amount of EBNA-1 is directly proportional to the comet length. EBNA-1 induced damage is accompanied by the phosphorylation of the histone H2AX and its kinase ATM.

The observed damage in EBNA-1 expressing cells may be induced either by endogenous stress such as ROS and replication stress or by impaired DNA damage response. Replication stress does not seem to be present in EBNA-1 expressing cells since the histone H2AX is phosphorylated throughout the cell cycle and not decreased by an arrest in G1 (unpublished observation). Once the replication stress was excluded, the levels of intracellular ROS were measured in EBNA-1 expressing cells. The cells were treated with membrane-permeable compound 2,7-dichlorofluorescein-diacetate that becomes fluorescent upon oxidation. EBNA-1 positive cells demonstrated an increase in ROS, which was reproduced in EBV+ cells compared to EBV– cells (Paper II). This observation confirms earlier studies that report high levels of ROS during primary EBV infection [192] and in EBV carrying tumors [193,194]. High levels of ROS in EBNA-1 expressing cells led to the oxidation of guanine to 8-oxoguanine, a direct effect of ROS on DNA (Paper III). Quenching of ROS by scavengers decreased the levels of phosphorylated H2AX and abnormal metaphase plates suggesting that the EBNA-1 dependent induction of ROS plays a direct role in the initiation of genomic instability.

The generation of ROS involves a cascade of reactions starting with the production of superoxide. The very unstable superoxide is rapidly converted to hydrogen peroxide by superoxide dismutase. There are two major ways how ROS are generated in cells, either by enhanced oxidative metabolism or by deregulation of enzymes involved in the production of ROS. Therefore the activity of the enzymes involved in the oxidative metabolism was inhibited. A decrease in ROS levels was observed in EBNA-1 expressing cells as well as in parental cells, however the EBNA-1 expressing cells possessed, independent on the treatment, significant higher levels of ROS than the parental cell lines. To investigate the eventual deregulation of proteins involved in ROS pathways, gene expression profiles, including only proteins known to produce or scavenge ROS, were analysed using public databases. Datasets, containing gene expression arrays from 18 EBV positive and negative datasets, revealed four proteins differentially regulated in EBV+ cell lines compared to EBV– cell lines. Only one out of these four genes, namely NADPH Oxidase 2 (Nox2), could be validated in our model system. Nox2 mRNA as well as protein levels were highly upregulated in EBNA-1 expressing cells. Soon after expression of EBNA-1 by removal of tetracycline in the tet-off regulated EBNA-1 cell line, the expression of Nox2 increased, suggesting a direct correlation between the expression of EBNA-1 and Nox2. To test whether Nox2 is transcriptionally regulated by EBNA-1, a reporter plasmid was constructed containing the Nox2 and the luciferase gene. If EBNA-1 regulates Nox2 transcriptionally, the expression of the luciferase genes should be increased. After transfection of EBNA-1 together with the construct the luciferase luminescence signal increased dependent on EBNA-1 in a dose dependent manner.

In contrast to the oxidative metabolism, where ROS occurs as a byproduct, the NADPH oxidase complex generates ROS as a primary function. The complex was first described in phagocytes, in which ROS attack foreign microbes. Seven members of the family of NADPH Oxidases were found in different tissues. All Nox family members are transmembrane proteins transporting electrons across biological membranes to reduce oxygen to superoxide. This common feature requires conserved structural properties including an NADPH-binding site at the very COOH terminus, a FAD-binding region in

proximity of the most COOH-terminal transmembrane domain, six conserved transmembrane domains, and four highly conserved heme-binding histidines. Nox2 consists of six transmembrane domains with its C-terminus and N-terminus facing the cytoplasm. Nox2 itself is the catalytic subunit of a whole complex containing $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac1. The subunit $p22^{phox}$ is constitutively associated with Nox2 in the cytoplasm. The other cytosolic factors need to be recruited to the membrane. Once all subunits, including Rac1, are assembled at the cell membrane [195], the complex is active and generates superoxide by transferring an electron from NADPH in the cytosol to oxygen on the luminal or extracellular space [196].

The expression of the subunits was not influenced by EBNA-1 expression, the activation of Rac1 however was elevated in the presence of EBNA-1 (Paper II). Inactivation of Nox2 with Nox2 inhibitors or the downregulation of Nox2 with recombinant lentiviruses expressing Nox2 specific shRNAs led to decrease of ROS, comet length and phosphorylation of H2AX. These results indicate that the activation of Nox2 is responsible for the increased levels of ROS and the subsequent induction of genomic instability in EBNA-1 expressing cells (Paper II).

EBNA-1 is the only protein consistently expressed in all EBV associated malignancies and sufficient to induce chromosomal aberrations and DNA strand breaks. These results link EBNA-1 to the induction of DNA damage via ROS and a direct contribution to tumorigenesis in BL. Increased levels of ROS have been demonstrated during primary EBV infection [192] and in EBV-carrying tumor cells [168,193]. Elevated levels of ROS in EBNA-1 expressing cells may play an important role in EBV transformation. This possibility is substantiated by the finding that EBV growth transformation is promoted by ROS [197] and inhibited by antioxidants [198]. ROS have been shown to oxidize lipids and proteins leading to the activation of several signalling cascades. EBNA-1 may promote EBV transformation by initiating signalling cascades, which are further activated by other viral latent genes. Taken this into consideration, the induction of ROS in EBNA-1 may be an important feature of infected B-cells to be able to colonize in the B-cell compartment, while the induction of DNA damage may be considered a tolerable side effect.

4.3 EBNA-3C AND LMP-1 ALSO INDUCE GENOMIC INSTABILITY

Latency I expressing cells demonstrated an increase in abnormal metaphase plates, which was more enhanced in latency III expressing cells (Paper I). EBNA-1 was identified as an inducer of genomic instability. However, in addition to EBNA-1, other protein(s) must be responsible for the increase of genomic instability in latency III expressing cells. The ability of all latent viral antigens to induce genomic instability in stable transfected cell lines was systematically investigated. Three viral antigens, namely EBNA-1, EBNA-3C and LMP-1, showed significant more abnormal metaphase plates than the parental cell line and the cell line transfected with the control vector (Paper III). Interestingly EBNA-1 and LMP-1 positive cell lines demonstrated a prevalence of dicentric chromosomes and chromosome fragments, whereas in EBNA-3C expressing cells dicentric chromosomes were less frequent. The induction of DNA damage in EBNA-1, EBNA-3C and LMP-1 expressing cells was confirmed by increased levels of phosphorylated H2AX and increased comet size compared to the parental cell line (Paper II and Paper III).

4.4 LMP-1 INHIBITS DNA REPAIR

The mechanism of how the cells accumulate DNA damage has been found for EBNA-1 expressing cells. EBNA-3C and LMP-1 expressing cells were tested for their endogenous ROS levels. Increased ROS levels in EBNA-1 expressing cells confirmed earlier results, however the ROS levels in EBNA-3C expressing cells remained unchanged compared to the untransfected parental cell line. LMP-1 expressing cells showed a moderate increase in ROS levels. The most common nucleotide alteration by ROS is guanine to 8-oxoguanine. The levels of 8-oxoguanine were measured and found that EBNA-1 expressing cells, as expected, showed significant higher levels of 8-oxoguanine, while the 8-oxoguanine levels in EBNA-3C expressing cells are similar to the levels in the parental cell line. LMP-1 expressing cells show a moderate increase in 8-oxoguanine, confirming that there are low levels of ROS present in these cells (Paper III).

To test, whether this moderate increase of ROS levels induces DNA damage in LMP-1 expressing cells, the cells were treated with antioxidants and stained for the phosphorylated version of H2AX. As expected and seen before, the phosphorylation of H2AX is decreased in EBNA-1 expressing cells after antioxidant treatment. However, no differences in the levels of phosphorylated H2AX with or without treatment of antioxidants were observed, suggesting that other mechanisms beside high levels of ROS induce the observed DNA damage.

If DNA damage is not induced by endogenous insults, it may be accumulated through impaired DNA damage response including altered DNA repair and deregulated cell cycle. Consequently the DNA repair capacity in EBNA-1, EBNA-3C and LMP-1 expressing cells was tested. Two different methods were used, one indirect and one more direct. Using the indirect test, the cells were treated with gamma radiation and allowed to repair the damage. At indicated time points, cells were sampled and the comet length was measured. While in parental EBNA-1 and EBNA-3C expressing cells no residual damage was observed 24h after the treatment, LMP-1 positive cells showed 30% residual damage. Using the more direct method these results were reproduced. Gamma and UV irradiated luciferase plasmids were transfected into the cells and harvested 24h later. Also with this direct test, parental and EBNA-1 expressing cell lines repaired efficiently all the damage. EBNA-3C expressing cells showed 5-10% residual damage and LMP-1 20% residual damage confirming that LMP-1 expressing cells inefficiently repair induced DNA damage.

Proper DNA repair relies on DNA damage sensing, which starts with the phosphorylation of ATM and is followed by a number of downstream events such as the phosphorylation of Chk2 and a G2 arrest. Consequently, expression and phosphorylation levels of ATM and Chk2 were tested. Parental, EBNA-1 and EBNA-3C expressing cells showed similar levels of ATM, while LMP-1 expressing cells clearly demonstrate lower levels of ATM. This downregulation was confirmed using two tet-off regulated LMP-1 cell lines. This is in line with the downregulation of ATM in EBV positive nasopharyngeal carcinoma [199]. The downstream signalling of the DNA damage response was tested. Whereas in parental, EBNA-1 and EBNA-3C expressing cells, ATM and Chk2 are phosphorylated after gamma irradiation, LMP-1 expressing cells do not show this phenotype. Furthermore, the DNA damage-induced G2 arrest, mediated by

Chk2, is abrogated in LMP-1 expressing cells, while the G2 arrest was activated in parental, EBNA-1 and EBNA-3C expressing cells (Paper III).

Inhibition of DNA repair by LMP-1 may promote oncogenesis, however it might also help the virus in viral replication. A number of viruses, such as herpes simplex virus (HSV), adenovirus, SV40 and HIV have been shown to activate the host damage response pathway [200]. Single- or double-stranded un-integrated DNA may trigger a DNA damage response. In the life cycle of EBV, there are two points that could lead to the activation of DNA damage response, first during initial infection, since the DNA exists as linear strand and the ends could be recognized as DSBs. The second point may be during lytic replication, when newly synthesized DNA is present as linear genome. Indeed, EBV lytic infection activates the DNA damage response by phosphorylating ATM. However the downstream target signalling was blocked [201]. In our results, the ability of LMP-1 to downregulate ATM and thereby inhibit the downstream signalling might have a function in virus replication. By blocking the cells from recognizing the viral DSB during replication, the virus evades the host checkpoint security system and enhances the environment for lytic replication. Other viruses, such as the human T-lymphotropic virus, have shown to decrease the ATM-dependent DNA damage response [202].

4.5 EBNA-3C INACTIVATES THE SPINDLE CHECKPOINT

EBNA-3C does not induce genomic instability by ROS and does not lead to accumulation of DNA damage by inefficient repair mechanism. Analysis of the number of chromosomes revealed a disposition to aneuploidy in all transfected cell lines, however in EBNA-3C a remarkable broader distribution of chromosome numbers with a significant number of chromosomes lower than 42 was observed (Paper III). These differences in chromosome number and aberration distribution suggest different mechanisms for the induction of the observed chromosome aberrations. Aneuploidy is known to be caused by improper chromosome segregation mostly due to mitotic spindle aberrations. An active spindle checkpoint leads to a halt of progression into metaphase if not all chromosomes are properly attached. Therefore, the spindle checkpoint in EBNA-3C expressing cells was tested by treating the cells with the spindle poison nocodazole. Clearly EBNA-3C expressing cells compared to parental, EBNA-1 and LMP-1 expressing cells overcome the spindle checkpoint and progress into the next cell cycle (Paper III). The expression of the components of the spindle checkpoint were tested by western blotting and one component, namely BubR1, in contrast to cdc2, Mad2, Ubc10 and Gadd45, is significantly downregulated in EBNA-3C expressing cells compared to parental, EBNA-1 and LMP-1 expressing cells. BubR1 transcripts, measured by Q-PCR are also significantly downregulated showing that EBNA-3C expression is associated with the transcriptional downregulation of BubR1 (Paper III). Suppression of BubR1 has been shown to deactivate the spindle checkpoint [203] and a mouse model with haplo-insufficiency of BubR1 demonstrates genomic instability [204] and tumorigenesis [205]. It is possible to imagine that EBNA-3C expressing cells do not induce genomic instability directly but accumulate structural chromosome aberrations due to a missing mitotic spindle control. This may not be the only way through which EBNA-3C expressing cells accumulate damage since a recent study shows that EBNA-3C interacts with Chk2 and thereby deregulates other cell cycle checkpoints [50].

The deregulation of the cell cycle by EBNA-3C may be associated with accumulation of DNA damage, however it is possible that the disruption is advantageous for the virus. Another product of a virus, the SV40 large T antigen has been shown to disrupt the spindle checkpoint [206]. The same study described that the large T antigen binds to the component of the spindle checkpoint Bub1 and Bub3. This interaction leads to an override of the spindle checkpoint upon nocodazole treatment resulting in chromosomal aberrations and aneuploidy. The viral advantage may be increased growth and proliferation, since the cells do not stop to align all chromosomes properly but proceed through M phase.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The work described in this thesis emphasizes the role of EBV in tumorigenesis. We have shown that three latent viral proteins, EBNA-1, EBNA-3C and LMP-1, enhance genomic instability by three different mechanisms. EBNA-1 induces DNA damage by increasing the intracellular levels of ROS, while EBNA-3C and LMP-1 lead to the accumulation of DNA damage by either impairing cell cycle checkpoints or diminishing DNA repair. This "mutator phenotype" may drive EBV infected cells into tumor initiation and progression.

The capacity of EBNA-1 to induce elevated levels of ROS is likely to increase the risk of mutations that may constitute a first step towards malignant transformation. It would be therefore important to understand why the virus has adopted this seemingly dangerous strategy and how its consequences are kept under control during viral infection. Numerous studies describe ROS as a second messenger for several signalling pathways including NFκB, AP-1, Hif1-alpha and NFAT. Several of these pathways are activated during EBV induced growth transformation. It would be interesting to study whether the effect of EBNA-1 on ROS production is essential for their activation. If so, one may expect that treatment with antioxidants will have a major impact on EBV induced B-cell immortalization and, more generally, on the establishment of persistent infections and perhaps even virus reactivation. It remains to be seen, whether antioxidants have protective effects in immunosuppressed transplant patients.

The ability of LMP-1 to block the DNA damage response is likely to play a primary role during virus reactivation by protecting linear viral genomes from the cellular repair mechanisms that would inhibit the assembly of infectious virus particles. The capacity of host cells to respond to nascent DNA viruses by activation of DNA repair pathways to concatemerization of the viral genome has been described for example in adenovirus infected cells. The same virus counteracts this cellular defence mechanism by downregulating the DNA-PK that regulates non-homologous end joining of DSBs [207]. It remains to be seen whether ATM, and possibly other DNA damage sensing kinases, are downregulated during the productive virus cycle in a LMP-1 dependent fashion. If so, inhibition of LMP-1 expression and restoration of the activity of the kinases could provide interesting new means to interfere with the production of infectious virus. Increased antibody titers to antigens of the productive cycle precede by several years the clinical manifestations of NPC and have a prognostic role in the follow-up of the tumor [208]. Virus production was also shown to play a yet uncharacterized role in the establishment of EBV carrying lymphomas in immunosuppressed and humanized mice [209]. Thus, inhibition of LMP-1 could be an effective strategy to counteract the effect of this protein in different stages of malignant transformation.

The capacity of EBNA-3C to promote the accumulation of DNA damage by transcriptional downregulation of BubR1 and functional inactivation of the spindle checkpoint is more difficult to reconcile with a physiological role of this protein in the context of EBV infection. The EBNA-3 family of proteins was shown to regulate the activity of EBNA-2 that drives B-cell growth transformation through activation of the Notch signalling pathway and essential viral genes such as LMP-1. It is important to stress

that, in addition to the effects described in our work, EBNA-3C was shown to interfere with numerous aspects of the regulation of the cell cycle, partly through contrasting effects on the activity of components of the ubiquitin-proteasome system. It remains to be seen whether the disparate activities of EBNA-3C reflect a common, yet unidentified function of this protein.

Although EBV appears to possess several means for promoting the initiation and progression of malignant transformation EBV carrying tumors are extremely rare compared to the wide spread of the virus in all human populations. This points to the essential tumorigenic role of additional events such as immunosuppression and the activation of cellular oncogenes. Interestingly the use of the immunosuppressive drug cyclosporine A (CsA) to reduce the risk of Graft-Versus-Host-Disease after transplantation has increased the risk of EBV-associated PTLD [210]. CsA is known to induce oxidative stress and may therefore cooperate with EBNA-1 in promoting genomic instability in a situation where the lack of effective responses could allow the expansion of cells where DNA damage has occurred. The malignant progression of these cells may be further supported by the expression of other viral proteins that interfere with DNA repair (e.g., LMP-1) or deregulation of cell cycle checkpoints (e.g., EBNA-3C) ultimately leading to full-blown PTLD. Clearly, a better understanding of the molecular mechanisms regulating these events would improve treatment of symptomatic EBV infection and EBV associated malignancies

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