Advances in Experimental Medicine and Biology 879

Janos Minarovits
Hans Helmut Niller *Editors*

Patho-Epigenetics of Infectious Disease

Springer

Advances in Experimental Medicine and Biology

Volume 879

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Patho-Epigenetics of Infectious Disease

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-319-24736-6 ISBN 978-3-319-24738-0 (eBook)
DOI 10.1007/978-3-319-24738-0

Library of Congress Control Number: 2015957434

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

Foreword

During recent years, the study of epigenetic phenomena in genetics has attracted increasing interest in all fields of biology and medicine. In epigenetics, we are dealing with a gamut of regulatory mechanisms based on the methylation of DNA, multiple histone modifications, the activity of small RNA's, and additional, so far, incompletely understood biochemical reactions. While there is solid evidence to support the role of these functions as participants in the regulation of genetic activities, it remains to be elucidated which modulations epigenetic regulators are subject to. Today, epigenetic mechanisms are held at least partly responsible for the causation of:

- Complex human diseases (from tumor to psychiatric) with evident or surmised genetic background
- Genetic imprinting and its defects important in the clinic
- Environmental effects on the genome
- Modulations in the course of infectious diseases
- Hitherto poorly investigated phenomena in developmental biology or decisive events during evolution
- Genome-wide sequelae of genome manipulations, e.g., by the insertion of foreign DNA

The similarity of DNA sequences, e.g., between chimpanzees and humans (about 95 %), and the obvious differences between these organisms have raised numerous tantalizing questions about the importance of regulatory mechanisms during evolution and the evolving phenotypes of different species.

The patho-epigenetics of infectious diseases is an important case in point. For decades, students of *Mycobacterium tuberculosis*, to name just one example, had to cope with the vagaries of this disease, its variability in pathology and enigmatic susceptibility of humans exposed to it, as well as the unpredictable response of patients to this infection and its treatment. In this context, epigenetics immediately comes to mind and is now being investigated in a number of laboratories.

In the volume *Patho-Epigenetics of Infectious Diseases* edited by Janos Minarovits (Szeged) and Hans Helmut Niller (Regensburg), major aspects of the role of epigenetic mechanisms have been addressed in a series of model infections, both viral – human immunodeficiency virus and Epstein-Barr virus – and bacterial. To set the stage for the informed reader, basic mechanisms have been discussed in an introductory chapter on *Epigenetic*

Regulation authored by the editors and their colleagues. In the following sections which are dealing with specific pathogens, the emphasis has been placed on the epigenetic consequences of infections in the host genomes.

Both, specialist in infectious diseases and the newcomer with an interest in epigenetics, will be attracted to this volume which has been edited by experts in the field. For many years, the authors of individual chapters have made important contributions to epigenetic aspects of the infectious diseases which they had specialized in. This book will become a valuable addition to many researchers' library.

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Preface

Epigenetic regulatory mechanisms ensure the heritable alterations of cellular states without affecting the nucleotide sequence of the DNA. In multicellular organisms belonging to the taxon *Eukarya* or *Eukaryota*, epigenetic control of transcription forms the basis for the phenotypic and functional diversity of various cell types that carry identical or nearly identical genomes. Epigenetic regulators affect chromatin structure and promoter activity by depositing stable, but reversible, marks on DNA or DNA-associated proteins. Such epigenetic marks ensure the faithful transmission of gene expression patterns to each progeny cell upon division (*epigenetic memory*).

The diverse epigenetic regulators act in concert to establish cell type-specific gene expression patterns in multicellular organisms. Disturbances in epigenetic control mechanisms, elicited by a variety of agents, may result, however, in pathological changes and disease development, as overviewed earlier (*Patho-Epigenetics of Disease. Eds*. Minarovits J. and Niller H.H. Springer Science and Business Media, New York, 2012). One of the pioneering observations connected viral DNA integration with epigenetic alterations and tumorigenesis in model organisms (reviewed by Doerfler 2012). This volume focuses on epigenetic dysregulation and patho-epigenetic processes caused by microorganisms, mainly viruses and bacteria infecting humans.

In the first chapter *Janos Minarovits*, *Ferenc Banati*, *Kalman Szenthe*, *and Hans Helmut Niller* briefly outline the major, "classical" epigenetic regulatory mechanisms that include DNA methylation, modifications of core histone proteins, as well as polycomb group (PcG) and trithorax group (TrxG) protein complexes that may also modify histones or affect chromatin compaction directly. This chapter also deals with novel epigenetic regulators such as variant histones, pioneer transcription factors, long noncoding RNA molecules, and proteins controlling long-distance chromatin interactions; it also gives a brief characterization of various chromatin types.

In Chap. 2, *Enass A. Abdel-Hameed*, *Hong Ji*, *and Mohamed Tarek Shata* summarize how the HIV provirus, i.e., the DNA copy of the human immunodeficiency virus (HIV) genome, undergoes epigenetic modifications in host cells and how the viral proteins affect the cellular epigenome and gene expression pattern. They also discuss the potential use of epigenetic drugs, in combination with highly active antiretroviral therapy (HAART), to eradicate latent HIV genomes from the cells of HIV-infected and AIDS patients.

In Chap. 3, *Hans Helmut Niller*, *Ferenc Banati*, *Daniel Salamon*, *and Janos Minarovits* describe the host cell-dependent epigenotypes of Epstein-Barr virus (EBV), the first human tumor virus which is associated with a series of malignant human tumors. EBV, a gammaherpesvirus, infects both lymphoid and epithelial cells, and epigenetic dysregulation caused by the latent, growth transformation-associated viral oncoproteins plays a role in the initiation and progression of EBV-associated neoplasms.

Epigenetic reprogramming of host cells by oncoviruses appears to be a general phenomenon. In Chap. 4, *Janos Minarovits*, *Anett Demcsák*, *Ferenc Banati*, *and Hans Helmut Niller* overview the complex epigenetic changes caused by human tumor viruses or tumor-associated viruses including Kaposi's sarcoma-associated herpesvirus (KSHV), hepatitis B virus (HBV), hepatitis D virus (HDV), hepatitis C virus (HCV), human papillomavirus (HPV), Merkel cell polyomavirus (MCPyV), and human T-cell lymphotropic virus type I (HTLV-I).

Recently, there were significant efforts to elucidate the epigenetic alterations caused by bacterial pathogens in infected cells and organisms. In Chap. 5, *Lorenzo Chiariotti*, *Lorena Coretti*, *Raffaela Pero*, *and Francesca Lembo* evaluate the data demonstrating that lipopolysaccharide (LPS), the major component of the outer membrane of most Gram-negative bacteria, elicits not only inflammatory reaction but also short-term and long-term epigenetic changes in innate immune cells and epithelial cells. The latter phenomenon, i.e., LPS-mediated epigenetic reprogramming of human monocytes, monocyte-derived macrophages, dendritic cells (DCs), and neutrophils, may result in LPS- or endotoxin tolerance (ET). ET is characterized by the incapacity to produce proinflammatory cytokines and by other dysfunctions that may influence the host response to further encounters with microorganisms.

In addition to LPS, other bacterial products including various toxins, surface proteins, and effector proteins produced by obligate or facultative intracellular bacteria also elicit epigenetic alterations in their target cells. In Chap. 6, *Hans Helmut Niller and Janos Minarovits* describe how these bacterial products elicit histone modifications, i.e., alter the "histone code." Certain bacterial pathogens induce alterations of host cell DNA methylation patterns, too. Such changes in the host cell epigenotype and gene expression pattern may hinder the antibacterial immune response and create favorable conditions for bacterial colonization, growth, or spread. In addition, chronic inflammation caused by bacterial pathogens may also affect the epigenotype of host cells indirectly, via the enhanced production of inflammatory mediators.

Szeged, Hungary — Janos Minarovits
Regensburg, Germany — Hans Helmut Niller

Reference

Doerfler W (2012) The impact of foreign DNA integration in tumor biology and evolution via epigenetic alteration. In: Minarovits J, Niller HH (Eds) Patho-epigenetics of disease. Springer Science and Business Media, New York, pp 1–14

Contents

Abbreviations

5-azadC	5-Aza deoxycytidine
5caC	5-Carboxyl cytosine
5fC	5-Formyl cytosine
5hmC	5-Hydroxymethyl cytosine
5mC	5-Methyl cytosine
ACH	Active chromatin hub
AIDS	Acquired immunodeficiency syndrome
ATL	Adult T-cell leukemia/lymphoma
BAHD1	Bromo-adjacent homology domain-containing protein 1
BALF5	BamHI-fragment A leftward frame 5
BARF	BamHI-fragment A rightward frame
BART (=CST)	BamHI-fragment A rightward transcripts
BCBL (=PEL)	Body cavity-based lymphoma
BHRF1	BamHI-fragment H rightward frame 1
BL	Burkitt lymphoma
BMDC	Bone marrow-derived stem cell
BRLF1	BamHI-fragment R leftward frame 1
BZLF1	BamHI-fragment Z leftward frame 1
CagA	Cytotoxicity-associated antigen
CBP	CREB-binding protein
ccc	Covalently closed circular
CCR5	C-C chemokine receptor type 5
CGI	CpG island
CHD1	Chromodomain helicase DNA-binding protein 1
CIMP	CpG island methylator phenotype
CIN	Cervical intraepithelial neoplasia
CLL	Chronic lymphocytic leukemia
CMV	Cytomegalovirus
COX-2	Cyclooxygenase-2
Cp	C promoter
CpG	A 5′-cytosine-phosphate-guanine-3′ dinucleotide
CST (=BART)	Complementary strand transcripts
CTCF	CCCTC-binding factor
DC	Dendritic cell
DLBCL	Diffuse large B-cell lymphoma
DMR	Differentially methylated region
DNMT	DNA methyltransferase

DNMTI	DNMT inhibitor
DUB	Deubiquitinase
E6, E7	HPV early region proteins 6, 7
EBER	Epstein-Barr-encoded small RNAs
EBNA	Epstein-Barr nuclear antigen
EBNA-LP	EBNA-leader protein
EBV	Epstein-Barr virus
EBVaGC	EBV-associated gastric carcinoma
EC	Elite controller
EMT	Epithelial mesenchymal transition
ET	Endotoxin tolerance
EZH1	Enhancer of zeste homolog 1 (PRC2 component)
EZH2	Enhancer of zeste homolog 2 (a HKMT, PRC2 component)
FoxA	Forkhead box protein A (a pioneer transcription factor)
GC	Germinal center
H1	Histone 1
H2A	Histone 2A
H2A.X	Histone 2A, variant family member X
H2A.Z	Histone 2A, variant family member Z
H2AK119ub1	Histone 2A mono-ubiquitinated at lysine 119
H2B	Histone 2B
H3	Histone 3
H3K27ac	Histone 3 acetylated at lysine 27
H3K27me3	Histone 3 tri-methylated at lysine 27
H3K4me3	Histone 3 tri-methylated at lysine 4
H3S10ph	Histone 3 phosphorylated at serine 10
H4	Histone 4
HAART	Highly active antiretroviral therapy
HAT	Histone acetyl transferase
HBV	Hepatitis B virus
HBx	HBV X-gene/protein
HBZ	HTLV-I basic leucine zipper factor
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HDACI	HDAC inhibitor
HDV	Hepatitis delta virus
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HKMT	Histone lysine methyl transferase
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
HMG	High mobility group protein
HMT	Histone methyl transferase
HNSCC	Head and neck squamous carcinoma
HOX	Homeobox
HP1a	Heterochromatin protein 1a

HPV	Human papilloma virus
HSV	Herpes simplex virus
hTERT	Human telomerase reverse transcriptase
HTLV	Human T-cell lymphotropic virus
HUS	Hemolytic uremic syndrome
HVS	Herpesvirus saimiri
IE	Immediate early
IEC	Intestinal epithelial cells
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
InlB	(Listeria) Internalin B
ISGs	Interferon-stimulated genes
IκB	Inhibitor of NF-κB
JMJD	Jumonji domain
Kcr	Crotonylated lysine
kDA	Kilodalton
KDM	(Histone) Lysine demethylase
KS	Kaposi sarcoma
KSHV (=HHV-8)	Kaposi sarcoma herpes virus
L1-TRp	Terminal repeat promoter for LMP1
LAD	Lamina-associated domain
LANA	Latency-associated nuclear antigen
LC	Lymphoblastoid cell
LCL	Lymphoblastoid cell line
LCR	Locus control region
LCV	Legionella-containing vacuole
LECA	Last eukaryotic common ancestor
lincRNA	Long intergenic noncoding RNA
LINE	Long interspersed nuclear element
LLO	Listeriolysin O
LMP	Latent membrane protein
LMP1p	LMP1 promoter
LMP2Ap	LMP2A promoter
lncRNA	Long noncoding RNA
LntA	Listeria nuclear targeted protein A
LOCK	Large organized chromatin lysine (K9) modified regions
LPS	Lipopolysaccharide
LSD1	Lysine-specific histone demethylase 1
LTNP	Long-term nonprogressor
LTR	Long terminal repeat
MAR	(Nuclear) Matrix attachment region
MBD	Methyl-CpG-binding domain
MBP	Methyl-binding protein
MCD	Multicentric Castleman's disease
MCPyV	Merkel cell polyoma virus

MeCP2	Methyl-CpG-binding protein 2
MeDIP-chip	Methylated DNA immune precipitation-microarray hybridization
MeDIP-seq	Methylated DNA immune precipitation-sequencing
MHC	Major histocompatibility complex
miR-155	Micro RNA-155
miRNA	Micro RNA
MTase	Methyl transferase
NaBT	Sodium butyrate
ND10	Nuclear domain 10
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells
NPC	Nasopharyngeal carcinoma
NUE	(Chlamydia) Nuclear effector
OAMZL	Ocular adnexal marginal zone B-cell lymphoma
OGT	O-linked N-acetylglucosamine [GlcNAc] transferase (a PRC1 component)
PARP	Poly-ADP-ribose polymerase
PBMC	Peripheral blood mononuclear cell
PC	Polycomb (a PRC1 component)
PcG	Polycomb group
PCNA	Proliferating cell nuclear antigen
PD	Periodontal disease
PEL (=BCBL)	Primary effusion lymphoma
PKR	Protein kinase R
PML-NBs	Promyelocytic leukemia-nuclear bodies
PPIase	Peptidyl prolyl cis-, trans-isomerase
PRC	Polycomb repressive complex
PRMT	Protein arginine *N*-methyltransferase
PSI	Post-septic immunosuppression
PTLD	Posttransplant lymphoproliferative disease
Qp	Q promoter
ROS	Reactive oxygen species
SAM	S-adenosyl-L-methionine
SENP	Sentrin-specific protease
SET	Suppressor of variegation 3-9 [Su(var)3-9], enhancer of zeste and trithorax (a HKMT prototype)
SUMO	Small ubiquitin-like modifier
SUZ12	Suppressor of zeste 12
TAD	Topologically associated domain
Tax	Transactivator from the HTLV-I X-gene region
TCGA	The Cancer Genome Atlas Research Network
TET	Ten-eleven translocation
TGF-β	Transforming growth factor beta
Th cell	T helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha

TR	Terminal repeat
TrxG	Trithorax group
TSA	Trichostatin A
VacA	Vacuolating cytotoxin A
VPA	Valproic acid
VZV	Varicella zoster virus
Wp	W promoter
XLP	X chromosome-linked lymphoproliferation

Epigenetic Regulation

1

Janos Minarovits, Ferenc Banati, Kalman Szenthe, and Hans Helmut Niller

Abstract

Some of the key epigenetic regulatory mechanisms appeared early during evolution, and the acquisition of novel epigenetic regulators apparently facilitated certain evolutionary transitions. In this short review we focus mainly on the major epigenetic mechanisms that control chromatin structure and accessibility in mammalian cells. The enzymes methylating CpG dinucleotides and those involved in the active demethylation of 5-metylcytosine (5mC) are outlined together with the members of the methyl binding protein (MBP) family that bind to and "interpret" the 5mC mark. The enzymes involved in reversible, covalent modifications of core histone proteins that affect chromatin structure are also described briefly. Proteins that build up Polycomb group (PcG) and Trithorax group (TrxG) protein complexes may also modify histones. By establishing heritable chromatin states, PcG and TrxG complexes contribute – similarly to cytosine methylation – to the transmission of cell type-specific gene expression patterns from cell generation to cell generation. Novel players involved in epigenetic regulation, including variant histones, pioneer transcription factors, long noncoding RNA molecules and the regulators of long-distance chromatin interactions are introduced as well, followed by the characterization of various chromatin types.

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J. Minarovits, H.H. Niller (eds.), *Patho-Epigenetics of Infectious Disease*, Advances in Experimental Medicine and Biology 879, DOI 10.1007/978-3-319-24738-0_1

Keywords
DNA methylation • Histone modifications • Polycomb and Trithorax complexes • Variant histones • Pioneer transcription factors • Long noncoding RNAs • Chromatin loops

1.1 Introduction: Epigenetic Regulation in the Domains of Life

Epigenetic regulatory mechanisms ensure the heritable alterations of cellular states without affecting the nucleotide sequence of the DNA. In multicellular organisms belonging to the taxon *Eukarya* or *Eukaryota*, epigenetic control of transcription forms the basis for the phenotypic and functional diversity of various cell types that carry identical, or nearly identical genomes.

Epigenetic regulators interact with a DNA protein complex, called chromatin, that is organized into repeating units (Kornberg 1974). The basic chromatin unit is the nucleosome composed of a segment of 147 bp long DNA wrapped around a core of eight histone proteins (Richmond et al. 1984). Nucleosomes are connected by free linker DNA segments of 20–60 bp. The histone octamer is formed by the core histones, H2A, H2B, H3 and H4, each in two copies. In many eukaryotes, a single molecule of the linker histone, histone H1 stabilizes the nucleosome and plays a role in the formation of a higher-order chromatin structure by binding to the linker DNA (Harshman et al. 2013). The nucleosome and the linker histone form the chromatosome and resembles a "beads-on-a-string" structure. It is worthy to note that the eukaryotic genes coding for the core (invariant) histones were apparently generated by gene duplication events from archaeal genes (see below; Volle and Dalal 2014).

Epigenetic regulators affect chromatin structure and promoter activity by depositing stable but reversible marks on DNA or DNA-associated proteins. Such epigenetic marks ensure faithful transmission of gene expression patterns to each progeny cell upon division (*epigenetic memory*) (Strahl and Allis 2000; Jenuwein and Allis 2001; Bird 2002; Rohlf et al. 2012). In addition, incorporation of variant histone molecules into the nucleosomes in selected chromatin regions or high-affinity binding of certain nonhistone proteins to the mitotic chromatin may "bookmark" the genes to be expressed or silenced in the daughter cells (Zaret et al. 2008; Kelly et al. 2010; Zaidi et al. 2011; Follmer et al. 2012). Thus, there are distinct forms of epigenetic memory that do not necessarily rely on the covalent modifications of chromatin constituents. Even more, the accessibility of distinct chromatin domains to transcription factors, RNA polymerases and recombinases depends on their location to various nuclear subcompartments as well, and switching of the nuclear position from one compartment to another may change the epigenetic marks and activity of coregulated promoters (Gyory and Minarovits 2005). Such a complexity of epigenetic regulatory mechanisms is characteristic for multicellular eukaryotes (Aravind et al. 2011; Jin et al. 2011; Jurkowski and Jeltsch 2011; Jeltsch 2013). Epigenetic modifications are also well documented, however, in unicellular eukaryotes. They are of clinical importance, because protozoan pathogens control the expression of virulence genes and differentiation related gene sets by epigenetic regulators that can be targeted by epigenetic drugs (epigenetic therapy) (Aravind et al. 2011; Fisk and Read 2011; Coyne et al. 2012; Hoeijmakers et al. 2012; Niller et al. 2012).

Most eukaryote genomes encode DNA methyltransferases (DNMTs) that recognise CpG dinucleotides and modify the C5 position of cytosine within that sequence. The wide distribution of DNA-(cytosine C5)-methyltransferases in eukaryote species suggests that they appeared early during evolution and that the genome of the last eukaryotic common ancestor (LECA) possibly encoded at least one DNA-(cytosine C5)-methyltransferase (Jurkowski and Jeltsch 2011).

Certain proteins encoded by a single gene in invertebrate genomes and by several genes in vertebrates preferentially bind to DNA sequences containing one or more symmetrically methylated CpG dinucleotides (reviewed by Hendrich and Tweedie 2003). In vertebrates, such methyl-CpG binding domain (MBD) proteins may increase the fidelity of DNA methylation mediated gene silencing by "reading" the methylation mark and "interpreting" it via their association with histone deacetylases, nucleosome remodelling complexes and histone methyltransferases (reviewed by Hendrich and Tweedie 2003; Van Emburgh and Robertson 2008; Hashimoto et al. 2010).

Phylogenetic analysis of the gene coding for the histone methyltransferase enzyme, H3K9 HMTase Su(var)3-9, suggests that histone modification also appeared early during evolution: the HMTase coding sequence fused to a functionally unrelated gene, the γ subunit of the translation initiation factor eIF2 approximately 400 myears ago (Krauss et al. 2006).

Acquisition of novel genes coding for epigenetic regulators possibly facilitated evolutionary transitions. It was suggested that the horizontal transfer of a histone methyltransferase gene from an animal host enabled the free living ancestor of the malaria parasite *Plasmodium falciparum* and other apicomplexans to change life style and evolve to an obligate parasite (Kishore et al. 2013). Acquisition of the novel regulator presumably allowed epigenetically controlled, coordinated expression of immune-evasion genes.

The social amoeba *Dictyostelium discoideum* but not other amoebae, also acquired a histone methyltransferase gene from animals, an event possibly related to the appearance of important characteristics of *D. discoideum*, i.e. complex cellular communication and differentiation (Kishore et al. 2013).

Similarly to eukaryotes, genetically identical unicellular organisms belonging to the domain *Bacteria* may also undergo differentiation or display heritable phenotypic heterogeneity. It is well documented that these morphological and physiological changes are frequently controlled by epigenetic regulatory mechanisms, especially DNA methylation (reviewed by Casadesus and Low 2013).

Putative chromatin modifying enzymes, related to the epigenetic regulators encoded by eukaryote genomes were also detected in organisms of the domain *Archaea* (Manzur and Zhou 2005; Niu et al. 2013). One of the two main archaeal phyla, *Euryarchaea* synthesize tetrameric histone homologues, and archeal histone genes were detected in certain *Crenarchaea*, too (Reeve 2003; Cubonova et al. 2005). These data suggested that histones appeared quite early during evolution, probably after the divergence of *Bacteria* – that lack histone-like sequences – and *Archaea*, but before the separation of *Archaea* and *Eukarya*. In addition, the demonstration of acetylated and methylated nucleoid proteins or archeal chromatin proteins raised the idea that in *Archaea*, similarly to *Eukarya*, chromatin modification may play an active role in transcriptional silencing (Bell et al. 2002; Wardleworth et al. 2002; Reeve 2003).

1.2 Epigenetic Regulatory Mechanisms

Epigenetic marks determine chromatin structure and accessibility by interacting with "*reader*" factors including methylcytosine (^{m}C) binding proteins, transcription factors, and chromatin remodeling complexes that affect and regulate transcription (Jin et al. 2011). Active promoters are usually located to domains of open chromatin or euchromatin, whereas silent promoters are typically situated in closed, condensed chromatin domains or heterochromatin (Fig. 1.1).

1.2.1 CpG Methylation

In *Eukarya*, DNA methyltransferases are epigenetic regulators that typically methylate the C-5 position of cytosines within CpG dinucleotides. With some exceptions, CpG methylation is associated with promoter silencing: it was observed that in vertebrate cells the control regions of inactive promoters are frequently methylated and located to "closed" chromatin domains suppressing transcription (reviewed by Robertson 2001). In addition, in somatic cells, a high level of CpG

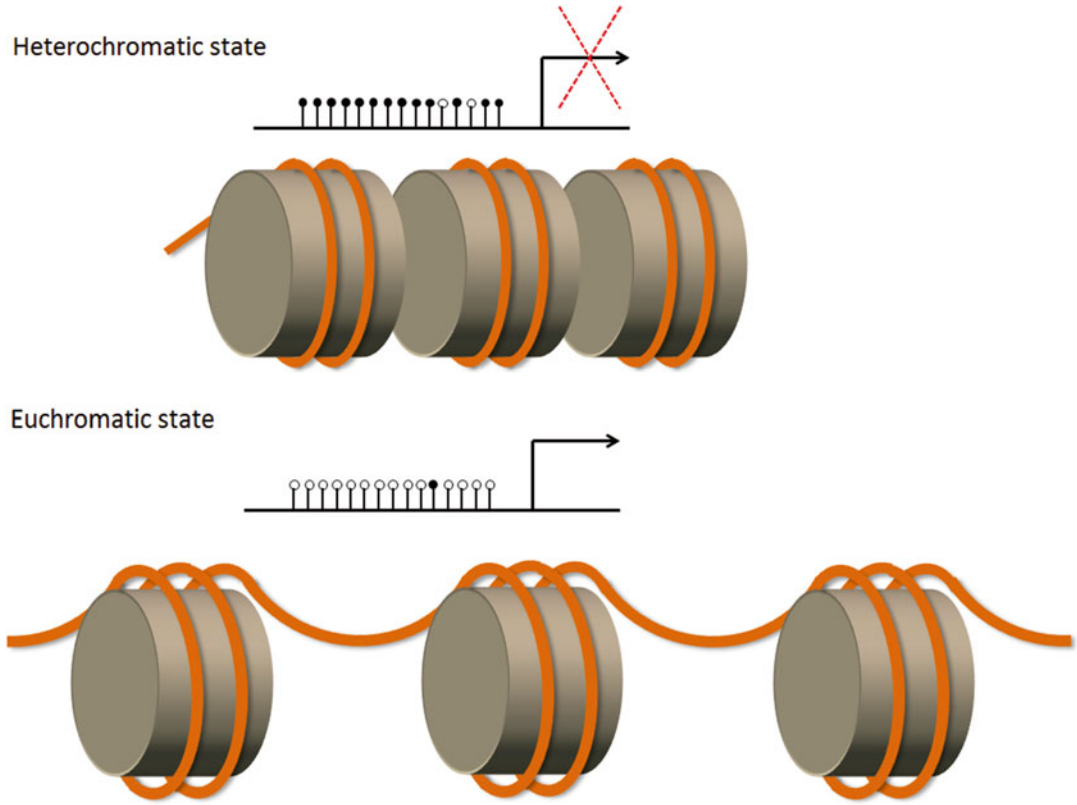

Fig. 1.1 Chromatin states and promoter activity. Nucleosomes are formed by stretches of DNA (indicated by *orange threads*) wrapped around histone octamers (depicted as *gray cylinders*). In the heterochromatic state (*top*) the chromatin is condensed; a silent promoter located to heterochromatin is indicated by an *arrow* with a *red X*. The regulatory regions of silent promoters are frequently hypermethylated as shown by *filled lollipop* symbols corresponding to methylated CpG dinucleotides. In the euchromatic state (*bottom*) the chromatin is loose, decondensed; an active promoter located to euchromatin is indicated by an *arrow*. The regulatory regions of active promoters are frequently unmethylated or hypomethylated as shown by *open lollipop* symbols corresponding to unmethylated CpG dinucleotides

methylation was typically observed at retrotransposons, including endogenous retrovirus genomes flanked by long terminal repeat (LTR) sequences and other repetitive elements lacking LTRs (e.g. long interspersed nuclear elements, LINEs) (reviewed by Ooi et al. 2009). Tandem repetitive sequences (e.g. pericentromeric minor and major satellite sequences), were also found to be densely methylated, similarly to the silent X chromosome in females (reviewed by Ooi et al. 2009).

In humans, cytosine methylation patterns are *maintained* by DNA methyltransferase 1 (DNMT1) that has a high affinity to hemimethylated DNA substrates generated during semiconservative DNA replication (Van Emburgh and Robertson 2008, Table 1.1, Fig. 1.2). DNMT1 restores the methylation pattern of the parental DNA strands on the initially unmethylated daughter strands by transferring a methyl group from the universal methyl donor S-adenosyl-L-methionine to the 5-position of cytosine. There are five conservative motifs in the C-terminal catalytic domain of DNMT1 that are also present in the prokaryotic DNA-(cytosine-C5) methyltransferases involved in restriction/modification phenomena (Posfai et al. 1989; Bestor 2000). In contrast, there are no significant sequence homologies with bacterial DNA methyltransferases at the N-terminal domain: thus, the origin of that regulatory domain is unknown at present. It is

Table 1.1 Human DNA methyltransferases and their relatives lacking DNA methyltransferase activity

Enzyme	Function	Note
DNMT1	Maintenance methylase	Critical for embryonic development
DNMT2	Lacks DNA methyltransferase activity; tRNA methyltransferase (?)	Sequence similarity with bacterial DNMTs
DNMT3A	*De novo* methylase development	Critical for embryonic development
DNMT3B	*De novo* methylase	Critical for embryonic development
DNMT3L	Lacks DNA methyltransferase activity	Sequence similarity with bacterial DNMTs; enhances the activity of de novo DNMTs; forms repressive complexes with HDACs

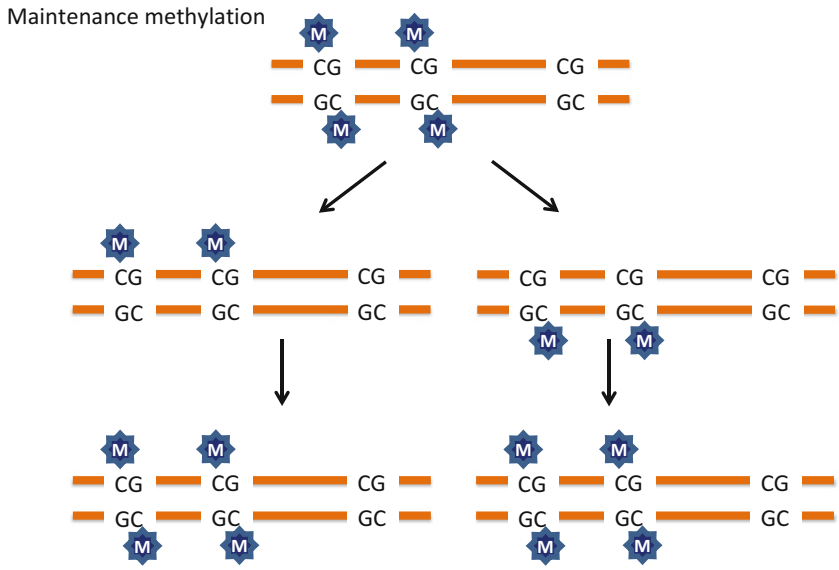

Fig. 1.2 Maintenance DNA methylation. A stretch of double stranded DNA with CpG dinucleotides (CG) either methylated (M, *blue*) or unmethylated on both strands is shown (*top*). During DNA replication (*arrows*), two hemimethylated DNA molecules are generated (one strand methylated, the other unmethylated) (*middle*). Finally, the original methylation pattern is restored (*arrows*) by DNMT1 (*bottom*)

apparently unique to eukaryotes and it is involved in the nuclear import of the enzyme. In addition, the N-terminal regulatory domain targets DNMT1 and the related murine enzyme, Dnmt1, to the replication foci during S phase and coordinates DNA replication with CpG methylation (reviewed by Bestor 2000).

Maintenance DNA methyltransferases are targeted to replication foci by interacting with PCNA (proliferating cell nuclear antigen) and UHRF1 (ubiquitin-like protein containing PHD and RING finger domains 1); alternatively, they could be recruited by a series of transcription factors, too (Araujo et al. 2001; Iida et al. 2002; Spada et al. 2007; Hervouet et al. 2010, 2012). The SRA (SET and RING-associated) domain of UHRF1 contacts both the major and minor grooves of DNA containing a hemimethylated CpG site – where only one DNA strand is methylated – by two loops. Both loops penetrate into the middle of the DNA helix that causes flipping out of the parental strand 5-methylcytosine (Hashimoto et al. 2008). The SRA-DNA interaction serves as an anchor keeping UHRF1 at the hemimethylated CpG site. By contacting UHRF1, DNMT1 is guided to the opposite unmethylated CpG dinucleotide located in the daughter strand. Recruitment of DNMT1 by UHRF1 is followed

by the transfer of a methyl group to the unmethylated cytosine of the target sequence (Hashimoto et al. 2010).

DNMT2, a protein that shares conserved motifs with bacterial DNA methyltransferases, has no DNA methyltransferase activity and it is not known to be involved in the regulation of CpG methylation in mammals (Defossez 2013, Table 1.1). DNMT2 can function, however, as a tRNA methyltransferase (Goll et al. 2006).

De novo DNA methyltransferases (DNMT3A, DNMT3B in humans) are involved in the establishment of DNA methylation patterns during embryonic development. They act preferentially on completely unmethylated DNA strands. In addition, by anchoring strongly to nucleosomes in highly methylated regions of the genome DNMT3A and DNMT3B play a role in maintenance DNA methylation as well, via *de novo* methylating the sites missed by DNMT1 in densely methylated, heterochromatic domains (Jones and Liang 2009; Gatto et al. 2012, Table 1.1). Similarly to DNMT1, the C-terminal catalytic domain of DNMT3A and DNMT3B also harbour conserved methyltransferase domains. However, the N-terminal regulatory domain differs from that of DNMT1: it consists of a cysteine-rich region, the ADD (ATRX/DNMT3/DNMT3L) domain and a proline-tryptophan motif (PWWP domain, reviewed by Gatto et al. 2012). These regions mediate interactions with a series of transcription factors, histone-modifying proteins, histone H3 tails with unmodified lysine 4 residues (H3K4me0) and histone H3 trimethylated at lysine 36 (H3K36me3). Both DNMT3A and DNMT3B form tetrameric complexes with DNMT3L molecules. The latter do not have enzymatic activity, but stimulate DNMT3A and DNMT3B function (reviewed by Hashimoto et al. 2010; Gatto et al. 2012, Table 1.1).

Methylated DNA sequences are specifically recognized by methyl-CpG binding proteins that are also referred to as members of the methyl binding protein (MBP) family (reviewed by Parry and Clarke 2011). MBPs "read", i.e. selectively bind methylated CpG dinucleotides (mCpGs) and "interpret" this epigenetic mark by recruiting histone-modifying enzymes that favour the establishment of a repressive chromatin structure silencing promoter activity. MeCP2 (methyl CpG binding protein 2), a chromosomal protein, contains a methyl-CpG binding domain (MBD) of 85 amino acids that recognizes a single symmetrically methylated CpG dinucleotide and a transcriptional-repression domain associating with mSin3A, a transcriptional repressor, and histone deacetylases (Nan et al. 1993, 1998; Free et al. 2001, Fig. 1.3). In addition, MeCP2 may

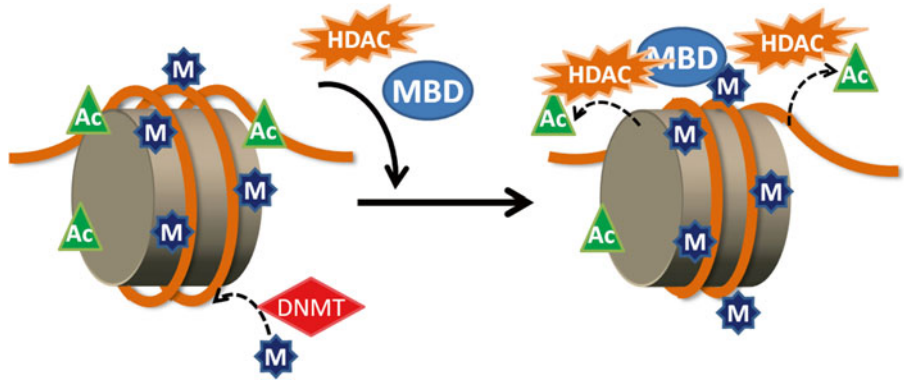

Fig. 1.3 Recruitment of histone deacetylase to methylated DNA sequences. The *left* side of the figure shows a nucleosome formed by a stretch of DNA (indicated by an *orange thread*) wrapped around a histone octamer (depicted as a *gray cylinder*) modified by acetylation (Ac, *green*). A DNA methyltransferase (DNMT, *red*) adds methyl groups (M, *blue*) to CpG dinucleotides. The *right* side of the figure shows that after binding of MBD (methyl-CpG-binding domain) family proteins (MBD, *blue*) to the methylated DNA, histone deacetylase enzymes (HDAC, *orange*) are recruited to the region and stabilize the heterochromatic state by removing the acetyl moieties from histones

reinforce transcriptional repression by tethering a histone methyltransferase activity to methylated DNA sequences (Fuks et al. 2003; Subbanna et al. 2014). In addition to MeCP2, other nuclear proteins (MBD1 to MBD6) also contain the MBD domain. MBD1, 2, 3 and 4 associate with repressor complexes. MBD4 that has a glycosylase activity interacts with the DNA repair machinery as well (reviewed by Parry and Clarke 2011). MBD 5 and 6 do not bind to methylated DNA, but interact with PcG proteins (Baymaz et al. 2014). MBD1 forms a stable complex with SETDB1 (SET domain, bifurcated 1), a histone H3-K9 methylase at DNA replication foci, coupling CpG methylation to histone methylation and heterochromatin formation (Sarraf and Stancheva 2004).

Non-methylated DNA sequences are specifically recognized by a family of proteins containing a zink finger-CxxC domain (ZF-CxxC domain) (Long et al. 2013). ZF-CxxC proteins may contribute to the maintenance of euchromatic histone marks at CpG islands and may prevent *de novo* CpG methylation by recruiting protein complexes with histone H3K4 methyltransferase (see Sect. 1.2.2.2) and DNA demethylase activities (see below), respectively. CFP1 (CxxC finger protein 1), a component of the mammalian SETD1 (SET domain 1) complex involved in histone H3K4 methylation, regularly associates with CpG islands (Thomson et al. 2010). There is a rigid CpG recognition loop within the CFP1 CxxC domain, consisting of a three amino acid residues (IRQ), which is unable to accomodate methylated CpG, but allows the binding of the non-methylated CpG dinucleotide (Xu et al. 2011). In addition, the H3K4 methyltransferases MLL1 and MLL2 also contain a zink finger-CxxC domain (reviewed by Long et al. 2013). It was also observed that TET2 and TET3 proteins that maintain the unmethylated state of CpG islands by converting 5-methylcytosine to 5-hydroxymethylcytosine, co-localize with HCF1 at active promoters and promote binding of the SET1 H3K4 methyltransferase complex to chromatin (Deplus et al. 2013).

Cytosine methylation is reversible, it can be removed by active or passive "*eraser*" mechanisms. The Tet (10–11 translocation) family of dioxygenases converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) followed by base excision repair (active demethylation; reviewed by Wu and Zhang 2011). Passive demethylation may occur when the recruitment of maintenance DNA methyltransferase DNMT1 is inefficient or the activity of the enzyme is inhibited during DNA replication (reviewed by Smith and Meissner 2013).

Tet proteins belong to the oxoglutarate and iron-dependent dioxygenase enzymes that are encoded by *Tet1*, *Tet2* and *Tet3* genes, derivatives of an ancestral gene of jawed vertebrates (Iyer et al. 2009). In addition to oxoglutarate and iron binding domains, the human TET1 and TET3 proteins contain a zink finger (CxxC) DNA binding domain as well that can recognize unmodified, methylated and hydroxymethylated sequences (reviewed by Branco et al. 2012). Dependence of Tet proteins on oxoglutarate and oxygen suggested that they may function as sensors of metabolic and oxidative cell states (Chia et al. 2011).

During development, Tet proteins are involved in global DNA demethylation events that result in epigenetic reprogramming in the early zygote and later in the migrating primordial germ cells (Hill et al. 2014). The rapid loss of 5-methylcytosine is attributed to Tet-mediated iterative oxidation followed by excision and repair in both cases.

Although most of the CpG dinucleotides are methylated in mammalian genomes, certain regions called CpG islands are devoid of methylation due to high levels of a modified histone (H3K4me3, histone H3 trimethylated at lysine 4; see Sect. 1.2.2) preventing the recruitment of *de novo* DNA methyltransferases. In addition, binding of Tet1 to unmethylated CpG rich sequences may also contribute to the protection of CpG islands from stochastic, aberrant methylation by converting the newly methylated 5mC to 5hmC (Williams et al. 2011, 2012). 5hmC may interfere with DNMT1, the maintenance methyltransferase, resulting in passive demethylation, or it may be converted to higher oxidative products targeted by thymine-DNA glycosylase. The lesion created

by the removal of 5fC and 5caC is processed through the base excision repair pathway (BER), restoring the unmethylated state (Williams et al. 2012; Hill et al. 2014). Thus, Tet proteins may function as the "guardians of CpG islands" (Williams et al. 2012). It is also worthy to note that conversion of 5mC to 5hmC may affect the association of 5mC-binding proteins and methylation-sensitive transcription factors with their recognition sequences, influencing thereby promoter activity (Williams et al. 2011). In addition, Tet proteins interact with SIN3A, a co-repressor complex, and OGT (O-linked N-acetylglucosamine transferase), an enzyme promoting transcription by glucosylating histone H2B (Williams et al. 2011; Vella et al. 2013; Dehennaut et al. 2014; Hill et al. 2014).

1.2.2 Histone Modifications

Two molecules each of the core histone proteins, histone H2A, H2B, H3 and H4 assemble to an octamer around which a stretch of 146 bp DNA is wrapped, forming a nucleosome which is the structural and functional unit of chromatin. Core histone proteins are characterized by a central α-helix flanked by shorter loops and helices and an N-terminal tail which is an unstructured region frequently undergoing chemical modifications (reviewed by Horikoshi 2013). Various types of covalent modifications affecting the core histone molecules constitute epigenetic marks that influence the structure and accessibility of chromatin as well as promoter activity. Mitotically heritable histone marks may be deposited by histone acetylases, protein arginine methyltransferases and histone lysine methyltransferases. Histone lysine methyltransferases are components of Polycomb group (PcG) and Trithorax group (TrxG) multiprotein complexes as well (reviewed by Jin et al. 2011). It is important to note that certain TrxG and PcG proteins not only covalently modify histones but also remain associated with mitotic chromatin and influence the activity of neighbouring promoters in postmitotic cells (Aoto et al. 2008; Blobel et al. 2009).

Similarly to DNA methylation, histone modifications are reversible: they are removed by histone deacetylases, histone lysine demethylases or the histone arginine demethylase JMJD6 (jumonji domain-containing 6 protein) (Cloos et al. 2008; Haberland et al. 2009). It is noteworthy that both demethylation of lysine and active demethylation of cytosine depends on oxygenases (reviewed by Jeltsch 2013). Thus, Jeltsch argued that the major reversible epigenetic systems could possibly appear only in the Cambrian period, after an increase in atmospheric oxygen. He suggested that such a change could have been a precondition for the generation of different classes of oxygenases, the enzymes permitting the use of stable but reversible covalent chromatin modifications for gene regulation, a key factor in the evolution of multicellular organisms (Jeltsch 2013).

In addition to epigenetic regulation, histone modifications play an important role in DNA damage responses and chromatin restoration, too (reviewed by Zhu and Wani 2010). The various types of histone modifications are listed in Table 1.2. All of them may affect the structure of the chromatin. It was suggested that histone acetylation and methylation may contribute to epigenetic inheritance.

1.2.2.1 Histone Acetylation

Acetylated histones are regularly associated with active promoters. Histone acetyltransferases (HATs) that are frequently associated with multiprotein complexes in the nuclei, transfer an acetyl group from acetyl CoA to the ε-amino group of lysine side chains (reviewed by Bannister and Kouzarides 2011; Horikoshi 2013, Table 1.3). This modification results in weakening of histone-DNA interaction as well as in the formation of potential binding sites for bromodomain proteins that specifically recognize acetylated lysine residues and mediate transcription and anti-silencing functions. In addition to histones, non-histone proteins are also modified by HATs.

Histone acetylation at the ε-amino group of lysine residues is reversible, the acetyl groups are removed by histone deacetylases (HDACs)

Table 1.2 Histone modifications

Type	Amino acid modified	Writer	Reader	Eraser
Acetylation	Lysine	HATs	Brd2, Brd4	HDACs
Methylation	Lysine	HKMTs	CHD1, HP1, PC	KDMs
	Arginine	PRMTs		JMJD6
Phosphorylation	Serine	Ser/Thr kinase family	14-3-3	Phosphatase enzymes
	Threonine	Tyrosine kinase		
	Tyrosine			
Ubiquitination	Lysine	Ubiquitin ligase	?	DUBs
SUMOylation	Lysine		?	SENP1
ADP ribosylation	Glutamate	PARP1	?	?
Deimination	Arginine (to citrulline)	Peptidylarginine deaminase		?
Proline isomerisation	Proline	Proline isomerase		Proline isomerase
O-GlcNAcylation	Serine	OGT, *O*-linked β-D-*N*acetyl-glucosamine transferase		OGA, *O*-GlcNAc- ase
	Threonine			
Crotonylation	Lysine	?	?	?

Based on Bannister and Kouzarides (2011), Tan et al. (2011), and Xu et al. (2014)

Table 1.3 Histone acetyltransferases, acetylated histone-binding proteins and histone deacetylases in mammals

Protein	Function	Note
GNAT family	Histone acetylation	C-terminal bromodomain
MYST family	Histone acetylation	Chromodomain, zinc fingers
P300/CBP	Histone acetylation	Bromodomain, zinc fingers, binding of transcription factors, co-activator function
Basal transcription factor family	Histone acetylation	Related to TAFII250
Nuclear receptor cofactor family	Histone acetylation	
Brd2	Reader	Two bromodomains binding histone H4K12ac; co-activator
Brd4	Reader	Two bromodomains, co-activator, chromatin insulator
HDAC	Histone deacetylation	
Class I		Constitutive expression
Class II		Tissue specific expression
Class III		Sirtuins, NAD^+ dependence
Class IV		Tissue specific expression

Based on Roth et al. (2001), Yang (2004), Bannister and Kouzarides (2011), Josling et al. (2012), and Bassett and Barnett (2014)
Abbreviations: *GNAT* Gcn5-related *N*-acetyltransferase, *Gcn5* general control non-derepressible 5 (yeast), *MYST* named for its founding members: MOZ, Ybf2/Sas3, Sas2, and Tip60, *p300* E1A-associated 300 kDa protein, *CBP* CREB-binding protein, *TAFII250* TBP-associated factor of 250 kDa, *TBP* TATA-binding protein, *Brd2* bromodomain-containing protein 2, *Brd4* bromodomain-containing protein 4, NAD^+ nicotinamide adenine dinucleotide, oxidized

(Yang and Seto 2008; Bagui et al. 2013; Lakshmaiah et al. 2014, Table 1.3). Whereas active promoters are typically located to acetylation islands, i.e. chromatin regions enriched in acetylated histones, deacetylated histones are usually located to transcriptionally repressed, heterochromatic domains. Thus, HDACs function as transcriptional corepressors and they are

divided into four classes based on sequence homology. Class I (HDACs 1, 2, 3, 8), class II (HDACs 4, 5, 6, 7, 9, 10), class III (the sirtuins) and Class IV (HDAC11) (Yang and Seto 2008).

Because the level of histone acetylation decreases during the mitosis, and most of the acetylated histone-binding proteins are not associated with mitotic chromatin, histone acetylation was not considered as a putative mechanism transmitting epigenetic information from cell generation to cell generation. Recent data however suggest that certain gene loci remain associated with acetylated histones bound by a bromodomain protein in mitotic chromatin ((Shibata and Nishiwaki 2014), see Sect. 1.2.3). Thus, acetylated chromatin domains may also play a role in epigenetic memory.

1.2.2.2 Histone Methylation

Methylation of lysine (K) or arginine (R) residues of histone H3 or H4 may activate or repress the activity of nearby promoters. Conserved sequence motifs of histone lysine methyltransferases (HKMTs) bring S-adenosyl-L-methionine (SAM) in the proximity of the ε-amino group of the target lysine residue and create mono-, di-, or trimethylated lysines. Depending on the position of the methylated lysine residue, such modifications may favour the formation of euchromatin, a loose chromatin structure favouring promoter activity, or may result in a more condensed, repressive chromatin structure (heterochromatin). A typical euchromatic mark around active promoters is histone H3K4me3 (histone H3 trimethylated on lysine 4). In contrast, histone H3K9me3 and histone H3K27me3 (histone H3 trimethylated on lysine 9 and lysine 27, respectively) usually associate with silent promoters.

Lysine-specific histone methyltransferases typically contain a SET domain of approximately 130 amino acids that forms a catalytic core observed in the prototypical HKMTs **S**u(var)3-9, **E**nhancer of Zeste and **T**rithorax (reviewed by Dillon et al. 2005, Table 1.4). The non-SET domain containing DOT1 (disruptor of telomeric

Table 1.4 Human histone lysine methyltransferases (HKMTs)

Enzyme	Specificity	Target residue	Function
(A) SET domain HKMTs			
EZH1 (PRC2 member)	Histone H3	K27	Transcriptional silencing
EZH2 (PRC2 member)	Histone H1	K26	Transcriptional silencing
	Histone H3	K27	Transcriptional silencing
G9a	Histone H3	K9	Transcriptional silencing
GLP1 (EuHMT1)	Histone H3	K9	Transcriptional silencing
MLL1 (ALL-1, HRX)	Histone H3	K4	Transcriptional activation
MLL2 (ALR-1)	Histone H3	K4	Transcriptional activation
MLL3 (HALR)	Histone H3	K4	Transcriptional activation
SET1	Histone H3	K4	Transcriptional activation
SET7/9	Histone H3	K4	Transcriptional activation
SET8	Histone H4	K20	Transcriptional silencing
SETDB1	Histone H3	K9	Transcriptional silencing
SUV4-20H1	Histone H4	K20	Transcriptional silencing
SUV4-20H2	Histone H4	K20	Transcriptional silencing
SUVAR39H1	Histone H3	K9	Transcriptional silencing
SUVAR39H2	Histone H3	K9	Transcriptional silencing
(B) Non-SET domain HKMT			
DOT1 and DOT1L	Histone H3	K79	Demarcation of euchromatin

Based on Dillon et al. (2005)

Table 1.5 Protein arginine methyltransferases (PRMTs) targeting histone tails

Enzyme	Targeted histone	Methylation mark	Function
PRMT1	Histone H4	R3me2a	Trancriptional activation
	Histone H2A	R3me2a	Trancriptional activation
PRMT6	Histone H4	R3me2a	Trancriptional activation
	Histone H2A	R3me2a	Trancriptional activation
	Histone H3	R2me2a	Transcriptional repression
PRMT4 (CARM1)	Histone H3	R17me2a	Trancriptional activation
	Histone H3	R26me2a	Trancriptional activation
PRMT5	Histone H4	R3me2s	Transcriptional repression
	Histone H2A	R3me2s	Transcriptional repression
	Histone H3	R8me2s	Transcriptional repression
PRMT6	Histone H4	R3me2a	Trancriptional activation
	Histone H2A	R3me2a	Trancriptional activation
	Histone H3	R2me2a	Transcriptional repression
PRMT7	Histone H4	R3me2s	Transcriptional repression
	Histone H2A	R3me2s	Transcriptional repression

silencing) and DOT1L (DOT1-Like) proteins target the globular domain (K79) of histone H3 and may play a role in the demarcation of euchromatic domains and transcriptional elongation (reviewed by Dillon et al. 2005; Nguyen and Zhang 2011).

Protein arginine *N*-methyltransferases (PRMTs) also use SAM as a methyl donor to mono- or dimethylate the guanidinium side chain of arginine. Dimethylation can be either asymmetrical (N,N-dimethylation) or symmetrical (N,N′-dimethylation) (Kouzarides 2007, Table 1.5). Such modifications targeting histone H3 and H4 may either activate or repress transcription. PRMTs modify, however, a series of nonhistone proteins as well at glycine- and arginine-rich patches within their substrates (Bedford 2007). Only a subset of protein arginine methyltransferases (PRMTs) modify the tails of histone H3, H4 or H2A (reviewed by Di Lorenzo and Bedford 2011). PRMT1 and PRMT6 generate transcriptional activation marks by methylating histone H4 and H2A at arginine 3 (R3me2a; a: asymmetric) whereas PRMT4 (CARM1) targets histone H3 producing the euchromatic marks H3R17me2a and H3R26me2a. In contrast, PRMT5 and PRMT6 leave heterochromatic marks on histone H3 (H3R8me2s and H3R2me2a, respectively; s: symmetric) (Di Lorenzo and Bedford 2011). PRMT5 and PRMT7 target arginine 3 of histone H4 and H2A yielding R3me2s marks repressing transcription.

The chromodomain protein CHD1 (chromodomain helicase DNA binding protein 1), an ATP-dependent chromatin remodeler, binds to the euchromatic histone mark H3K4me3 whereas methylated H3K9me3 and H3K27me3 associate with other chromodomain proteins HP1 (heterochromatin protein 1) and PC (Polycomb), respectively (Cao et al. 2002). PC is a component of the polycomb repressor complex 1 (PRC1) involved in transcriptional repression and in the establishment of three dimensional, long-range chromatin interactions (reviewed by Schwartz and Pirrotta 2007; Lanzuolo et al. 2012; Cheutin and Cavalli 2014).

Histone lysine demethylases (KDMs) are either amine oxidases that may associate with a repressor complex and utilize FAD (flavin adenine dinucleotide) as a co-factor (e.g. LSD1, lysine-specific demethylase 1, also known as KDM1A), or they are Jumonji C (JMJC)-domain containing proteins that use Fe(II), α-ketoglutarate and molecular oxygen as co-factors, similarly to the Tet family of dioxygenases that target 5mC (Labbe et al. 2013). The only arginine demethylase described so far is JMJD6 (Jumonji domain-containing 6 protein). JMJD6 acts as a

dioxygenase and demethylates histone H3 dimethylated at arginine 2 (H3R2me2) and histone H4 dimethylated at arginine 3 (H4R3me2) (Chang et al. 2007). It also acts on methylated estrogen receptor alpha (metERα) and affects the estrogen-activated signal transduction cascades outside of the nucleus by reducing methylation at the asymmetrically dimethylated arginine R260 (Poulard et al. 2014).

1.2.2.3 Histone Phosphorylation

The genome-wide histone phosphorylation resulting in chromosome condensation during mitosis is mediated by the serine/threonine kinase Aurora-B targeting histone H3 at serines 10 and 28 (Goto et al. 2002; Sugiyama et al. 2002). It was observed that Aurora B was also active in the late G2 phase of the cell cycle when it was localized to the heterochromatin and phosphorylated serine 10 of histone H3 (Goto et al. 2002). During interphase, histone H3 phosphorylation by signal transduction kinases ensures rapid transcriptional induction of immediate early genes, including *c-fos* and *c-jun*, that respond to mitotic signals (reviewed by Sawicka and Seiser 2012). In addition, histone H3 phosphorylation mediates induction of a wide variety of genes upon stimulation with extracellular signals in different cell types and may play a role in transient derepression of promoters silenced by histone H3K9me3 (Sawicka and Seiser 2012). Histone H3 phosphorylation at serine 10 (H3S10ph) recruits members of the 14-3-3 protein family to the chromatin, especially when the neighbouring K9 or K14 lysine residue is acetylated, and such a dual modification ensures full transcriptional activation of the *Hdac1* and *p21* genes (reviewed by Sawicka and Seiser 2014). In resting B lymphocytes Aurora B phosphorylates histone H3S28 at active promoters (Frangini et al. 2013). This modification may block the E3 ubiquitin ligase activity of Ring1B, a Polycomb protein which is deposited to the same set of promoters (Frangini et al. 2013). Histone phosphorylation can be reverted by histone phosphatases. Histone H3S10ph is dephosphorylated by protein phosphatase 1 (PP1) (Murnion et al. 2001).

1.2.2.4 Histone Ubiquitination

Ubiquitin, a 8.5 kDa polypeptide of 76 amino acids is added to proteins by the concerted action of ubiquitin activating, conjugating and ligating enzymes. Ubiquitination affects the cellular location and interactions of proteins and typically signals their proteasomal degradation. Histones are the most abundant ubiquitinated proteins: the core histones, especially histone H2A and H2B, but also histone H1, the linker histone can be mono- or polyubiquitinated (Cao and Yan 2012). Polyubiquitination of histones is usually induced by DNA damage, and polyubiquitinated histones provide binding sites for the mediators of the DNA damage response.

Both histone ubiquitination and deubiquitination were implicated in silencing of *HOX* genes (homeotic genes) by Polycomb group (PcG) proteins (reviewed by Schuettengruber and Cavalli 2010; see also Sect. 1.2.3). The PcG protein RING1B monoubiquitinates histone H2A at lysine 119, whereas other PcG proteins, RING1B and BMI1 upregulate the activity of RING1B (Cao and Yan 2012). Histone H2AK119ub1 is involved in Polycomb-mediated silencing (Wang et al. 2004). Other members of the PcG complex contain a H2A deubiquitinase (DUB) activity, that cleaves the isopeptide bond between ubiquitine and lysine (reviewed by Schuettengruber and Cavalli 2010). Monoubiquitination of histone H2B at lysine 120 occurs during transcription and stimulates histone H3K4 di- and trimethylation, i.e. the establishment of an euchromatic mark (Kim et al. 2009). This suggests that histone ubiquitination, indirectly, may play a role in the transmission of epigenetic information from cell generation to cell generation.

RNF8 is a RING-finger ubiquitin ligase that facilitates the assembly of DNA repair proteins at DNA double-strand breaks (Mailand et al. 2007). Recently it was demonstrated that RNF8-mediated ubiquitination of histone H2A on the sex chromosomes during meiosis is necessary for dimethylation of histone H3 at lysine 4 (H3K4me2), an epigenetic mark that persists throughout meiotic division and may serve as the epigenetic memory in post-meiotic spermatids

where further RNF8-dependent epigenetic modifications occur (Sin et al. 2012). These include histone H3K4me3, histone crotonylation and deposition of the variant histone H2A.Z at the promoters of "escape" genes to be activated on inactive sex chromosomes in post-meiotic, round spermatids (Sin et al. 2012). Thus, histone ubiquitination may ensure the transmission of epigenetic information from meiotic cells to post-meiotic cells.

1.2.2.5 Histone SUMOylation

Small ubiquitin-like modifiers (SUMO proteins) are 92–97 amino acid long, approximately 10 kDa molecules that share structural similarities with ubiquitin. Their conjugation to protein targets is mediated by an enzymatic machinery resembling the one involved in the ubiquitination pathway: so called E1, E2 and E3 enzymes perform activation, conjugation and finally ligation of SUMO proteins to core histones at lysine residues (Gareau and Lima 2010). There are multiple E3 ligases involved in the last step, the formation of an isopeptide bond between the carboxy-terminal carboxyl group of SUMO and an ε-amino group of a substrate acceptor Lys residue (Yang and Chiang 2013). SUMOylation could possibly block the establishment of other covalent modifications including acetylation and ubiquitination. It was demonstrated that SUMOylation of histone H4 repressed promoter activity in cultured cells by the recruitment of histone deacetylase and heterochromatin protein 1 (HP1) (Shiio and Eisenman 2003).

SUMOylation appears to be an important modulator of transcription factor function (Yang and Chiang 2013). It was observed that SUMOylated nonhistone proteins were associated with the transcription start site on many of the most active housekeeping genes in HeLa cells, and SUMO-1 distribution correlated with that of H3K4me3, an euchromatic mark (Liu et al. 2012). In contrast, SUMOylation of the transcription factor Elk-1 repressed Elk-1 regulated promoters by the recruitment of histone deacetylase (Yang and Sharrocks 2004). SUMO isoforms may be removed by enzymes belonging to the sentrin-specific protease (SENP) family. The role of SUMOylation in epigenetic memory remains to be established.

1.2.2.6 Histone ADP Ribosylation

ADP-ribosyltransferases modify glutamate residues of histones by the transfer of ADP-ribose from NAD^+, a process reverted by the action of poly-ADP-ribose polymerases (PARPs, reviewed by Bannister and Kouzarides 2011). The contribution of ADP ribosylation to the transfer of epigenetic information from cell generation to cell generation remains to be elucidated.

1.2.2.7 Histone Deimination

In addition to demethylation by the dioxygenase JMJD6, the methyl group from arginine can also be removed in a reaction called demethylimination or deimination that generates citrulline, which can't be methylated (Cuthbert et al. 2004). Deimination is carried out by peptidylarginine deiminase 4 (PADI 4) and results in an irreversible alteration of histone H3 and H4 structure. Its role in the establishment of epigenetic memory is unknown at present.

1.2.2.8 Histone Proline Isomerization: A Noncovalent Histone Modification

In histone H3 of *Saccharomyces cereviseae* the peptidyl-prolyl isomerase (PPIase) Fpr4 interconverts the *cis* and *trans* isomers of the alanine 15-proline 16 peptide bond, and it was observed that acetylation at the neighbouring lysine 14 (K14) residue promotes the *trans* conformation (Howe et al. 2014). Proline isomerization at proline 38 of histone H3 affects the ability of Set2 to methylate lysine 36 (K36) that suggests a role for a noncovalent histone modification in epigenetic regulation (Nelson et al. 2006).

1.2.2.9 Histone *O*-GlcNAcylation

N-acetylglucosamine, briefly GlcNAc, may form a covalent linkage with serine or threonine residues of proteins when transferred from UDP-GlcNAc to them by OGT (*O*-GlcNAc transferase). This modification occurs on cytosolic, nuclear and mitochondrial proteins and marks also the core histones. *O*-GlcNAcylation of histone tails

may activate or repress promoter activity, depending on the residue modified. OGT belongs to the polycomb group of proteins, and it is a component of Polycomb Repressive Complex 1 (PRC1). In fact, OGT was found to be identical with the polyhomeotic (PH) protein that interacts with EZH2 (Enhancer of Zeste Homolog 2), the lysine methyltransferase of the other PcG complex, PRC2 (reviewed by Dehennaut et al. 2014). In addition, OGT is also a partner of the TET proteins of dioxygenases that demethylate 5mC (see Sect. 1.2.1). TET2 and TET3 may recruit OGT to the chromatin (Chen et al. 2013). The *O*-GlcNAc mark is removed by OGA (*O*-GlcNAcase). Because UDP-GlcNAc is a product of the hexosamine biosynthetic pathway, it was suggested that the nutritional state of the organism may affect the epigenotype and result in metabolic diseases and cancer due to epigenetic reprogramming (Dehennaut et al. 2014). However, the exact role of *O*-GlcNAcylation in epigenetic inheritance remains to be clarified.

1.2.2.10 Histone Crotonylation

Histone lysine crotonylation (Kcr) is a newly identified chromatin mark enriched at active promoters and potential enhancers (Tan et al. 2011). In male germinal cells Kcr marks sex chromosome-linked genes activated following meiosis.

1.2.3 Polycomb and Trithorax Complexes

Polycomb group (PcG) and Trithorax group (TrxG) protein complexes are capable to establish heritable chromatin states and transmit cell type specific gene expression patterns from cell generation to cell generation. PcG and TrxG complexes were discovered in *Drosophila melanogaster* as regulators of *Hox* (homeotic) genes (reviewed by Schuettengruber et al. 2007). TrxG complexes were characterized as activators of *Hox* genes both in *Drosophila* and in mammals, a phenomenon attributed to the histone lysine methyltransferase members of the TrxG complex that deposit the euchromatic mark H3K4me3 to the chromatin. In mammals, the enzymes involved in histone H3K4 methylation are the SET1A, SET1B, and mixed lineage leukemia (MLL) proteins 1–4 (Shilatifard 2012). These proteins require additional subunits for activity, similarly to the yeast methylase Set1, and the *com*plex of *p*roteins *ass*ociated with the histone H3K4 methylases is termed COMPASS (Miller et al. 2001). TrxG proteins in humans also form COMPASS-like multisubunit complexes with SET1A/B and MLL1–4 (Shilatifard 2012).

PcG proteins were identified as silencers of Hox genes in *Drosophila* (reviewed by Schuettengruber et al. 2007; Simon and Kingston 2009). Similarly to TrxG proteins, PcG proteins also form multisubunit complexes called Polycomb-repressive complex 1 and 2 (PRC1 and 2) (Margueron and Reinberg 2011; Simon and Kingston 2013; Voigt et al. 2013; Scelfo et al. 2015). Gene silencing by PRC2 was attributed to the deposition of H3K27me3, a heterochromatic mark, to the chromatin by the histone methyltransferases EZH2 and EZH1 (enhancer of zeste homologs). Subsequently, H3K27me3 is bound by Polycomb (PC), a member of PRC1 in *Drosophila*, or its mammalian and human homologs Cbx2, −4, −6, −7, and −8 (chromobox protein homolog 4, 6, 7, and 8), although the latter proteins bind with lower affinity to H3K27me3 peptides and they can't distinguish between H3K27me3 and H3K9me3 marks (Kaustov et al. 2011). There are at least five distinct PRC1 subcomplexes that contain, in addition to the RING1A/B ubiquitin ligase that monoubiquitinates histone H2A, different PCGF (Polycomb group RING finger) proteins, in addition to the core components EED (Embryonic Ectoderm Development), SUZ12 (Suppressor of Zeste 12), and Retinoblastoma binding proteins 46 and 48 (also designated as RBBP7 and RBBP4) (Gao et al. 2012; Tavares et al. 2012; Simon and Kingston 2013). Histone H2AK119 monoubiquitination plays an important role in Polycomb mediated silencing of the *Ubx* gene in *Drosophila* (Wang et al. 2004).

An alternative model suggests that noncanonical PRC1 complexes can be recruited to the targeted chromatin regions first, followed by

ubiquitination of histone H2A. Such an H3K27me3-independent pathway would rely on an unknown signal recognized by the RYBP protein. RYBP is a partner of RING1B, the catalytic subunit of PRC1. Subsequently, H2Aubq deposited by RING1B would induce PRC2 activity, resulting in H3K27 trimethylation (Tavares et al. 2012; Scelfo et al. 2015).

In addition to creating repressive histone marks, either directly by monoubiquitination or indirectly by recruiting histone lysine methyltransferases, PRC1 can also affect chromatin structure and silence gene expression by direct compaction of chromatin (Francis et al. 2004; Eskeland et al. 2010). The Ring1B-mediated compaction of nucleosomal arrays is independent of Ring1B enzymatic activity and occurs even on nucleosomes assembled from tail-less histones (Francis et al. 2004). In the absence of PRC1, i.e. in Ring1B null cells, there is a large-scale decompaction of chromatin that can be detected at the level of chromosomes using FISH (Eskeland et al. 2010).

PcG proteins were originally identified as gene silencers. Recent data suggest, however, that they may play a role in promoter activation, too. Although the heterochromatic histone mark H3K27me3 is deposited by PRC2 to CpG-rich inactive promoters, it was observed that in embryonic stem cells the monomethylated H3K27me1 – that is also deposited by PRC2 – accumulates within transcribed genes (Ferrari et al. 2014). In addition, EZH1, a PRC2 member, was associated with transcriptionally competent euchromatic regions in mouse skeletal muscle myoblasts, and it was recruited to transcriptionally active promoters in differentiating myocytes (Mousavi et al. 2012). It is worthy to note, however, that in another cell type, i.e. in differentiated osteoblasts, EZH1 is recruited to silent promoters marked with a dual histone modification, H3K9me3/S10ph (histone H3 lysine 9 trimethylated and serine 10 phosphorylated, Sabbattini et al. 2014).

In resting B lymphocytes, the PRC1 subunit Ring1B is deposited to active promoters marked by Aurora kinase B that phosphorylates histone H3S28 (Frangini et al. 2013). Ring1B apparently functions as a coactivator of transcription at these promoters. One may speculate that the function of the PRC1 subunit Ring1B is context dependent: it may activate promoters when associated with Aurora kinase B that blocks its E3 ubiquitin ligase activity, but acts as a transcriptional repressor in other chromatin environments (Frangini et al. 2013). Aurora kinase B also enhances binding and activity of the USP16 deubiquitinase at transcribed genes, further decreasing the level of monoubiquitinated histone H2A (Frangini et al. 2013).

It is remarkable that the histone specific lysine methyltransferases (KMTs) of TrxG and PcG complexes are active only when incorporated into large protein complexes and their activity and specificity is defined by the subunits of the respective complexes (Del Rizzo and Trievel 2011). Whereas TrxG and PcG proteins may exert antagonistic effects during interphase, it was observed that in living transgenic lines of *Drosophila* a small fraction of both TrxG and PcG proteins, fused to enhanced green fluorescent protein (EGFP), interact in a cooperative manner and remain bound to mitotic chromatin (Fonseca et al. 2012; Steffen et al. 2013). In *Drosophila* embryos, both PcG and TrxG proteins remain associated with newly replicated chromatin as well (Petruk et al. 2012). These data suggest that similarly to variant histones and pioneer transcription factors (see Sects. 1.2.4 and 1.2.5), PcG and TrxG proteins bound to newly replicated DNA molecules and to mitotic chromatin may bookmark certain promoters for repression or activation in the daughter cells, establishing thereby heritable chromatin states (epigenetic memory).

1.2.4 Variant Histones

In addition to covalently modified tails of core histones, certain histone variants that replace invariant histones in histone octamers may also convey epigenetic information (Volle and Dalal 2014). In contrast to core histones which associate with newly replicated DNA, variant histones can be incorporated into the chromatin through-

out the interphase in a replication-independent manner. Thus, it was observed that the histone variant H2A.Z marked transcriptional start sites of active genes in interphase cells and retained its position even in highly condensed mitotic chromosomes (Kelly et al. 2010). Its nucleosome occupancy changed in mitotic chromatin. H2A.Z acted like a bookmarking protein that permitted promoter activation in the daughter cells after chromosome decondensation. Based on these data, Kelly and Jones suggested that altered nucleosome occupancy may form a novel epigenetic mechanism (Kelly et al. 2010; Kelly and Jones 2011).

In *Caenorhabditis elegans*, the histone variant H2A.Z, also called HTZ-1, acts in concert with BET-1, a member of the bromodomain and extra terminal (BET) family of acetylated histone binding proteins to maintain the expression status of selector genes that govern the fates of cell groups (Shibata and Nishiwaki 2014). Although the level of histone acetylation decreases during the mitotic phase and most of the acetylated histone-binding proteins do not bind to mitotic chromatin, BET-1 remains associated with selector gene loci enriched in acetylated histones where H2A.Z is deposited. In this case H2A.Z represses transcription, but maintains the poised state of RNA polymerase II at the promoters (Shibata and Nishiwaki 2014). Shibata and Nishiwaki (2014) suggested that, upon receiving a proper differentiation signal, H2A.Z is released permitting the transcription of selector genes that specify cell, tissue or regional identity.

Variant histones function not only as epigenetic marks, but also as transcriptional regulators, although their deposition may facilitate chromation access of both activating and repressive regulatory complexes (Weber and Henikoff 2014). In addition, the variants H2A.X and H2A.Z are involved in the repair of double-strand DNA breaks (Volle and Dalal 2014). A subset of active promoters, enhancers and insulator regions is enriched in two distinct variant histones, H3.3 and H2A.Z (Jin et al. 2009). Nucleosome core particles containing H3.3/H2A.Z double variant histones are instable, a feature facilitating the access of transcription factors to such areas ("nucleosome-free regions") (Jin et al. 2009). It is worthy to note that histone chaperones play an important role in nucleosome assembly (reviewed by Burgess and Zhang 2013), as well as in the regulation of histone modifications (Begum et al. 2012; Stevenson and Liu 2013; Wang et al. 2013).

1.2.5 Pioneer Transcription Factors

It was observed that in living cells transcription factors are regularly unable to access the majority of their consensus binding sites in the genome due to the nucleosomal structure of the chromatin and the folding of nucleosomes into higher order structures (reviewed by Zaret and Carroll 2011). Cooperative binding with other transcription factors may circumvent this problem and a unique category of nuclear regulatory proteins, called *pioneer transcription factors* can access their target sites even on nucleosomes located to heterochromatic regions. Their binding to their recognition sites is usually stable and preceeds the binding of other transcription factors to promoter regulatory sequences. The association of pioneer transcription factors with enhancer regions may speed up inductive responses or alter the local chromatin structure that enables the binding of other transcription factors (reviewed by Zaret and Carroll 2011).

Heterochromatin binding, the remarkable capacity of the pioneer transcription factors belonging to the FoxA (forkhead box protein A) family is due to their structural similarity to the linker histone: there is a winged helix motif present in both FoxA proteins and histone H1 that facilitates nucleosome binding. In addition, the C-terminal domain of FoxA proteins contributes to chromatin opening, whereas the N-terminal transactivating domain may recruit coregulator proteins.

In addition to variant histones, direct binding of distinct non-histone proteins to regulatory regions of the genome may also constitute epigenetic marks that can be inherited to daughter cells. It was demonstrated that "*pioneer*" *transcription factors* or "bookmarking" proteins remain bound to chromatin even in mitotic chro-

mosomes, and accelerate transcriptional reactivation following mitosis (Zaret et al. 2008; Caravaca et al. 2013). Not only can pioneer factors bind to highly methylated DNA sequences and occupy the position of histone H1 at nucleosomes, but they can establish euchromatic regions as well by inducing local cytosine demethylation. As described above, their binding to *tissue-specific enhancers* preceeds transcriptional activation of the genes associated by such pre-marked enhancers (Zaret et al. 2008).

1.2.6 Long Noncoding RNAs

Long noncoding RNA (lncRNA) molecules are potential carriers of epigenetic information. They may directly interact with PRC2 and target the activity of the histone lysine methyltransferase EZH2 to selected genomic loci, resulting in trimethylation of histone H3 at lysine 27 (H3K27me3), chromatin condensation, and promoter silencing. LncRNAs interact with other chromatin remodeling complexes as well (reviewed by Nie et al. 2012).

In principle, lncRNAs may affect chromatin structure by acting as tethers, scaffolds, allosteric regulators or decoys, and may directly interfere with transcription as well (reviewed by Keller and Buhler 2013). Tethering or recruitment of chromatin-modifying complexes by lncRNAs may either silence or activate gene expression. Approximately 3,300 large intergenic noncoding RNAs (lincRNAs) – encoded in genomic regions located between genes – were detected in various human cell types (Khalil et al. 2009). In addition to the well-characterized lincRNA HOTAIR (HOX transcript antisense RNA) that binds both the PRC2 (Rinn et al. 2007) and the lysine-specific histone demethylase LSD1 (Tsai et al. 2010), a series of other lincRNAs also associated with PRC2 or other chromatin modifying complexes. Thus, it was suggested that certain lincRNAs may guide chromatin-modifying complexes to specific genomic loci and control promoter activity (Khalil et al. 2009). The role of lncRNAs in the transmission of epigenetic memory remains to be established.

1.2.7 Long-Distance Chromatin Interactions

DNA methylation and histone modifications may spread from their primary sites of deposition to neighbouring chromatin areas resulting in the establishment of extended heterochromatic or euchromatic regions, i.e. nuclear subcompartments repressing or facilitating transcription, respectively (reviewed by Gyory and Minarovits 2005; Doerfler 2012). Long-distance chromatin interactions mediated by CCCTC binding factor (CTCF) and cohesin proteins may insulate chromatin domains and allow coregulation of promoters within the loops by preventing the spread of chromatin modifications from adjacent areas.

Burke et al. observed that CTCF was bound to mitotic chromosomes and found that a chromatin loop at the Igf2/H19 locus could also be detected in mitosis (Burke et al. 2005). They also observed the loss of a neighbouring chromatin loop in mitotic chromatin. This finding indicated, however, that certain chromatin loops may be preserved in mitotic chromosomes and could possibly contribute to epigenetic memory (Burke et al. 2005). In contrast, the insulator upstream of the c-MYC gene changed its structure in mitotic HeLa cells in parallel with the disappearance of the sequence-specific direct binding of CTCF (Komura et al. 2007). This observation suggested that the nucleoprotein complex involving this particular insulator element must be reassembled *de novo* after cell division (Komura et al. 2007). The potential contribution of long distance chromatin interactions to epigenetic memory needs further studies.

1.3 Chromatin Types

1.3.1 Euchromatin (Active Chromatin)

Euchromatic regions are characterized by a relaxed, decondensed structure that permits the access of transcription factors, RNA polymerases and recombinases. Transcribed housekeeping genes and cell type or tissue specific genes are

preferentially located to such active chromatin domains. Promoters and regulatory sequences located to euchromatic domains are typically unmethylated or hypomethylated and they are frequently marked by euchromatic, activating histone modifications including acetylated histone H3 and histone H4 or histone H3K4me2/me3. Active chromatin is organized in three dimensions, forming topologically associating domains (TADs) that are flanked by insulator elements and characterized mainly by internal chromatin interactions (Ciabrelli and Cavalli 2015). The 3D folding of the chromatin, i.e. the formation of DNA loops is mediated by insulator binding proteins including CTCF and cohesin complexes that may interact with coactivator proteins to connect enhancer and promoter sequences; such interactions may generate cell type-specific or developmental stage-specific looping, and corresponding gene expression patterns (Rubio et al. 2008; Kagey et al. 2010). It was observed that transcriptionally active chromatin regions and euchromatic domains involved in recombination are preferentially located in the nuclear interior and translocation of inactive, heterochromatic domains from the nuclear periphery to more central regions facilitates the switching-on of silent promoters and creates a favourable environment for the gene recombination in cells of the adaptive immune system (reviewed by Gyory and Minarovits 2005).

1.3.2 Hererochromatin (Repressive Chromatin)

Heterochromatic regions are characterized by a condensed structure that usually hinders the access of transcription factors, RNA polymerase complexes and recombinases to their recognition sequences. Silent genes are frequently located to such inactive chromatin domains. The promoters and regulatory sequences of silent genes are typically hypermethylated and are frequently marked by heterochromatic, repressive histone modifications including deacetylated histone H3 and histone H4 or histone H3K9me3 and H3K27me3. Depending on the epigenetic marks enriched in heterochromatic domains, one can distinguish between various subtypes of heterochromatin.

1.3.2.1 Polycomb-Repressed Chromatin

Polycomb-repressed chromatin corresponds to genomic regions silenced by PRC1 and PRC2 repressive complexes. These compacted chromatin domains are typically enriched in histone H3K27me3. Long-range interactions between TADs may displace Polycomb-repressed chromatin from active chromatin regions or lamina-associated nuclear areas (reviewed by Ciabrelli and Cavalli 2015).

1.3.2.2 Null Chromatin, Lamin-Associated Domains

The so called null chromatin apparently lacked typical histone marks in genome-wide studies. It was highly enriched, however, in lamin proteins that form a meshwork just below the nuclear membrane and frequently silence the lamin associated domains (LADs) of chromatin at the nuclear periphery. LADs are insulated by CTCF from the neighbouring chromatin areas that frequently contain transcribed housekeeping genes (reviewed by Ciabrelli and Cavalli 2015). It is worthy to note that in addition to its location at the nuclear periphery, lamin B1 is also present in the nucleoplasm as an internal lattice, and it is associated with matrix attachment regions (MARs), i.e. DNA sequences acting as epigenetic insulators (Luderus et al. 1992; Arope et al. 2013). MARs correspond to LADs and typically contain AT-rich elements (AT core) that may unwind when exposed to superhelical strain. The AT core is relatively histone-poor but enriched in RNA polymerase II, CTCF and the euchromatic mark histone H3K4me3 (Arope et al. 2013). MARs may augment gene expression, whereas depletion of lamin B1 may severely inhibit both RNA polymerase II and RNA polymerase I mediated transcription (Tang et al. 2008; Arope et al. 2013).

1.3.2.3 Constitutive Heterochromatin

Constitutive heterochromatin is located to centromeric and telomeric regions that are usually

marked by H4K20me3 and H3K9me2/me3. The latter mark is typically bound by HP1a (heterochromatin protein 1a). Constitutive heterochromatin is highly compacted and it is devoid of euchromatic histone modifications. The repressive mark H3K27me3 is also absent (reviewed by Ciabrelli and Cavalli 2015).

1.4 Coregulation of Gene Batteries

Coordinated expression of tissue- or cell-type-specific genes could be achieved by repetitive regulatory sequences situated at the control regions of coregulated genes. Alternatively, the organization of chromatin into higher order structures, e.g. chromatin loops may ensure the coordinated activation or silencing of gene batteries (reviewed by Gyory and Minarovits 2005). Clustering of genes may arrange them into domains enriched in transcription factors, and such co-transcribed genes located to "transcription factories" may be located quite far from each other, even on different chromosomes in interphase nuclei (reviewed by Osborne 2014, see also Sect. 1.4.2). Although RNA polymerase II may reside in transcription factories only transiently, proteomics studies support the existence of such stable, lamin-associated compartments for active transcription (Melnik et al. 2011). Active tranacription may affect the three-dimensional organization of the genome, in concert with CTCF and cohesins that establish and stabilize chromatin loops (Choi and Feeney 2014; Osborne 2014).

1.4.1 Locus Control Regions, Chromatin Loops, Topologically Associated Domains

Locus control regions (LCRs) typically contain enhancer sequences and insulator elements that separate the genes located within a chromatin loop from the surrounding chromatin domains and regulatory elements. A locus control region may bind tissue-specific transcription factors that may contribute to regional DNA hypomethylation and demethylation of methylated histones, too (for review see Gyory and Minarovits 2005). The boundaries of TADs are determined by the insulator binding protein CTCF that prevents spreading of heterochromatin and blocks enhancer activity. CTCF binding is frequently associated with the attachment of cohesin subunits that stabilize the chromatin loops formed by CTCF-CTCF interactions (Choi and Feeney 2014). The architecture of chromatin loops at the major histocompatibility comlex (MHC) is affected by the matrix attachment region (MAR)-binding protein SATB1 (special AT-rich sequence binding protein 1). SATB1 interacts with PML nuclear bodies, a subnuclear structure first described in promyelocytic leukemia cells (Kumar et al. 2007).

1.4.2 Nuclear Subcompartments

Switching of the nuclear environment may facilitate or suppress both transcription and genetic recombination. Relocation of V, D, and J gene segments of the active IgH allele occurs during B lymphocyte development: in pro-B cells they move away from the nuclear periphery to an euchromatic domain before recombination and subsequent transcription (reviewed by Gyory and Minarovits 2005). It is interesting to note, however, that active genes may also associate with the nuclear periphery: Recently, using three-dimensional imaging and chromatin immunoprecipitation (ChIP)-chromosome conformation capture (3C) techniques, Park et al. observed that in murine plasma cells active immunoglobulin genes were located in the vicinity of the nuclear periphery (Park et al. 2014). In addition, active *IgH*, *Igκ* and *IgJ* genes, coding for immunoglogulin heavy and light chains and for J (joining) chain, a component of IgM and IgA molecules secreted to the mucosa, were preferably colocalized in transcription factories (see below, Sect. 1.4.2). Because *IgH*, *Igκ* and *IgJ* are located on three different chromosomes, such an arrangement may facilitate *trans*-chromosomal emhancer interactions and the utilization of shared transcription factors (Park et al. 2014).

In parallel with the nuclear subcompartment switch, the B cell and T cell antigen receptor loci undergo large-scale structural changes (locus contraction). The rosette-like chromatin loops formed by CTCF and cohesins collapse into a single globule that permits recombination even between variable (V), diversity (D) and joining (J) gene segments located at a long distance from each other (Choi and Feeney 2014). These 3D changes are facilitated by transcription of non-coding RNAs (ncRNAs) encoded in euchromatic regions that are brought into juxtaposition by a long-range enhancer to the same transcription factory (Choi and Feeney 2014).

1.4.3 Transcription Factories, Active Chromatin Hubs

Transcription factories are nuclear foci that contain two or more DNA-dependent RNA polymerase enzymes active on at least two different templates (Osborne et al. 2004; Papantonis and Cook 2013). Active promoters may interact with multiple cis-regulatory elements located at a considerable distance from the promoter. Looping out of DNA may bring the regulatory elements in the vicinity of promoters. Active chromatin hubs (ACHs) are defined as spatial units of regulatory DNA elements interacting with an active promoter as an ACH (de Laat and Grosveld 2003; Harmston and Lenhard 2013). An ACH may contain more than one transcribed genes, and a transcription factory may be composed of several ACHs. Transcription factories and active chromatin hubs appear to be transient three-dimensional chromatin domains formed in interphase nuclei that do not play a role in epigenetic inheritance.

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HIV-Induced Epigenetic Alterations in Host Cells

2

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Abstract

Human immunodeficiency virus (HIV), a member of the *Retroviridae* family, is a positive-sense, enveloped RNA virus. HIV, the causative agent of acquired immunodeficiency syndrome (AIDS) has two major types, HIV-1 and HIV-2 In HIV-infected cells the single stranded viral RNA genome is reverse transcribed and the double-stranded viral DNA integrates into the cellular DNA, forming a provirus. The proviral HIV genome is controlled by the host epigenetic regulatory machinery. Cellular epigenetic regulators control HIV latency and reactivation by affecting the chromatin state in the vicinity of the viral promoter located to the 5′ long terminal repeat (LTR) sequence. In turn, distinct HIV proteins affect the epigenotype and gene expression pattern of the host cells. HIV-1 infection of $CD4^+$ T cells *in vitro* upregulated DNMT activity and induced hypermethylation of distinct cellular promoters. In contrast, in the colon mucosa and peripheral blood mononuclear cells from HIV-infected patients demethylation of the *FOXP3* promoter was observed, possibly due to the downregulation of DNA methyltransferase 1. For a curative therapy of HIV infected individuals and AIDS patients, a combination of antiretroviral drugs with epigenetic modifying compounds have been suggested for the reactivation of latent HIV-1 genomes. These epigenetic drugs include histone deacetylase inhibitors (HDACI), histone methyltransferase inhibitors (HMTI), histone demethylase inhibitors, and DNA methyltransferase inhibitors (DNMTI).

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J. Minarovits, H.H. Niller (eds.), *Patho-Epigenetics of Infectious Disease*, Advances in Experimental Medicine and Biology 879, DOI 10.1007/978-3-319-24738-0_2

Keywords

HIV latency • *de novo* methylation • T cell specific genes • "Shock and kill" therapy • Histone deacetylase inhibitors

2.1 Introduction

The meaning of epigenetics keeps revolving ever since Waddington first coined the word to describe the idea that phenotype arises from genotype through programmed changes in development (Waddington 1953). A more recent definition of an epigenetic trait that it is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence (Berger et al. 2009). To date, epigenetic mechanisms include DNA methylation, histone modification, histone variants, nucleosome positioning, non-coding RNA and others, which are largely unknown. All of these epigenetic mechanisms closely intertwine with each other and regulate the gene expression programs of a cell to ensure its proper cellular function. They are responsive to changes in the environment of a cell, including all the developmental signals and environmental cues that lead to diseases, which is particularly evident during cellular differentiation and cancer development (Timp and Feinberg 2013; Boland et al. 2014; Cullen et al. 2014). A reasonable hypothesis is that environmental cues associated with diseases might initiate or influence the epigenetic processes of host cells, leading to epigenetic reprogramming of host cells to favor their pathogenic function and contributing to the development of the disease. One of these pathogenic environmental factors is human immunodeficiency virus (HIV) infection. HIV often targets several immune and non-immune cells including T helper lymphocytes, macrophages, dendritic cells and brain resident microglia, leading to changes in epigenetic modifications such as DNA methylation, histone modification, and microRNA (miRNA) to facilitate provirus integration, disease progression and viral latency. The highly environment-responsive and heritable nature of epigenetic modifications suggests that they can modulate the course of HIV-1 infection and play important roles in chronic diseases associated with HIV and its effect on T cells. Even with the great advance of highly active antiretroviral therapy (HAART) in controlling HIV replication, the treatment does not associate with a complete restoration of the T cell count and functions. Therefore understanding the epigenetic mechanisms utilized by HIV during infection may benefit the treatment and eradication of the virus.

In this book chapter, we summarized recent progress in characterizing how HIV infection utilizes epigenetic mechanisms in provirus integration, virus replication and establishment of viral latency in host cells. The clinical implications of these findings and the potential therapeutic drugs to interfere with epigenetic changes will be discussed. As regards miRNA in HIV infection, it is reviewed extensively elsewhere (Sun et al. 2012; Tan Gana et al. 2012; Swaminathan et al. 2014), and will be discussed briefly in this chapter.

2.2 Background on Epigenome

DNA methylation is the chemical modification of cytosine by covalently adding a methyl group to its 5-carbon. Most of the time methylated cytosine is found in the context of CpG dinucleotide. The CpG dinucleotides tend to cluster in regions called CpG islands, defined as regions of more than 200 bases with a G+C content of at least 50 % and a ratio of observed to an expected frequency of at least 0.6. Genome-wide profiling of DNA methylation by next-generation sequencing in several species demonstrated DNA methylation at promoters and 3′ end of a gene is negatively associated with gene expression levels, whereas gene body methylation seems to be positively relevant (Feng et al. 2010; Zemach et al. 2010). In mammalian cells, DNA methylation is maintained by DNA methyl-transferases. Different DNA methyl transferases had been characterized. For example, DNMT1 methylates hemi-methylated parent-daughter duplexes

during DNA replication and DNMT3a and 3b *de novo* methylate DNA.

Studies on the mechanism for demethylation were very controversial and inconsistent. Recently TET proteins and 5-hydroxymethylcytosine were proposed as a promising mechanism to demethylate DNA (Hill et al. 2014). Active methylation by DNMTs and demethylation by TETs may result in the dynamic DNA methylation changes during development by controlling gene expression. Proteins that bind to 5mC including methyl-DNA binding domain proteins (MBD1-3), methyl-CpG binding protein 2 (MeCP2) can recruit histone modification enzymes to regulate transcription (Klose and Bird 2006).

Post-translational modification of histone tails is another epigenetic mechanism that is broadly studied and it results in a combinatorial readout that affects gene expression through the regulation of local chromatin structure. Several most commonly studied histone modifications include histone 3 (H3), specifically H3 lysine 9 methylation (H3K9me), H3 lysine 4 methylation (H3K4me), H3 lysine 27 methylation (H3K27me), H3 lysine 27 acetylation (H3K27ac), and H3 lysine 36 methylation (H3K36me3). They have been shown to predict and correlate accurately with chromatin/transcriptional states of regulatory elements in many different cell types.

High-resolution, genome-wide studies have elucidated common themes of gene regulation by modified histones. Genomic regulatory regions that respond to developmental and environmental stimuli (i.e., enhancers and promoters) are marked by histone modifications that confer transcriptionally permissive (euchromatin) or repressive (heterochromatin) chromatin states, which are mediated by the Trithorax group proteins and polycomb group proteins, respectively. Common euchromatin modifications are H3K4me3, H3K9ac, H3K27ac, and H3K36me3. They are found primarily at active enhancers (H3K9ac and H3K27ac), promoters (H3K4me3), and within the bodies of actively transcribed genes (H3K36me3) (Ernst et al. 2011). H3K4 methyltransferase Setd1 generates H3K4me3, which displays a punctate localization pattern within 1–2 kb near promoters of active genes (Bernstein et al. 2006). It promotes transcription by recruiting nucleosome remodeling complexes and histone acetylases. Histone deacetylases (HDACs) can remove such active marks and repress gene expression. In addition, repressed chromatin often acquires two repressive marks H3K9me3 and H3K27me3. The histone lysine methyltransferase (HKMT) that catalyzes H3K9me3 and H3K9me2 is G9a. The HKMT that catalyzes H3K27me3 is enhancer of zeste homolog 2 (EZH2), which is a member of the multisubunit polycomb repressive complex 2 (PRC2). PRC2 is targeted to genomic regions in response to environmental cues to generate H3K27me3. This modification recruits another multiprotein complex, PRC1, which contains members of the chromobox family that recognize H3K27me3. Another component of PRC1 is the ring finger protein, RING1B, an ubiquitin ligase that catalyzes monoubiquitination of histone H2A, a modification that impedes RNA polymerase II (RNAPII) elongation, resulting in transcriptional repression. The enzyme that demethylates H3K4me1/2 and H3K9 is lysine-specific demethylase 1 (LSD1, encoded by KDM1A), while the Jumonji domain 2 (JMJD2, encoded by KDM4A) demethylates H3K9me3 and H3K36me3 and JMJD3 (encoded by KDM6B) demethylates H3K27me3.

Many cellular differentiation processes, including immune cell differentiation, are accompanied by dynamic changes in DNA methylation and histone modifications, which often occur at key transcription factor sites and at genomic locations encoding functional molecules such as cytokines to control their lineage commitment (Ji et al. 2010; Zhu et al. 2010; Cullen et al. 2014; Zhang et al. 2014). Protein components in epigenetic machinery such as DNA methyltransferases (DNMTs), DNA methyl-group binding proteins and histone modification enzymes, often bind to these cytokine signature gene loci through interaction with key transcriptional factors, setting up local epigenomic structure and controlling their expression (Zhu et al. 2010). In addition, environmental cues including viral infection also can directly regulate the expression levels of DNMTs (Pion et al. 2013; Abdel-Hameed et al. 2014) or accumulation of histone modifiers (HDACs and HKMTs) at targeted genes (Friedman et al. 2011).

Recently, non-coding RNAs, including long non-coding RNAs (lncRNAs) (Saayman et al. 2014) and microRNAs, have emerged as important regulators of gene expression in diverse biological contexts. They generally control gene expression by modulating transcription, or via post-translational mechanisms targeting the splicing, stability or translation of mRNA. LncRNAs are long non protein-coding transcripts larger than 200 nucleotides and they are the most abundant non-coding RNA species in the mammalian genome (13,870 in the human and 4,074 in the mouse genome). The mechanism(s) employed by lncRNAs differ depending on their cytosolic or nuclear location. In the cytosol, lncRNAs directly interact with target mRNAs (or miRNAs) to control their expression and to regulate mRNA translation or interact with specific signaling proteins to regulate pathway-specific gene expression programs such as in T cells and dendritic cells (Willingham et al. 2005; Wang et al. 2014). In contrast, nucleus-localized lncRNAs largely function by modulating epigenetic processes such as histone modifications to alter gene expression by acting as a guide, decoy, or scaffold (Guttman and Rinn 2012; Rinn and Chang 2012). In addition to a variety of biological processes, a functional role of lncRNAs has started to emerge in controlling gene expression in the immune system, including innate cell development, adaptive immunity (T and B cells) and host defense against microbial infection such as HIV infection (Atianand and Fitzgerald 2014). Different from LncRNA, miRNAs is a group of single-stranded short non-coding RNAs (~22–25 nt) that complement the 3′-untranslated regions of their messenger RNA targets to regulate their expression. In mammalian cells, miRNAs are generated from multiple intron specific regions or can be transcribed from their own promoters, which is dependent on two RNase III enzymes Drosha (from primary miRNA to pre-miRNA in the nucleus) and Dicer (from pre-miRNA to mature miRNA in the cytoplasm). Approximately 400 miRNAs have been identified in humans, and more than half of mammalian transcripts are predicted to be targets of miRNAs. Not surprisingly, many reports indicate that miRNAs play important roles in regulating virtually all biological processes including hematopoiesis and the functions of immune cells, including T cells (Baltimore et al. 2008).

In order to evade the immune responses of their host, viruses will target host genes involved in cell cycle progression, senescence, survival, inflammation, and immunity for epigenetic manipulation. In the following section we will discuss HIV integration into host DNA, HIV latency, and the effect of epigenetic modification in HIV progression.

2.3 HIV Integration

HIV is a plus-sense RNA virus that requires reverse transcription of its RNA to DNA and then DNA integration to establish a chromosomal provirus as an obligate replication intermediate. Linear HIV DNA is cleaved at each 3′ end to generate the precursor ends for integration. The resulting recessed 3′ ends are inserted into target DNA by a subsequent DNA strand transfer reaction (Engelman et al. 1991). Briefly, HIV DNA integration involves a coordinated set of DNA cutting and joining reactions. The reverse transcriptase enzyme of HIV synthesizes linear double-stranded cDNA, which is the template for the viral enzyme integrase. Integrase catalyzes two separate enzymatic reactions in the infected cells: an initial 3′ processing of the cDNA ends, which is followed in the nucleus by their covalent interactions to the 5′ phosphates of a double-stranded staggered cut in chromosomal DNA. HIV integration favored active transcription units, however, the mechanism underlying HIV-1 selectivity is largely unknown (Serrao et al. 2014). There is strong evidence that HIV favors particular bases at sites of integration, mainly near transcription-associated histone modifications, such as H4 acetylation, and H3 K4 methylation and H3 acetylation. It disfavored regions rich in transcription-inhibiting modifications, which include DNA CpG methylation and H3K27me3 (Wang et al. 2007). Recently, a total of 1610 unique HIV-1 integration sites were identified. In general, most

of these sites have overlapping flexible Purine (R)/pyrimidine (Y) dinucleotides at the center of the integration site (Serrao et al. 2014). Due to the initial role of HIV integration in the life cycle of the virus, HIV-1 integrase is an important therapeutic target in the fight against HIV. Integrase inhibitors target the enzyme active site and they have been used in multiple clinical trials, but the generation of drug resistance poses a great challenge (Engelman et al. 2013).

2.4 HIV Latency and Reactivation

After HIV integrates into the host genome, the HIV viral genome starts regulating its replication. Viral gene expression is regulated by the HIV LTR, which interacts with multiple viral and cellular DNA-binding proteins. In latently infected T cells, the viral life cycle is arrested following integration. Latency is a complex process that involves several factors, including epigenetic modification (Williams et al. 2006; Easley et al. 2010), promoter occlusion (Duverger et al. 2009), a lack of necessary transcription factors in resting cells (Williams and Greene 2007), and restriction of translation by viral and host miRNAs (Huang et al. 2007). In latent $CD4^+$ resting T cells, the lack of active forms of key cellular transcription factors (Nabel and Baltimore 1987; Bohnlein et al. 1988; Kinoshita et al. 1997; West et al. 2001; Ganesh et al. 2003; Shan et al. 2011), and of the HIV Tat protein and its cellular cofactors (Selby and Peterlin 1990; Jones and Peterlin 1994; Tyagi et al. 2010), limits viral transcription (Lassen et al. 2004; Williams and Greene 2007; Shan et al. 2011). Additionally, DNA methylation and histone modifications have been postulated to promote transcriptional silencing of integrated proviruses (Coull et al. 2000; Williams et al. 2006; Blazkova et al. 2009; Kauder et al. 2009).

During latency, the HIV-1 promoter is hypermethylated at two CpG islands surrounding the HIV-1 transcriptional start site, and DNMTs are most likely recruited to the HIV-1 promoter (Fig. 2.1). Additionally, nuc-0 and nuc-1, nucleosomes located around the HIV-1 promoter region of latent HIV proviruses, blocks transcriptional elongation. Thus, only short transcripts containing TAR (trans-activation response element, a binding site of the viral transactivator Tat) are produced. Nuc-1 maintains its hypoacetylation by HDACs recruited to the 5′-LTR via several transcription factor such as YY1, CTIP-2, p50-p50 homodimer, CBF-1 (Fig. 2.1). The co-repressor CTIP-2 recruits HDACs and the HMT Suv39h1 and interacts with the Sp1 transcription factor at the HIV-1 5′-LTR. Other repressive histone marks such as H3K27me3 or H3K9me2 are also implicated in HIV-1 latency.

NF-κB, a key transcription factor that regulates genes involved in anti-apoptotic (survival) and pro-inflammatory pathways, is one of multiple cellular proteins that interacts with the HIV LTR to regulate gene expression and disease progression (Gilmore 2006). NF-κB contributes substantially to maximal viral replication (Leonard et al. 1989; Chen et al. 1997; Chene et al. 1999), and increases the pathogenic potential of retroviruses (Dollard et al. 1994). During latency reactivation, the active form of NF-κB is sequestered in the cytoplasm by the inhibitor of nuclear factor κB (IκB), while NF-κB p50-p50 homodimers occupy the NF-κB sites at the viral LTR region.

Foxp3+ regulatory T cells (T_{reg}) play an important role both in HIV replication and in immune responses to HIV. Over-expression of *Foxp3* in T cells resulted in a decrease in basal levels of NF-κB activation and subsequent suppression of the transcription of HIV LTR (Grant et al. 2006). The role of T_{reg} cells in HIV infection is still controversial. HIV gp120 binds to T_{reg} and enhances its immunosuppressive function (Christman 2002). In contrast, others have reported a beneficial effect of T_{reg} by decreasing HIV infection in conventional T cells through direct contact and activation of a c-AMP dependent mechanism (Irizarry et al. 2009). Mechanisms of HIV latency in T_{reg} are not thoroughly investigated. However, there is evidence that HIV uses epigenetic modifications to evade host immunity and ensure its latency (Williams et al. 2006; Williams and Greene 2007; Colin and Van Lint 2009; Easley et al. 2010).

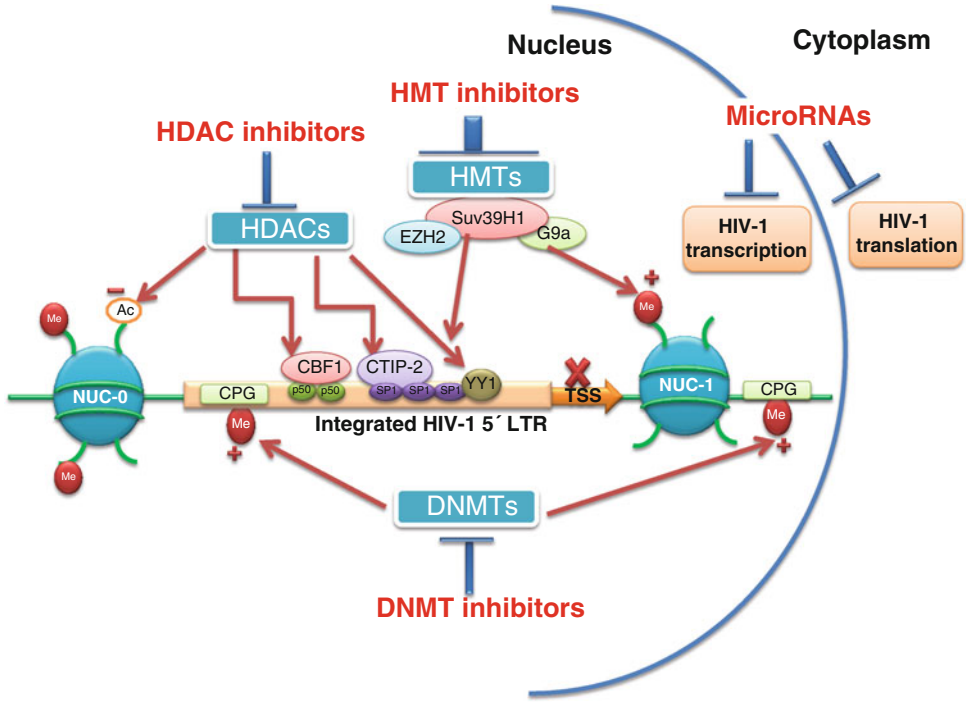

Fig. 2.1 Mechanisms by which drugs target epigenetic changes in HIV-1 infection. The figure above is a schematic representation of epigenetic modifications that play roles in the nucleosome organization of the HIV-1 genome 5′ LTR and maintain a repressed chromatin structure in a latent HIV-1. HDACs recruitment to HIV-15′-LTR via several transcription factors such as YY1, CTIP-2, p50-p50 homodimer, and CBF-1 results in de-acetylation of histones at nuc-0 and nuc-1 nucleosomes. HMTs, such as Suv39h1, EZH2 and G9a methylate histones at nuc-0 and nuc-1 nucleosomes. The transcription co-repressor CTIP-2 recruits HDACs and the HMT Suv39h1 and interacts with the Sp1 transcription factor at the HIV-1 5′-LTR. Recruitment of DNMTs leads to hypermethylation of two CpG islands surrounding the HIV-1 transcriptional start site. HDAC, HMT and DNMT Inhibitors can inhibit these suppressive mechanisms and restore suppressed HIV-1 promoter with consequent gene expression. MicroRNAs as therapeutic targets can block HIV-1 receptor binding, transcription or translation

2.5 HIV Progression and Epigenetic Modification

Several factors are involved in the control of HIV transcription/replication, including epigenetic modifications at the promoter level. Analysis of the HIV long terminal repeat (LTR) methylation status in infected patients showed a higher degree of DNA methylation in the 5′-LTR of long-term non-progressor and elite controller (LTNP/EC) versus progressor patients. There was also a positive correlation between the level of DNA methylation and time of infection, indicating a certain contribution of HIV LTR silencing in reducing the number of replicating viruses which may account for a delayed progression (Palacios et al. 2012). Epigenetic modifications can modulate HIV-1 integration, transcription and latency of infection. Integration of HIV-1 into the host genome is not likely to occur in a transcriptionally inactive, methylated region (Bushman et al. 2005). In latently infected $CD4^+$ T cells, the transcription of the integrated HIV-1 is usually suppressed by methylation of its promoter (Kauder et al. 2009). Epigenetic modification of host genes can presumably affect HIV-1 transcription and replication. Furthermore, HIV-1 can induce *de novo* methylation of the host T cell-specific genes through the induction of DNA methyltransferase 1 (DNMT1) *in vitro*

(Youngblood and Reich 2008). Recent data suggested that methylation of 5′-LTR of HIV is associated with the control of viral replication in a subset of patients characterized as LTNP/EC (Palacios et al. 2012). In our laboratory we studied the role of HIV-1 infection in the aberrant methylation of the genome, particularly in immune-related genes by which the virus can evade the host immune system. Specifically, we measured the levels of DNA methylation within the *FOXP3* promoter (as a biomarker for T_{reg}). We also investigated the effect of aberrant DNA methylation level of the *FOXP3* gene and protein expression. Additionally, we examined the relationship between *FOXP3* methylation and the clinical profile of HIV-1 infected patients and its correlation with immunological and virological status. A significant positive correlation between the level of *FOXP3* methylation and blood $CD4^+$ counts was found (Abdel-Hameed et al. 2014). To investigate possible mechanisms of aberrant methylation of *FOXP3* by HIV-1 infection, gene expression patterns of DNA methylation enzymes were tested by Microarray analysis and quantitative rt-PCR. Expression of *DNMT1*, *DMAP1*, *METTL7B*, and *METTL10*, were significantly down regulated in HIV-infected patients compared to controls and had a significant positive correlation to *FOXP3* promoter methylation (Abdel-Hameed et al. 2014). We concluded that a lower level of *FOXP3* promoter methylation was significantly associated with changes in the gene and protein expression of *FOXP3* and a higher frequency of $T_{reg,}$ which may be caused by an aberrant methylation process associated with HIV infection. Furthermore, HIV-1 can induce increased methylation of the host T cell-specific genes through the induction of DNA methyltransferase 1 (*DNMT1*) *in vitro*. Mikovits et al. showed that acute infection of T lymphocytes with HIV-1 results in increased expression of DNA methyltransferase enzyme with subsequent overall increase in DNA methylation in infected cells (Mikovits et al. 1998). The virus infection specifically increased *IFN-γ* gene promoter methylation, resulting in the subsequent down regulation of expression of this cytokine. The increased methylation effect on the methyltransferase gene transcription of the T cell and on IFN-γ was reversed by the introduction of antisense methyltransferase construct into the cells. In another study, HIV transfection of T lymphocytes has led to increased promoter methylation and transcription repression of the gene GNE, a key enzyme in the sialic acid biosynthesis pathway, which might lead to the cell-cell recognition and consequently trafficking of T cells (Giordanengo et al. 2004).

It is worth mentioning that most of the published work is related to the effect of HIV on epigenetic modifications of T-cells *ex vivo*, which does not necessarily reflect the extent or the mechanisms of epigenetic change that HIV might induce in T cells *in vivo. In vivo* studies are more comprehensive but are technically more challenging because HIV-1 infects a very small fraction of T cells. Currently, limited data is available to describe changes in host genes after *in vivo* infection and more comprehensive global studies are warranted. With the advancing techniques of single cell analysis, it is possible to isolate several types of immune cells representing the immune system interacting with the virus and compare several paradigms of gene and protein expression alterations together with epigenetic modifications.

To study the role of global histone modifications during acute infection of HIV-1 in highly proliferating T cells, the gene expression data of HIV-infected cells with their accompanying changes in protein histone post-translational modifications were examined. There were substantial changes in the histone post-translational modification that were linked to considerable fluctuations in the expression of associated chromatin enzymes. Compared to uninfected cells, there was a ninefold increase in the abundance of the histone H3K4me2 peptide that associated with a significant decrease in KDM1A, a K4 and K9 specific demethylase. However, few differences were found between HIV and HIV UV-inactivated infection (Britton et al. 2014).

The alteration in miRNA expression is by far the most frequently reported epigenetic change in T cells induced by HIV infection in *in vivo* studies. As reported earlier, miRNAs can inhibit

HIV-1 expression by either modulating host innate immunity or by directly interfering with viral mRNAs (Klase et al. 2012). Comparing the expression of 377 miRNAs in CD4⁺ T cells from HIV-1 elite LTNPs, naive patients, and exposed uninfected patients, Bignami et al. observed that 21 miRNAs significantly differentiated elite from exposed uninfected patients and 23 miRNAs distinguished naive from exposed uninfected patients, whereas only 1 miRNA (miR-155) discriminated elite from naive patients (Bignami et al. 2012). Analysis of miRNA expression after exposure of healthy CD4⁺ T cells to gp120 *in vitro* confirmed that miRNA profiles could be a signature of HIV infection in immune cells. Several other *in vivo* studies exploring miRNA expression in PBMCs of HIV infected subjects confirmed that down-regulation of miR-150, miR-29 and miR-125b family members in viremic patients (Klase et al. 2012). *In vivo* miRNA changes correlate, to some extent, with clinical disease parameters of CD4⁺ cell counts and viral loads, and therefore, could be useful biomarkers for HIV-1 disease progression.

2.6 Drugs Targeting Epigenetic Changes in HIV Infection

Current highly active antiretroviral therapies (HAARTs) for HIV infection rely on cocktails of potent antiviral drugs to reduce virus in the peripheral circulation below detectable levels. Unfortunately, this regimen fails to eradicate the virus. Even after decades of effective HAART, high levels of virus replication invariably resume when antiretroviral treatment is interrupted. The viral rebound appears to be due to reactivation of virus from a long-lived pool of latently infected cells, which is primarily in the pool of resting memory CD4⁺ T cells.

Recently, multiple epigenetic modification drugs are in clinical trials to address how to eradicate HIV latency (Table 2.1). To eradicate the viral reservoir, the latent reservoir should be removed using "shock and kill" strategy. This strategy depends on simultaneous treatment with HIV-activating agents to stimulate viral replication/production in latently infected cells and antiretroviral therapy to block new infections (Poveda 2014). Several compounds have been suggested for transcriptional reactivation of HIV-1 latency and could be divided into four main epigenetic drugs such as histone deacetylase inhibitors (HDACIs, including Vorinostat, Romidespin, Panobinostat, and Valproic acid to target the hypoacetylated state of nuc-1), histone methyltransferase inhibitors (HMTI, such as Quinazoline derivatives), histone demethylase inhibitors (such as Polyamine analogues), DNA methyltransferase inhibitors (DNMTI such as Azacitidine, Decitabine, S100, and CP-4200 to target 5′-LTR DNA methylation (Van Lint et al. 2013, Table 2.1, Fig. 2.1). In HIV-1-infected cells, treatment with HDACI reactivates the HIV-1 expression in latent reservoirs (Poveda 2014).

Table 2.1 Drugs target epigenetic changes in HIV infection

Drug	Compound	Study phase
DNMT inhibitors	Azacitidine	Preclinical
HDAC inhibitors	Vorinostat	Phase I/II
	Romidepsin	Phase I/II
HMT inhibitors	Deazaneoplanocin A (DZNep)	Preclinical
	Quinazoline derivatives	Preclinical
	Ellagic acid	Preclinical
Histone demethylase inhibitors	Polyamine analogues	Preclinical
Non-coding RNAs	miR155, miR-28, miR125b, miR150, miR223 and miR382	Preclinical
	HIV-expressed antisense ncRNA	Preclinical

HIV silencing is characterized by high levels of histone deacetylases (HDACs) and deacetylated histones as well as methylated histones at LTRs. HDACI romidepsin is the strongest HIV expression inducer of HIV in $CD4^+$ T cells from patients on suppressive antiretroviral therapy, compared to other HDACIs including vorinostat and panobinostat (Friedman et al. 2011; Wei et al. 2014). Thus, the use of HDACI as adjuvant to HAART can represent a new potential therapeutic strategy to eradicate the viral infection. Additionally, epigenetic aberrations can be reversed by the use of HDACI, or HMTI (Poveda 2014). Infections by HIV also induce expression of DNMTs, therefore DNMT inhibitors have been proposed to treat HIV latency. In addition to histone methylation, hypermethylation of CpG islands near the HIV-1 promoter correlates with silencing of HIV-1 transcription in both Jurkat cells and primary isolates from HIV-1-positive aviremic patients. Treatment of these cells with the DNA methylation inhibitor leads to enhanced reactivation and outgrowth of silenced proviruses (Blazkova et al. 2009; Kauder et al. 2009).

miRNAs represent excellent therapeutic targets due to their higher stability and interaction with several HIV proteins. Besides the fact that host miRNAs are differentially expressed in HIV-susceptible host cells ($CD4^+$, macrophages), or HIV-resistant cells (monocytes) (Fowler and Saksena 2013). *In vitro* studies have identified several host miRNAs that are directed against HIV proteins and could be potential anti-HIV therapeutic targets (Klase et al. 2012). Whether the virus itself encodes its own miRNA is still controversial. It has been shown that the viral proteins Tat, Nef and Vpr will suppress the miRNA processing machinery in the infected host cell. Small RNA molecules against previous HIV proteins can limit target expression and inhibit viral replication in T cells and primary lymphocytes (Fowler and Saksena 2013). In a recent study, a lentivirus gene vector was used to deliver several small RNA based molecules together with a ribozyme that cleaves the mRNA of chemokine receptor 5 (CCR5) protein, a co-receptor for HIV-1 infection on a subset of $CD4^+$ T cells (Burnett and Rossi 2012).

Most of the miRNA therapeutic targets are in the preclinical stage (Table 2.1). Specificity is an issue in designing miRNA genetic therapies due to the cooperative nature of those small RNA species. miRNA has the ability to bind several sites in multiple targets and can repress these targets at the same time. Overcoming those hurdles is imminent for a future successful therapy.

2.7 Conclusion

After HIV integration into the host genome, the HIV viral genome starts regulating its replication. Viral gene expression is regulated by the HIV LTR which interacts with multiple viral and cellular DNA-binding proteins. In latently infected T cells, the viral life cycle is arrested following integration. Epigenetic modification of host genes can presumably affect HIV-1 transcription and replication. Furthermore, HIV-1 can induce increased methylation of the host T cell-specific genes through the induction of DNA methyltransferase 1 (DNMT1) *in vitro*.

Current highly active antiretroviral therapies (HAARTs) for HIV infection rely on cocktails of potent antiviral drugs to reduce virus in the peripheral circulation to below detectable levels. Unfortunately, this regimen fails to eradicate the virus. When antiretroviral treatment is interrupted, high levels of virus replication invariably resume due to reactivation of virus from a long-lived pool of latently infected cells. To eradicate these viral reservoirs, one possible strategy is the simultaneous treatment with HIV-activating agents to stimulate viral replication/production in latently infected cells and antiretroviral therapy to block new infections.

Several epigenetic modifying compounds have been suggested for transcriptional reactivation of HIV-1 latency. Four groups of epigenetic drugs are proposed for this purpose including histone deacetylase inhibitors (HDACI), histone methyltransferases (HMT), histone demthylase inhibitors, and DNA methyltransferase inhibitors (DNMTI). Non-coding RNAs targeting viral proteins have also been proposed as therapeutic targets.

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Epigenetic Alterations in Epstein-Barr Virus-Associated Diseases

3

Hans Helmut Niller, Ferenc Banati, Daniel Salamon, and Janos Minarovits

Abstract

Latent Epstein-Bar virus genomes undergo epigenetic modifications which are dependent on the respective tissue type and cellular phenotype. These define distinct viral epigenotypes corresponding with latent viral gene expression profiles. Viral Latent Membrane Proteins 1 and 2A can induce cellular DNA methyltransferases, thereby influencing the methylation status of the viral and cellular genomes. Therefore, not only the viral genomes carry epigenetic modifications, but also the cellular genomes adopt major epigenetic alterations upon EBV infection. The distinct cellular epigenotypes of EBV-infected cells differ from the epigenotypes of their normal counterparts. In Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC) and EBV-associated gastric carcinoma (EBVaGC) significant changes in the host cell methylome with a strong tendency towards CpG island hypermethylation are observed. Hypermethylated genes unique for EBVaGC suggest the existence of an EBV-specific "epigenetic signature". Contrary to the primary malignancies carrying latent EBV genomes, lymphoblastoid cells (LCs) established by EBV infection of peripheral B cells *in vitro* are characterized by a massive genome-wide demethylation and a significant decrease and redistribution of heterochromatic histone marks. Establishing complete epigenomes of the diverse EBV-associated malignancies shall clarify their similarities and differences and further clarify the contribution of EBV to the pathogenesis, especially for the epithelial malignancies, NPC and EBVaGC.

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J. Minarovits, H.H. Niller (eds.), *Patho-Epigenetics of Infectious Disease*, Advances in Experimental Medicine and Biology 879, DOI 10.1007/978-3-319-24738-0_3

Keywords
Viral epigenotypes • Epigenetic signature • Genome-wide demethylation • Heterochromatic histone marks • Host cell methylome

3.1 Introduction

Epstein-Barr virus (EBV) is one of the eight currently known human pathogenic herpesviruses, together with herpes simplex virus (HSV) -1 and -2, varicella zoster virus (VZV), cytomegalovirus (CMV), human herpesvirus (HHV) -6, HHV-7, and HHV-8, also called Kaposi sarcoma-associated herpesvirus (KSHV). EBV was the first human tumor virus to be discovered, by electron microscopy in cultivated cells derived from Burkitt lymphoma (BL) biopsies (Epstein et al. 1964). Shortly after, seroepidemiology linked EBV with endemic BL (Henle and Henle 1966), the stunning ability of EBV to induce B cell growth (Henle et al. 1967; Moore et al. 1967) and to immortalize and growth-transform B cells (Pope et al. 1968) was recognized, and EBV was identified as the agent causing infectious mononucleosis (Henle et al. 1968). Localizing EBV to the malignant cells of anaplastic carcinomas of the nasopharynx (NPC), but not to the plenty of infiltrating lymphocytes, installed EBV as the first epitheliotropic human tumor virus (Wolf et al. 1973), a decade before high-risk papillomaviruses were identified in cervical carcinoma biopsies (Durst et al. 1983).

Meanwhile, EBV was also detected in between 30 and 40 % of classical Hodgkin lymphoma subtypes world-wide (Weiss et al. 1987, 1989), but close to 100 % of Hodgkin lymphoma in severely immunosuppressed hosts, and in a subset of gastric carcinomas (Burke et al. 1990; Shibata et al. 1991), encompassing approximately 8–10 % of this world-wide occurring sporadic tumor. Beyond BL, NPC and gastric carcinoma, EBV was also associated with T or natural killer (NK) cell lymphomas (Harabuchi et al. 1990), leiomyosarcoma (McClain et al. 1995), and post-transplant lymphoproliferative disease (Hamilton-Dutoit et al. 1993). All in all, EBV is probably co-responsible for about 200,000 new cases of cancer per year corresponding to between 1 and 2 % of all human neoplastic disease (Munz 2014). Furthermore, ever since elevated anti-EBV antibody levels were found in patients with systemic lupus erythematosus (Evans et al. 1971) and multiple sclerosis (Sumaya et al. 1980), EBV has been incriminated as a trigger of autoimmune diseases (reviewed in Niller et al. 2009, 2011). Therefore, the discovery of EBV, more than 50 years ago, has enormously stimulated tumor virology and cancer research in general, and high hopes have been pinned to it that EBV might turn out as a Rosetta stone for understanding multistep carcinogenesis, and also for better understanding the role of viruses in immunopathological disorders (de The 1984, 1985).

Because after primary infection EBV genomes persist in host cell nuclei as circular chromatin-structured mini-chromosomes in a latent mode, EBV has served as a very prolific model system for the development of large scale sequencing technology (Baer et al. 1984), and for studying the cellular mechanisms of DNA replication (Yates et al. 1985), and transcriptional and epigenetic regulation of gene expression (Li and Minarovits 2003). Altogether, EBV is one of the most comprehensively, if not the best-studied of all viruses. Therefore, we dedicate a separate book chapter to the epigenetic alterations in EBV-associated tumors. EBV gene expression is highly dependent on epigenetic regulatory mechanisms. In diverse cell types carrying latent viral genomes, genetically identical viral genomes bear different epigenetic marks, thereby leading to specific patterns of viral latent gene expression and establishing distinct viral epigenotypes (Minarovits 2006). However, also the host cell genomes of EBV-infected cancer cells carry distinct epigenetic marks differing from each other in dependence of the respective tumor type and differing from the epigenetic profiles of their

healthy counterparts (Niller et al. 2009). Before we review the current state of epigenetic alterations in selected EBV-associated lymphoid and epithelial malignancies, we give a brief description of the biology and clinical aspects of EBV infection, the viral latency gene products with an emphasis on their epigenetic impact, the establishment of latency in memory B cells, and the different epigenotypes that EBV genomes may assume.

3.2 Physiological Course of EBV Infection

EBV is the only known human member of the subfamily *Gammaherpesvirinae* that belongs to the genus *Lymphocryptovirus*, the name reflecting the viral property of homing to and "hiding" in the B lymphocyte compartment. Worldwide, primary EBV infection usually occurs very early in life with no or little symptoms so that at the age of 10 years most people have turned seropositive. In Europe and the USA, primary infection of up to 50 % of the population occurs later in life, i.e. in adolescence or early adulthood. In this case, symptomatic infection may ensue in up to 50 % of the infected youths, with the more severe cases presenting as infectious mononucleosis, also named kissing disease, typically including pharyngitis, tonsillitis, fever, malaise, lymphadenopathy. The disease is often accompanied with splenomegaly, a minority also with hepatomegaly, and characteristically with more than 50 % of atypical mononuclear cells in the blood smear which can be mistaken for leukemia, and from which the name mononucleosis is derived. In rare and extreme cases of mononucleosis, splenic rupture or fulminant hepatitis with liver failure may occur (reviewed in Longnecker et al. 2013).

Contrary to VZV, the virus causing chickenpox, the infectivity of EBV is not high enough to make airborne infection the regular way of transmission. Thus, EBV is transmitted by intimate contact, usually through saliva, mostly from asymptomatic mothers which are occasionally shedding the virus due to intermittent lytic replication from their tonsillar epithelia and their salivary glands, to their infants, or between adolescents. The virus is assumed to initially enter B cells at the surface of the tonsils or the oropharyngeal lymphoid tissue of Waldeyer's ring, through engagement of its major envelope glycoprotein gp350/220, with the CD21 surface receptor which is also known as the CR2 receptor for the complement component C3d (Fingeroth et al. 1984). Tonsillar epithelial cells most likely serve as helpful enhancers for initial high-level virus production and occasional virus shedding later-on, as they can take up the virus through direct cell to cell contact and sustain an efficient lytic viral replication more easily than lymphoid cells (Borza and Hutt-Fletcher 2002; Hadinoto et al. 2009).

In the course of primary infection, the lytic replication cycle is triggered by the expression of two major viral immediate early genes BRLF1 and BZLF1 which in turn activate transcription of the viral early proteins whose expression leads to viral DNA replication, and the sequential expression of viral late genes which mainly code for virus structural building blocks. During an incubation time of approximately 4 weeks, the lytic replication cycle leads to a massive local virus production and shedding within the oropharyngeal lymphoid tissue, with the virus spreading systemically to the blood stream. Within lymphoid tissues, naïve, germinal center and memory B cells are infected. A high percentage of the circulating blood memory B cell pool is infected in acute mononucleosis which may, in extreme cases, affect up to 50 % of circulating memory B cells. Contrary to naïve B cells within the lymphoid tissue, naïve B cells in the blood stream remain largely uninfected (Hochberg et al. 2004). In the course of mononucleosis the immune system mounts an efficient defense through both its cellular and humoral arms. The vigorous immune response more so than the cytolytic viral damage leads to the well-known clinical symptoms of mononucleosis.

When primary infection is finally overcome, the virus remains latent in a minority of 1–10 in a million of peripheral B cells, where no viral gene expression occurs at all, or only a very restricted set of viral latency genes is occasionally

expressed upon cell division (Qu and Rowe 1992; Miyashita et al. 1997; Hochberg et al. 2004). After primary infection, the latent virus persists invisibly to the immune system in the healthy host, and the lytic replication cycle is restricted to lymphoid surface or salivary gland epithelia to which the immune system has only limited access (reviewed in Longnecker et al. 2013).

3.3 EBV Latency Types and Tumors

In EBV-associated tumor biopsies and EBV-infected cultured cell lines, the gene expression patterns of latent viral genomes depend largely on the host cell phenotype or stage of B cell development. Based on the activity status of the C promoter (Cp), we discern two major latency groups, **Cp-on and Cp-off latency**. When the major B lymphoid-specific promoter Cp is on, the full set of latency gene products, including all six EBNA (Epstein-Barr nuclear antigen) proteins, two latent membrane proteins, the multiply spliced BART transcripts, the non-translated EBER1 and -2 RNAs and three clusters of BHRF1 and BART microRNAs, are expressed. This expression pattern is commonly called **latency type III**. Except in BL cell lines where genetic alterations have caused permanent growth and which have undergone an *in vitro* switch to latency type III, cells in latency III have been immortalized by EBV, and are driven to permanent lymphoblastoid growth. When undergoing growth-transformation, the complete EBNA-transcription program is first directed by the W promoter (Wp) which becomes progressively methylated, before upstream Cp takes over the transcriptional control of the EBNA genes (Woisetschlaeger et al. 1990; Jansson et al. 1992; Tierney et al. 2000).

The astonishing ability and efficiency of EBV to immortalize and growth-transform B cells from the peripheral blood (Pope et al. 1968; Nilsson et al. 1971) *in vitro* has captured the interest of tumor virologists since its discovery and has also dominated EBV research and tumor virology until a decade ago. However, this ability is not to be mistaken for bona fide oncogenesis, because the specific viral contribution to growth-transformation and BL-oncogenesis appear to be rather distinct (Niller et al. 2004b). In order to identify the impact of EBV-induced transformation on the methylome of primary human B cells, Hansen et al. (2014) performed whole genome bisulfite sequencing and gene expression analysis of uninfected quiescent B-cells, B-cells activated by CD40 Ligand (CD40L) plus interleukin 4 (IL-4) for 3 weeks, and EBV-infected B cells obtained from the same donors 3 and 6 weeks after the infection. Surprisingly, compared to normal B cells, large scale hypomethylated blocks comprising two thirds of the cellular genome were induced 6 weeks after EBV infection, but not by B cell activation or 3 weeks after infection. These hypomethylated blocks significantly overlap with the positions of lamina-associated domains (LADs) and large organized chromatin lysine (K9)-modified regions (LOCKs), previously described in lung and pulmonary fibroblasts (Guelen et al. 2008; Wen et al. 2009). Comparison with the data obtained from the ENCODE project on the tier 1 lymphoblastoid cell line (LCL) GM12878 suggested, that they may also be enriched in H3K27me3, while depleted of all transcription factors. Furthermore, these regions were associated with gene expression hypervariability, and largely corresponded to hypomethylated blocks previously described in human cancer (Hansen et al. 2011). These results suggest, that the formation of hypomethylated blocks may play a central role in the EBV induced transformation of B cells (Hansen et al. 2014). Accordingly, EBV-induced transformation led to a genome-wide decrease and redistribution of hetreochromatic marks (Hernando et al. 2014). Thus, the physiologically activated B-cell appears to be highly different from the growth-transformed B-lymphoblast. EBV-converted LCs can only expand in the absence of an anti-EBV directed immune response, i.e. if taken into *in vitro* culture or if the immune system is defunct, e.g. in XLP (X chromosome-linked lymphoproliferation) patients or in severely immune suppressed patients who suffer early-onset post-transplant lymphoproliferative disease (PTLD).

LCLs which can be EBV-transformed *in vitro* from the white blood cells of healthy donors, are assumed to correspond to early onset PTLD cells. Therefore, early onset PTLD does not constitute a primarily malignant tumor, because it may be resolved, if immune control kicks back in early enough. However, it may turn monoclonal in the course and regularly become malignant, if growing too long out of immune control. In support of a molecular distinction between BL and PTLD tumors, the molecular signatures of viral and cellular protein and microRNA expression indicated that the respective contribution of EBV to both tumor types was different in each case (Navari et al. 2014).

A special latency type, called **latency IIb** has recently been highlighted by the secondary *in vitro* infection of chronic lymphocytic B cell leukemia (B-CLL) cells which exhibited EBNA2, but not LMP1 expression, which use Wp or both Wp and Cp, and which do not proliferate (Rasul et al. 2014). Secondarily EBV-infected B-CLL cells have also been found *in vivo* (Lewin et al. 1991). Latency IIb has also been observed in mononucleosis (Niedobitek et al. 1997; Kurth et al. 2000), in PTLD disorders (Oudejans et al. 1995; Brink et al. 1997), and in a unique case of a threefold mixed-latency type endemic BL with type IIb expression from a single chromosomally integrated viral genome (Kelly et al. 2006). Most likely latency IIb represents a transitional stage which may have got frozen on the path to full-blown growth-transformation exhibiting Cp-on latency III, through constraints imposed on viral gene expression by the cellular phenotype, e.g. secondarily infected CLL cells. Cells exhibiting latency IIb may normally get deleted by the healthy immune response, after primary infection is resolved (reviewed in Klein and Nagy 2010; Klein et al. 2013).

When Cp does not become switched on, expression of the key viral replication protein EBNA1 is governed by the Q promoter (Qp) instead of Cp, and the key immortalizing EBNA2 protein and the other four EBNA proteins are not expressed. Cells from EBV-infected germinal center B cells (**latency I or II**) or memory B cells (**latency 0**), and also from EBV-associated tumor biopsies, e.g. Burkitt lymphoma (latency I), Hodgkin lymphoma (latency II), NPC (latency II, +/− LMP1, +BARF1 expression) and gastric carcinoma (latency I, +/− LMP2A, +BARF1, +BARF0 expression), exhibit **Cp-off expression patterns** (Table 3.1). In addition, the two epithelial cancers highly express the BART, but not the BHRF1 microRNAs (Kim et al. 2007; Zhu et al. 2009; Cancer Genome Atlas Research Network 2014). Cp-off tumors constitute primarily malignant lymphomas or cancers which do not require a grossly defunct immune system in order to develop, but may arise in the face of a more or less functional or, in the case of BL or Hodgkin lymphoma, an even hyperstimulated, yet possibly imbalanced immune system, while EBV-associated epithelial cancers can, in addition, be viewed as molecular accidents by which the virus entered and succeeded to persist in a non-physiological target cell.

Table 3.1 Host cell-dependent expression of latent Epstein-Barr virus proteins. Key viral latency proteins or so-called oncoproteins are differentially expressed in EBV-associated malignancies and transformed cell lines. Depending on the specific viral gene expression patterns, specific viral latency classes are defined

	Cell phenotype and latency type				
	Cp-off latency group		Cp-on latency		
	BL	HL	NPC	GC	LCL
Protein	Type I	Type II	Type IIa	Type Ib	Type III
EBNA1	+	+	+	+	+
EBNA2	–	–	–	–	+
LMP1	–	+	+/–	–	+
LMP2A	–	+	+	+/–	+
BART mRNAs	+	+	+	+	+
BARF1	–	–	+	+	–
BART microRNAs	+	+	++	++	+
BHRF1 microRNAs	–	–	–	–	++

Abbreviations: *BL* Burkitt lymphoma, *HL* Hodgkin lymphoma, *NPC* nasopharyngeal carcinoma, *GC* gastric carcinoma, *LCL* lymphoblastoid cell line, probably corresponding to early-onset PTLD (post-transplant lymphoproliferative disease), *EBNA* Epstein-Barr virus nuclear antigen, *LMP* latent membrane protein, *BART* BamHI A region transcripts, *BARF1 Bam*HI A rightward frame 1, *BHRF1* BamHI H right frame

The fundamental difference between Cp-off and Cp-on latency types is further underscored by the fact that both NPC and EBVaGC are not characterized by a massive genome demethylation, as EBV-immortalized cells are, but they belong to the group of CIMP cancers, i.e. they exhibit a CpG Island Methylator Phenotype. The importance of immune hyperstimulation for explaining the pathogenesis of endemic BL has, to our knowledge for the first time, been brought to attention and discussed by Bornkamm, Klein, and Lenoir in 1987 (Klein 1987; Lenoir and Bornkamm 1987). The possibility of a complex temporary composite between immune suppression and hyperstimulation in the same patient, i.e. in patients with HIV, solid organ transplant, parasitic infestation or holoendemic malaria, and the occasional confusion which may arise from this complex overlay has been clarified (Niller et al. 2004b). Recently, the discussion has been revisited under the aspect of novel epidemiological data, i.e. analysing BL incidence in equatorial Africa with a combination of both holoendemic malaria and HIV (Rickinson 2014). The disproportionate immune hyperstimulation may be provided by antigens of *Plasmodium falciparum* or through other parasitic infections (Araujo et al. 1996, 1999; Torgbor et al. 2014), together with hotspots of arboviral outbreaks, or by early-phase HIV-infection in the case of HIV-associated BL (Kalter et al. 1985; van den Bosch and Lloyd 2000; van den Bosch 2004). An immune response skewed from Th1 towards Th2 helper cells may further contribute to increase BL risk (Lubega 2007; van den Bosch 2012).

3.4 EBV Latency Gene Products

For a more extensive description of viral latency gene products, we refer to recent reviews (Niller et al. 2011; Tsao et al. 2015) and a textbook chapter (Longnecker et al. 2013), and for a quick overview to Table 3.1. In short, the **EBER** genes code for two small abundant nuclear RNAs. As a matter of routine, they serve histo-pathologists to identify EBV-infected cells by in situ hybridization. EBERs complex with the La autoantigen of systemic lupus and with the ribosomal protein L22, and bind dsRNA dependent protein kinase R, thereby blocking the pro-apoptotic interferon α dependent signaling pathway (Clarke et al. 1991). The EBERs exert tumorigenic and anti-apoptotic functions in addition to blocking dsRNA-PKR (Ruf et al. 2000). A significant amount of La-complexed EBERs can be secreted by infected cells which leads to TLR3 (Toll-like receptor) signaling in neighbouring cells. EBER-induced innate immune responses may be associated with EBV-induced autoimmune pathology (Iwakiri et al. 2009). Interestingly, although the EBERs are non-translated RNAs, both contain at least one hypothetical full length open reading frame, the significance of which has so far not been experimentally addressed (Niller et al. 2004c).

EBNA1 is the only viral transcription and replication protein required for oriP replication and nuclear maintenance through the nuclear matrix attachment region (Sugden and Warren 1989; Jankelevich et al. 1992; White et al. 2001) encompassing oriP. Within the viral genome, EBNA1 binds oriP and the latency promoter Qp, and EBNA1-binding sites within the cellular genome have been described as well (O'Neil et al. 2008; Canaan et al. 2009; Dresang et al. 2009; d'Herouel et al. 2010; Lu et al. 2010, 2011). EBNA1-bound oriP also acts as a long-distance enhancer within the viral genome for the mutually exclusive control of the viral latency promoters Cp and Qp and for additional viral promoters (Gahn and Sugden 1995; Zetterberg et al. 2004). EBV-specific epigenetic alterations may be due to viral gene products interacting with cis-acting regulatory elements and with cellular epigenetic regulators (Table 3.2). As oriP and Qp are constitutively unmethylated, EBNA1 keeps its binding sites in the viral genome free of CpG methylation (Lin et al. 2000; Salamon et al. 2000, 2001). EBNA1 may even serve as a Bookmarking protein which keeps cellular genes marked for activation throughout the cell cycle (Niller and Minarovits 2012). In gastric carcinoma cell lines, EBNA1 by itself kept the bidirectional promoter for the tumor suppressor gastrokine genes GKN1 and GKN2 methylation-

Table 3.2 Epigenetic modifiers encoded by EBV latent genomes and their potential actions

Latency product	Epigenetic mechanism	Putative outcome
EBNA1	Binding to EBNA1 binding sites; bookmarking, may act as pioneer factor	Hypomethylation, gene activation
EBNA2	Histone acetylation	Promoter activation
EBNA3A (EBNA3)	PRC2 recruitment	Promoter repression
EBNA3C (EBNA6)	PRC2 recruitment	Promoter repression
	HDAC1/2 recruitment	
EBNA-LP (EBNA5)	HDAC4 displacement	Coactivation
LMP1	DNMT1,-3A, -3B upregulation	Promoter silencing
	Bmi-1 (PRC1-part) upregulation	Promoter silencing/activation
	KDM6B upregulation	Promoter activation via H3K27me3 demethylation
	miR-155 upregulation	KDM3A downregulation
		Promoter silencing (via H3K9me2)
LMP2A	DNMT1,-3B upregulation	Promoter silencing
	miR-155 upregulation	KDM3A downregulation
		Promoter silencing (via H3K9me2)

free (Lu et al. 2014). Several transcriptional activators have been shown to cooperate with EBNA1 in activating cellular genes. In EBNA1 expressing B cell lymphoma cells, EBNA1 and Sp1 coactivated the antiapoptotic survivin promoter (Lu et al. 2011). The ability of EBNA1 to induce transcriptional activation may also be connected to its binding to Brd4, a bromodomain protein which preferentially interacts with acetylated chromatin (Lin et al. 2008). Further, nucleosome assembly protein NAP1 supports EBNA1 replicative and transcriptional functions (Wang and Frappier 2009). EBNA1 may play a larger chromatin modifying role than previously thought. It attracts a histone deubiquitylating complex to oriP (Sarkari et al. 2009), and interacts with multiple nuclear proteins, among them high mobility group (HMG) B2 (Jourdan et al. 2012), and HMGA proteins (Coppotelli et al. 2013).

EBNA2 is considered the main transforming viral protein (Hammerschmidt and Sugden 1989) which acts as major transactivator of promoters via indirect binding to RBP-Jκ/CBF1-recognition sequences involved in immortalizing and growth-transforming B cells *in vitro* (Ling et al. 1993). Besides CBF1, additional cellular transcription factors binding to sites for PU.1, AUF1, and CRE tether EBNA2 to its transcriptional targets (Johannsen et al. 1995; Fuentes-Panana et al. 2000). Specific targets of EBNA2 are viral promoters Cp, LMP2Ap and LMP1p, and promoters for B cell activation markers which push the resting B cell towards entering the cell cycle (reviewed in Gyory and Minarovits 2005). Although EBNA2 activity overlaps with Notch signaling, it governs its own extensive cellular transcriptional program and epigenetic reprogramming via recruitment of histone acetyl transferases (HAT) p300, CBP and PCAF to drive B cell proliferation (Wang et al. 2000; Zhao et al. 2011b). Further, EBNA2 interacts with human SNF-SWI-homolog chromatin modifier proteins in order to open up chromatin conformation (Wu et al. 1996).

EBNA-LP (**EBNA5** in the Stockholm nomenclature) is a protein containing the repetitive units of the internal viral W-repeat. During the course of growth-transformation, it is the first viral latency protein to be expressed and it greatly enhances transformation efficiency, as it serves as a transcriptional coactivator for EBNA2 (Mannick et al. 1991). Because the W-repeat is frequently recombined during lytic viral replication, EBNA-LP exhibits a considerable size variation (Finke et al. 1987). EBNA2 coactivation through EBNA-LP is mediated by displacing the PML-nuclear body protein Sp100 from PML-NBs (Ling

et al. 2005) and by displacing HDAC4 and HDAC5 from EBNA2-bound promoters (Portal et al. 2006).

Among viral latency proteins, the three members of the **EBNA3** family, EBNA3A (EBNA3), EBNA3B (EBNA4), and EBNA3C (EBNA6) dominate the cytotoxic immune response against EBV (reviewed in Taylor et al. 2015). The three genes are part of the multiply spliced EBNA transcription unit, are arranged in tandem and show distant homology to each other. EBNA3C is the best-studied of the three proteins. While EBNA3A and -3C are required for EBV-mediated growth-transformation of primary B cells (Tomkinson et al. 1993), EBNA3B is not (Tomkinson and Kieff 1992). By competing with EBNA2 for promoter-bound CBF1 they modulate the transcriptional effects of EBNA2 (Johannsen et al. 1996). Like EBNA2, EBNA3C interacts also with the cellular histone acetyl transferase (HAT) p300, thereby downmodulating the EBNA2 effect (Cotter and Robertson 2000). Furthermore, EBNA3C forms a complex with HDACs 1 and 2, prothymosin α, and the corepressors mSin3A and NCoR to downregulate transcription (Knight et al. 2003). EBNA3C-regulated genes overlap significantly with EBNA2-, EBN3A-, and EBNA3B-regulated genes (White et al. 2010; Zhao et al. 2011a; McClellan et al. 2013). The requirement for continuous EBNA3A and -3C expression for transformed B cell growth was explained with their binding of the transcriptional co-repressor CtBP, via which cell cycle checkpoint control proteins p16INK4A, p14ARF are epigenetically downregulated through the deposition of inhibitory histone modifications on their promoters (Skalska et al. 2010; Maruo et al. 2011). Recruitment of PRC2 and deposition of the repressive histone mark H3K27me3 on the promoter of pro-apoptotic Bcl-2 family member Bim is dependent on EBNA3A and -3C, too (Paschos et al. 2012).

The latent membrane protein **LMP1** is an integral membrane protein containing six transmembrane domains which can mimic a constitutively active TNF receptor and, like EBNA2, is essential for the growth-transformation of primary B cells. Its expression is governed by its terminal repeat promoter L1-TRp, while the bidirectional promoter LMP1p for LMP1 and LMP2B, is mainly active in growth-transformed B cells. In the germinal center, LMP1 signaling may replace and overlap with the CD40 signal transduction pathway (Uchida et al. 1999). Therefore, LMP1 may contribute to apoptosis resistance during the physiologically vulnerable and apoptosis-prone phase of the GC reaction, in case the contact between the B cell and the T helper cell is lost. However, LMP1 is not only expressed on EBV-transformed B cells and Hodgkin lymphoma (HL) cells, but variably also on the surface of NPC cells. In NPC cells, LMP1 induces the level of DNMTs 1, -3A and -3B leading to the epigenetic repression of several tumor suppressor genes (Tsai et al. 2002, 2006; Seo et al. 2008). In GC cells, however, and in GC-derived EBV-positive HL cells, only DNMT3A was upregulated by LMP1, while the other two DNMTs were downregulated (Leonard et al. 2011). In Hodgkin lymphoma cell lines, LMP1 induces NF-κB signaling which has broad effects on the transcriptional expression profile of the HL cell. One of the induced genes is the PRC1 component Bmi-I which contributes to the survival of HL cells through its influence on transcriptional regulation of many genes (Dutton et al. 2007). Independently of NF-κB, in GC and HL cells LMP1 induces KDM6B (JMJD3), an eraser enzyme removing the methyl groups of H3K27me3 repressive marks (Anderton et al. 2011). LMP1 induces several microRNAs, miR-155 and miR-146a (Motsch et al. 2007), miR-29b (Anastasiadou et al. 2010), and miR-10b (Li et al. 2010), thereby leading to the downregulation of several cellular genes or promoting metastasis. In NPC cells, LMP1 cooperates with LMP2A in upregulating miR-155 which on its part downregulates KDM3A (JMJD1A) expression, a histone demethylase for removing the repressive H3K9me2 mark (Du et al. 2011). In epithelial cells, LMP1 induces epithelial-mesenchymal transition (EMT), and LMP1 expression is associated with metastasis of clinical NPC tumors (Horikawa et al. 2007).

The other latent membrane protein **LMP2A** containing 12 transmembrane domains mimics a

constitutively active B cell receptor (Merchant et al. 2000; Mancao and Hammerschmidt 2007), and it is not as essential for the growth-transformation of primary B cells as LMP1 or EBNA2. Its expression is governed by the LMP2Ap and, in the case of the shorter splice variant in LCs, LMP2B, by the bidirectional promoter LMP1p for both LMP1 and LMP2B. The LMP2A mRNA can only be transcribed after an initial circularization of the viral genome upon infection (Laux et al. 1988). In the GC reaction, LMP2A showing strong homology to the cellular B cell receptor may replace, overlap and interfere with the B cell receptor signal transduction pathway. Therefore, LMP2A may contribute to apoptosis resistance during the GC reaction, in case the contact between the B cell and the antigen presenting cell is lost. In EBV-associated gastric carcinoma cells (EBVaGC) which do not express LMP1, LMP2A may induce DNMT1 or DNMT3B via STAT3 phosphorylation eventually leading to the widespread methylation of tumor suppressor genes, like PTEN (Hino et al. 2009) and a multitude of additional genes (Zhao et al. 2013b). In epithelial cells, LMP2A has transforming potential (Scholle et al. 2000; Fukuda and Longnecker 2007) and induces EMT (Kong et al. 2010).

The multiply spliced **BART RNAs**, also called CST (complementary strand transcripts), are transcribed in all viral latency forms, but at particularly high levels in NPC cells and also in EBVaGC (Smith 2001; Cancer Genome Atlas Research Network 2014). The BARTs contain reading frames potentially coding for proteins named BARF0, RK-BARF0, A73, and RPMS1 and introns encoding a set of 40 **BART microRNAs** in two clusters (Pfeffer et al. 2004). The reading frames have been expressed *in vitro* and their products analyzed for interactions and function. The proteins may modulate EBNA2 transcriptional regulation and modify Notch signaling pathways, if the proteins are actually expressed *in vivo* (Smith et al. 2000; van Beek et al. 2003; Thornburg et al. 2004). The BART microRNAs are preferentially expressed in epithelial cells (Cai et al. 2006) and in NPC and EBVaGC cell lines and tumor tissues (Kim et al. 2007; Zhu et al. 2009; Cancer Genome Atlas Research Network 2014). Many viral and cellular target genes have been identified in microRNA targetome studies (Dolken et al. 2010; Riley et al. 2012; Skalsky et al. 2012). Distinct BART microRNAs modulate viral LMP1 and LMP2A expression (Lo et al. 2007; Lung et al. 2009), expression of viral lytic genes BALF5, BRLF1, and BZLF1 (Barth et al. 2008; Jung et al. 2014), downregulate the expression of cellular pro-apoptotic genes PUMA, Bim and BID (Choy et al. 2008; Marquitz et al. 2011), genes involved in immune recognition (Nachmani et al. 2009; Dolken et al. 2010), tumor suppressor genes DICE1 and CDH1 (Lei et al. 2013; Hsu et al. 2014), and the epithelial cell-specific suppressor of metastasis NDRG1 in NPC cells (Kanda et al. 2015).

BARF1, a transforming gene product from the viral BamHI A region which was previously counted among the lytic genes, is expressed from latent viral genomes in the EBV-associated epithelial cancers, NPC and EBVaGC (Decaussin et al. 2000; zur Hausen et al. 2000; Seto et al. 2005). BARF1 shows homology with the human colony stimulating factor 1 receptor (CSF1R), the gene product of the *c-Fms* proto-oncogene (Strockbine et al. 1998) and appears to modulate the host immune response to EBV infection (reviewed in Takada 2012).

The **BHRF1** cluster of four microRNAs is mostly expressed in latency type III, but not in epithelial cells (Pfeffer et al. 2004; Cai et al. 2006; Kim et al. 2007). During the early phase of B cell infection, BHRF1 microRNAs may induce apoptosis resistance and cell proliferation (Seto et al. 2010). In primary lymphomas exhibiting latency type III, miR-BHRF1-3 may contribute to the downregulation of the T cell attracting chemokine CXCL11 (Xia et al. 2008).

3.5 Establishing EBV Latency in Memory B Cells

Ground-breaking work has established the blood memory B cell as the site of permanent EBV latency *in vivo* (Miyashita et al. 1997; Babcock

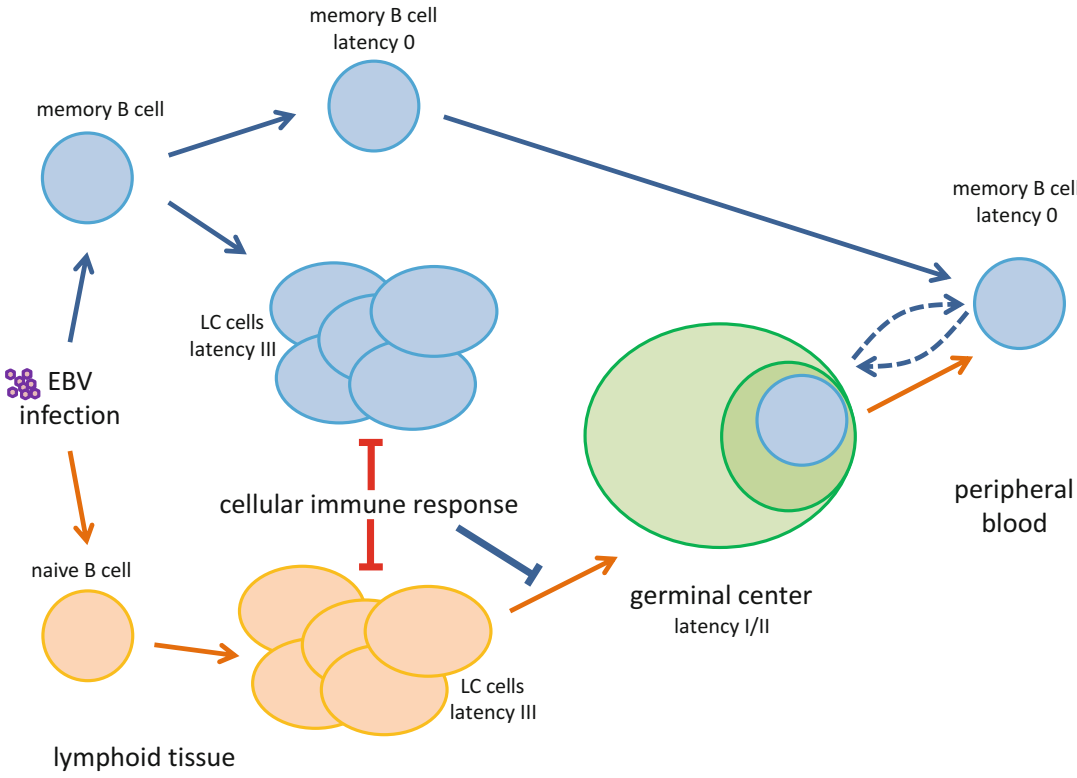

Fig. 3.1 Establishment of EBV latency in memory B cells of the peripheral blood. The traditional model (*below*, *brown arrows*) posits that upon EBV infection of naïve B cells in the tonsils, they become growth-transformed cells exhibiting a viral latency type III gene expression pattern, then some of them undergo a germinal center passage, where they switch from latency type III to latency type I or II, either by a promoter switch from Cp to Qp or by deletion of the EBNA2 reading frame, upon which they end up as memory B cells (Thorley-Lawson 2001). The alternative model (*above*, *blue arrows*) posits that upon EBV infection of memory B cells in the tonsils, they circularize the incoming viral genome, don't become growth-transformed, and remain in latency types 0, I, or II. All naïve or memory B cells which express the highly immunogenic latency type III pattern are extinguished by the cellular immune response. Generally, EBV-infected cells exhibiting latency III tend to avoid GC reactions. However, in cases of immune hyperstimulation, EBV-infected memory B cells may regularly enter or re-enter GC reactions (Araujo et al. 1999) where molecular accidents, like the Burkitt-translocation may occur (Niller et al. 2003; Rossi and Bonetti 2004)

et al. 1998). However, there is some uncertainty on which path permanent viral latency is established. The traditional view is certainly that upon EBV infection of naïve cells in the tonsils, the expanded and growth-transformed infected B cells undergo a germinal center passage, upon which they end up as memory B cells (Fig. 3.1). According to this model, the viral latency gene expression pattern would change in concordance with the B cell phenotype or the developmental stage on its path through the germinal center reaction. An implication of this model is that the EBV-transformed lymphoblast is the direct precursor of the BL cell. Specifically, a switch of the cellular phenotype accompanied by a switch in the expression profile from the extensive latency III pattern of growth-transformed lymphoblasts to the restricted latency I or 0 patterns of GC or memory cells, respectively, is required by this model (Hawkins et al. 2013), reviewed in (Rickinson 2014). Although such a latency switch has not been observed *in vivo*, a phenotypic switch of LCs, not accompanied by a latency type switch, could be engineered *in vitro* through overexpression of c-Myc in conditionally EBNA2-expressing cells (Polack et al. 1996;

Pajic et al. 2001). Partial latency type switches could be reconstructed *in vitro* by addition of IL-21 or by coculture of LCs with CD4+ T cells secreting soluble CD40L (Kis et al. 2010; Nagy et al. 2012). Furthermore, EBV superinfection of BL cells exhibiting a viral latency type I of their endogenous viral genomes silenced EBNA2 expression of the incoming viral genomes after an initial phase of Wp-on or Cp-on latency (Hughes et al. 2011, 2012).

Another view, first proposed more than 10 years ago, posits that the EBV transformed LC is fundamentally different from the physiologically antigen-activated B cell which undergoes a GC reaction (Fig. 3.1). In this model, the LC, whose epigenome was recently described by Hansen et al. (2014) is not the direct precursor of the BL cell, because EBV-transformed LCs exhibiting a latency class III expression pattern are mostly extinguished by the vigorous immune response which is mounted in the course of mononucleosis or also during asymptomatic primary infection (Niller et al. 2003, 2004a, b). Thus, a prior LC stage is not required for BL oncogenesis. Here, BL oncogenesis depends on c-Myc binding to its recognition site in the locus control region (LCR) of the viral genome which expresses latency I or II functions, but not on prior EBNA2 expression. A minority of BLs which exhibit a Wp-directed gene expression pattern close to latency type III, but without expressing EBNA2 (Kelly et al. 2002), then likely rose from a secondary latency type I to III switch in a pre-existing incipient BL tumor, made possible by bouts of immune suppression due to severe malaria tropica (Whittle et al. 1984). EBNA1 expression in this subset of BL tumors is governed by Wp instead of Cp or Qp, which may signify that an imminent EBV-driven growth-transformation was at its onset abrogated by deletion of EBNA2, before fully growth-transformed cells arose which would have been exterminated by the immune response Kelly et al. 2002). EBNA2 suppression in BL cells is then a tertiary event, achieved either through deletion of the EBNA2 reading frame in the minority of cases, or through extirpation of all BL cells that arrived at the highly immunogenic LC phenotype in the majority of BL cases, after the patients re-gained immune control (Kelly et al. 2002; Niller et al. 2004b). Cp-on latency type III as it occurs in LCs *in vitro* and in early-onset PTLDs *in vivo* is mostly regarded as a form of viral latency (Rowe et al. 1987). Latency III, however, may also be viewed as an alternative viral activation or re-activation form alongside the lytic replication cycle, but not as a form of latency in the strict sense, for which only Cp-off latency types 0, I or II would qualify. Thus, latency type III has been also named the **B cell growth-program** (Thorley-Lawson 2001). This notion is supported by the observation that upon demethylating treatment of a latency type I BL cell culture, lytic cycle transcription and the growth-program were activated in distinct cell subsets (Masucci et al. 1989).

The ability of EBV to sustain two distinct paths to viral activation, both lytic cycle and growth-program, seems to be due to the architectural intricacy of the EBV genome, in contrast to the HSV and CMV genomes (for review see Niller et al. 2007). How frequently the two distinct activation paths may overlap in one single cell subset (Ma et al. 2012), or how frequently the growth program may follow upon the lytic cycle program (Kalla and Hammerschmidt 2012) in EBV-associated malignancies, remains to be established.

Upon *in vitro* infection of isolated peripheral blood B cells, linear EBV genomes are circularized to become covalently closed circular (ccc) extrachromosomal episomes as early as 16 h post infection. Infected G0 cells do not need to pass through G1 or to enter S phase for EBV genomes to become circularized, however, a single G0 to early-G1 transition is sufficient for circularization (Hurley and Thorley-Lawson 1988). Interestingly, in already activated or cycling cells, incoming EBV was subject to a linear chromosomal integration, but not to circularization (Hurley et al. 1991), reviewed in (Morissette and Flamand 2010). Circularization occurs, before EBV-infected B cells express activation markers and start proliferating, and well before the viral episomes are amplified later-on in the course of growth-transformation (latency III) which would be the natural course of events *in vitro* (Hurley

and Thorley-Lawson 1988). The possibility of establishing viral latency *in vitro* before cellular activation and proliferation take place reflects on the *in vivo* situation during primary infection. During mononucleosis, several distinct patterns of latency gene expression are observed, including latency III (Niedobitek et al. 1997) which is the most prominent, due to the ease of *in vitro* culture and because growth-transformed B cells overwhelm the organism, if unchecked by the immune system. And indeed, the rare carriers of X chromosome-linked lymphoproliferative (XLP) disease, are overwhelmed by growth-transformed B cells after primary infection, due to a mutant *SH2D1A* gene coding for SLAM-associated protein (SAP) which is important for immune cell signaling, and mononucleosis may end deadly (Coffey et al. 1998), reviewed in (Latour and Veillette 2003). However, if kept at bay through an ever increasing immune response, infected B cells are basically able to establish EBV latency, even if they do not complete a cell cycle, or if they cycle only transiently and become resting again, and if they never reach a Cp-governed LC stage exhibiting latency III.

Correspondingly, during the systemic spread of EBV from Waldeyer's ring to the blood stream, many naïve, as well as resting cells, memory cells and germinal center cells are directly infected, but infected naïve cells do not expand or participate in GC reactions (Kurth et al. 2000), and the expanding EBV-infected GC and memory cells in tonsils of mononucleosis patients do not participate in GC reactions either (Kurth et al. 2003). This observation was confirmed by Araujo et al. (1999) who did not find any EBV-infected cells in GC reactions in a series of excised tonsils from children in Berlin (Araujo et al. 1999). Thus, under normal immune conditions, EBV infected cells do not even seem to regularly reach the germinal center. However, in tonsils from Northeast Brazil where children suffered a high load of parasitic infections at the time (Araujo et al. 1996), a GC passage of EBV-infected cells and the expansion of EBV-positive GC clones was indeed observed. However, the hyperstimulated GC environment permitted only latency type I or occasional type II viral gene expression, but not latency type III viral gene expression (Araujo et al. 1999). Because the viral growth-program which depends on EBNA2 expression is inhibitory for AID expression which is the key enzyme of the germinal center reaction, latency III is apparently incompatible with a physiological GC reaction (Panagopoulos et al. 2004; Tobollik et al. 2006; Boccellato et al. 2007). More recently, this was supported by Roughan et al. (2010) who found that EBV-infected cells in non-IM tonsils from persistently EBV-infected carriers which expressed GC markers did not express the latency III protein EBNA2 and did not expand in physiological GC reactions which would yield 100s or 1000s of cells, but achieved only abortive reactions of less than ten cells (Roughan et al. 2010). The general "avoidance behaviour" of GC reactions by EBV-infected cells is also in accordance with the observation made in transgenic mice that LMP1 inhibits GC formation (Uchida et al. 1999). If a regular GC cell suffers a *c-Myc*-translocation or becomes a Reed-Sternberg cell, it is designated for apoptosis. However, if such a "crashed" B cell is EBV-infected, it may have a bigger than remote chance to survive the GC reaction and become the founder cell of a hematological malignancy, like a BL or a Hodgkin lymphoma. This is then due to the viral or cellular anti-apoptotic functions induced by the latent virus or exerted by its gene products (Niller et al. 2003; Rossi and Bonetti 2004).

Altogether, after Hansen et al. (2014) described the massive demethylation taking place during EBV-transformation of peripheral blood B cells to the growth-program (Hansen et al. 2014), the evidence for the establishment of latency through direct infection of memory B cells (Kurth et al. 2000) and for the origin of BL without a general detour through an LC stage (Niller et al. 2003, 2004a, b; Rossi and Bonetti 2004), is further solidifying (Niller et al. 2014b). A point which still needs clarification is whether the cellular epigenomes and gene expression profiles of early-onset PTLDs are actually the same as or as close to the molecular profiles of the *in vitro* EBV-transformed LC (Hansen et al. 2014) as is generally assumed. A complete bisulfite

sequence map of early-onset PTLD genomes would be very helpful to clarify this point. Furthermore, complete methylomes of EBV-associated malignancies besides the endemic BL cell line Daudi (Kreck et al. 2013) are desiderata.

3.6 Epigenetic Regulation of EBV Latency Promoters

Cell-type specific and developmental stage-dependent viral gene expression patterns are achieved by the repertoires of viral and cellular transcription factors present in the respective cell types and by diverse epigenetic mechanisms acting on the major viral latency promoters thereby defining distinct viral epigenotypes (Minarovits 2006; Fernandez et al. 2009; Takacs et al. 2009; Arvey et al. 2013). Before the growth-program of Cp-on latency is established upon *in vitro* infection, Wp is governing latency III gene expression at first. However, Wp becomes gradually switched off by CpG-methylation, while Cp takes over (Tierney et al. 2000; Elliott et al. 2004). In *in vitro* EBV-infected tonsillar GC B cells, LMP1-induced DNMT3A binding to Wp correlates with the promoter methylation status (Leonard et al. 2011). In Cp-on latency, unmethylated Cp is located to an acetylation island, while in Cp-off latency, heterochromatic histone marks are deposited on the methylated Cp (Fejer et al. 2008). In this case, among the EBNA proteins, solely replication protein EBNA1 is expressed from Qp. In Cp-on latency, Qp is suppressed by heterochromatic histone marks and binding of repressor proteins, but not by CpG-methylation (Zhang and Pagano 1999; Salamon et al. 2001; Fejer et al. 2008). CTCF binding closely upstream contributes to the protection of Qp from methylation which stays constitutively unmethylated in all latency types (Tempera et al. 2010). The LMP promoters are also regulated by epigenetic modifications (reviewed in Takacs et al. 2009, 2010). LMP1p activity correlates with its methylation status, but not with transcription factor occupancy (Minarovits et al. 1994; Salamon et al. 2001; Takacs et al. 2001). LMP2Ap activity again is co-regulated by its methylation status, histone marks and transcription factor occupancy (Salamon et al. 2003; Gerle et al. 2007). The EBER genes closely upstream of constitutively unmethylated oriP (Salamon et al. 2000) are also constitutively unmethylated and expressed in all latency types (Minarovits et al. 1992), but their promoters are sensitive to CpG-methylation which prevents binding of transcription factors c-Myc and ATF (Niller et al. 2003; Banati et al. 2008).

Recently, the chromatin boundary protein CCCTC-binding factor (CTCF) has come into the focus of viral latency studies. CTCF mRNA and protein was expressed at higher levels in BL cells than in LCs, and it contributed to EBNA2 repression by downregulating Cp via its binding site between oriP and Cp (Chau et al. 2006). A knockdown of CTCF in BL cells did not lead to the activation of Cp, and a switch to latency III was not observed. However, deletion of that CTCF-binding site led to a prolonged transcription from Cp of incoming viral genomes in one superinfected EBV-positive BL cell line. Thus, CTCF was suggested to contribute, among other factors, to Cp silencing (Hughes et al. 2012). Contrary, Salamon et al. (2009) did not find a correlation between CTCF binding and Cp or Qp activity in several cell lines and, by genomic footprinting found hints for additional proteins besides CTCF playing a role in determining viral latency types (Salamon et al. 2009). CTCF binding at Qp may not serve a repressive function, but as a chromatin boundary separating methylated and unmethylated regions (Tempera et al. 2010). Holdorf et al. (2011) profiled the occupancy of CTCF, RAD21 (a cohesin component), and RNA polymerase II on the EBV episomes in Raji cells, a latency III type BL cell line. They found very prominent RNA pol II binding in the middle of oriP and at a couple of other locations which coincided with known promoter sequences. Raji is a BL cell line which does not use Wp or Cp, but a so far unknown promoter, in order to express a latency III transcriptional pattern (Woisetschlaeger et al. 1989; Walls and Perricaudet 1991). Thus, this large peak of RNA pol II binding within oriP may constitute the missing latency III promoter for the EBNA transcripts in Raji cells (Fig. 3.2). Further, they found CTCF sites not only at active

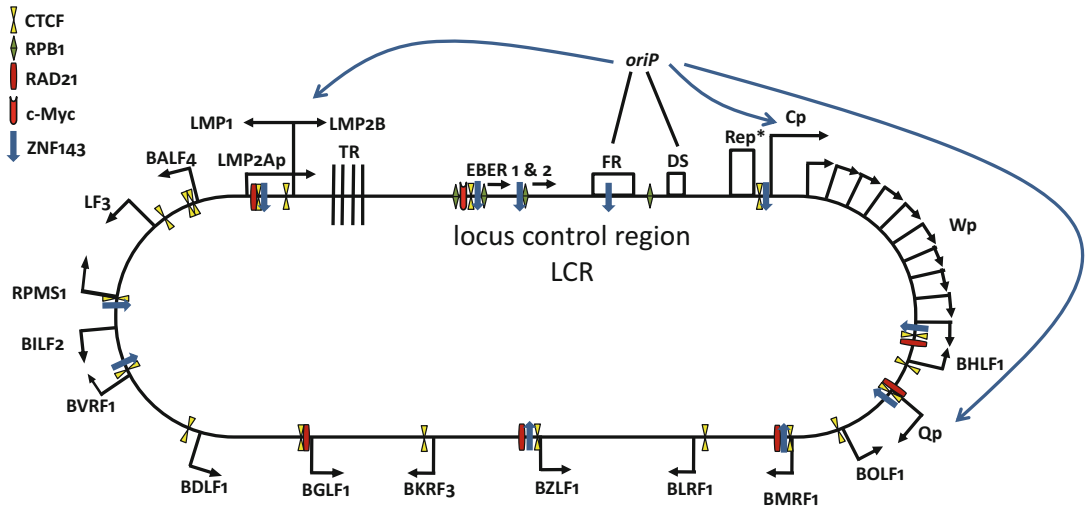

Fig. 3.2 Binding of CTCF, cohesin, RAD21, ZNF143, c-Myc to the latent EBV genome. The EBV genome (not drawn to scale) is depicted as an episome which was circularized at the terminal repeats (*TR*) immediately after infection. Binding sites for CTCF, cohesin, and RAD21 (a subunit of the RNA pol II complex) were determined by Holdorf et al. (2011) in Raji cells, the binding sites for CTCF, cohesin, RAD21 and the transcriptional activator ZNF143 were determined by Arvey et al. (2012, 2013) mainly in a metaanalysis from EBV-transformed lymphoblastoid cell lines. The binding site for c-Myc at the EBER locus was determined by Niller et al. (2003) in several cell lines, among them Raji. The locus control region encompassing the EBER locus and the origin of plasmid replication (oriP) with its elements FR (family of repeats) and DS (dyad symmetry) is crucial for regulating the latency type (Tempera et al. 2011). In latency type I, the LCR loops to and activates Qp, in latency III it loops to and activates Cp, and also acts as a transcriptional enhancer for the bidirectional LMP promoter for LMP1 and LMP2B (latent membrane proteins). Rep* is a sequence adding to oriP function. Cp, the repetitive Wps, and Qp are alternative latent promoters for the EBNA (Epstein-Barr nuclear antigen) transcripts. Lytic cycle promoters around the viral genome are labeled according to the EBV mapping nomenclature

and inactive latency control elements, but also at lytic promoters suggesting several distinct functions of CTCF, like repression, activation, or chromatin insulation, depending on the context of additional binding proteins. Some of the CTCF sites overlapped with cohesin binding, but not RNA pol II binding (Holdorf et al. 2011).

A recent metaanalysis was performed on data sets which were extracted from public databases containing total genomic chromatin data from large scale deep-seq projects that included EBV-transformed LCs. Thereby, a total of 19 CTCF binding sites was found around the EBV genome, mostly located to important regulatory regions (Arvey et al. 2012, 2013). One major role of CTCF may be to support distinct three-dimensional conformations of viral genomes depending on the latency type. In latency I, the locus control region (LCR) of EBV containing oriP looped to Qp, while in latency III the LCR was contacting Cp. Chromatin loop formation and maintenance between the LCR and other regions of the viral genome was dependent on intact CTCF binding sites (Tempera et al. 2011). However, how the selectivity of the looping is achieved remains to be established. One possibility is certainly that cohesin may play a distinguishing role. Thus, the knockdown of cohesin subunits RAD21 and SMC1 led to the loss of an LCR-LMP-region loop in LCs (Arvey et al. 2012). This is in concordance with the immunoglobulin H locus, where cohesin binding was associated with multiple chromatin looping and recombinatorial activity (Degner et al. 2009), and with KSHV, where chromatin looping was facilitated by CTCF-cohesin interactions (Kang et al. 2011). Remarkably, the viral LCR exhibited a partial non-specific stickiness for protein (Arvey et al. 2013), probably reflecting on the nuclear matrix attachment function of the LCR (Jankelevich et al. 1992).

Therefore, it is almost certain that many more factors than just CTCF and cohesin are involved in specific chromatin looping and viral 3D structure accompanying the regulation of viral latency (Fig. 3.2). For example binding of ZNF143, a most intriguing zinc-finger protein which mediates promoter looping in the cellular chromatin by directly binding to promoters and indirectly to CTCF-cohesin complexes (Bailey et al. 2015), was found just upstream of the EBERs and at a few additional locations of the viral genome, in each case colocalized with CTCF (Arvey et al. 2012). At the same location of the LCR, a binding site for c-Myc had been found in Raji cells by *in vivo* footprinting and chromatin immune precipitation which likely plays a crucial role in the origin of endemic BL tumors in the GC reaction (Niller et al. 2003, 2004a, b).

3.7 Epigenetic Profiles of Cellular Genomes in EBV-Associated Neoplasms

Cancers in general exhibit epigenomic profiles which are different from that of their healthy cellular counterparts (Baylin and Jones 2011) and from each other (Costello et al. 2000). As a rule, cancer cells carry stretches of hypomethylated DNA throughout their genomes, but exhibit also cancer type-associated hypermethylations at specific CpG islands which are frequently overlapping with tissue-specific regulatory elements and with developmentally regulated PRC-binding elements (Esteller 2007a, b; Baylin and Jones 2011). Thus, also EBV-associated neoplasms differ regarding their transcriptomes and, as far as partial or complete CpG-methylation maps have been established, also regarding their epigenomes. Furthermore, the EBV-infected counterparts of a specific tumor type exhibit transcriptomes and epigenetic profiles different from their EBV-negative counterparts. Lymphomas and epithelial cancers are two major groups of EBV-associated malignancies, whose epigenomes need to be individually studied in order to learn more about their individual modes of pathogenesis.

B cells transformed to the LC phenotype through EBV infection exhibit a massive hypomethylation affecting two thirds of their genome and one third of all genes in a complete genomic bisulfite sequence analysis. A total of almost 3000 small differentially methylated regions (DMRs), mostly located in the vicinity of promoters, amounting to a total of 1 MB, were also observed in LCs, approximately half of them hyper- and hypomethylated (Hansen et al. 2014). Hypomethylation of around 250 genes was observed in LCs using a 27K chip, but no hypermethylation (Hernando et al. 2013). Demethylation of most promoters associated with the transformation process was associated with binding of B cell specific transcription factors, half of them binding NF-κB (Hernando et al. 2013). Correspondingly, a major part of the epigenetic reprogramming in LCs and a set of different **DLBCLs** (Diffuse Large B cell Lymphomas) was due to NF-κB signaling (Vento-Tormo et al. 2014). Hypomethylated genes are involved in cellular proliferation, B cell signaling, chemotaxis, cell adhesion, immune response and inflammation (Hernando et al. 2013). An earlier study found hypomethylated genes involved in cell cycling, transcriptional regulation and the immune response (Caliskan et al. 2011). Parallel to demethylation, EBV transformation leads to a major redistribution and decrease of heterochromatic histone marks (Hernando et al. 2014).

A focused analysis of the p16INK4A and Bim loci found heterochromatic histone marks which were deposited through PRC2 mediated by EBNA3A and -3C (Skalska et al. 2010; Paschos et al. 2012). A major finding of both Hernando et al. (2013) and Hansen et al. (2014) is the clear distinction of epigenomes between EBV-transformed LCs, on the one hand, and CD40L/IL4-activated B cells, on the other hand. The methylation profile of activated B cells was very close to that of quiescent B cells, while that of LCs had drifted far away (Hansen et al. 2014). Correspondingly, the heterochromatin profile of transformed LCs could also be achieved through infection with EBNA2- or LMP1-deleted viruses, but not through CD40L/IL4-stimulation of B cells to proliferate. Thus, the transformation trait

could be clearly dissected from mere proliferation of activated B cells in those experiments (Hernando et al. 2013). This is a most intriguing observation which certainly deserves further in-depth analysis.

The epigenetic profile of BL tumors seems to considerably differ from that of LCs. So far, there is only one completed bisulfite sequence map of an EBV-positive endemic BL cell line, Daudi (Kreck et al. 2013). Sixty nine percent of all genomic CpG dinucleotides were methylated, and more than 90 % of 969 genes which are hypermethylated in mature aggressive B cell lymphomas including BL, were also silenced by methylation in Daudi cells (Martin-Subero et al. 2009b; Kreck et al. 2013). Nearly 60 % of hypermethylated genes in BL tumors were target genes of PRC2 in embryonic stem cells (Martin-Subero et al. 2009a). PRC2-targeted hypermethylation does probably not depend on EBV infection. Possibly, BLs are derived from precursor cells with stem-cell features (Martin-Subero et al. 2009b). In addition, c-Myc hyperactivity in BL tumors may contribute to their "stemness phenotype" (Takahashi et al. 2007). The overall tendency of BL is towards a high methylation profile, contrary to the hypomethylation-prone profile of LCs. A point which needs further clarification is whether primary BL tumors exhibit the same epigenetic profile as the cell line Daudi. Also in this case complete bisulfite maps would be helpful.

The epigenetic profiles of both NPC and EBV-associated GC suggest a CpG Island (CGI) Methylator Phenotype (CIMP) for both epithelial cancers (reviewed in Niller et al. 2007, 2012, 2014a, c; Kaneda et al. 2012; Lo et al. 2012). EBV gene expression patterns in NPC and GC belong mostly to latency I (or II) with a variable expression of LMP1 in the case of NPC, and with expression of BARF1, BARF0 and a variable expression of LMP2A in addition to EBNA1 and the BART microRNAs in the case of GC (Sugiura et al. 1996), reviewed in (Niller et al. 2012). Because EBV is usually monoclonal in malignant tissues, a causal role for the virus is assumed (Raab-Traub and Flynn 1986). Furthermore, a rather peculiar case of a very rapidly developing EBV-positive GC in an immune suppressed patient suggests a direct role for EBV in GC carcinogenesis (Au et al. 2005). However, the sequential order of carcinogenesis in both epithelial cancer types remains to be elucidated. Chromosomal aberrations have been found in NPC precursor lesions, i.e. in high grade dysplasia and carcinoma in situ, however, virus-infected normal epithelia have so far not been found for both NPC and GC (Kaneda et al. 2012; Lo et al. 2012).

Nevertheless, it is very likely that EBV infection is causal for a major epigenetic disruption of epithelial cells on their path to malignancy. One mechanism by which EBV causes the hypermethylation in **EBVaGC**, may be that LMP2A, in the cases where it is expressed, activates DNMT1 and DNMT3B expression which on their part lead to a global but non-random CGI-methylation in EBVaGC (Hino et al. 2009; Zhao et al. 2013b). How target specificity is achieved remains unclear at the moment. Candidate gene studies have reported a series of genes significantly more frequently hypermethylated in EBVaGC, but not in the other GC subtypes, e.g. *CDH1* (Wu et al. 2000; Sudo et al. 2004), *p16INK4A*, *PTEN*, *RASSF1A*, *GSTP1*, *MGMT*, *MINT2* (Kang et al. 2002), *p14ARF*, *p15INK4B*, *p16INK4A* (Chong et al. 2003), *p14ARF*, *p16INK4A* (Sakuma et al. 2004), *p73* (Ushiku et al. 2007), *HOXA10* (Kang et al. 2008). EBVaGC and microsatellite instability were mutually exclusive (Chang et al. 2003), which was reflected in constitutively unmethylated *MLH1* and *MSH2* genes in EBVaGC (Kang et al. 2002; Vo et al. 2002). An epigenetic field of cancerization, like in *Helicobacter pylori*-associated GC (Ushijima 2007), was not observed in the surrounding mucosa of EBVaGC (Enomoto et al. 2007; Ushiku et al. 2007). Further tumor suppressor genes *WNT5A* (Liu et al. 2013) and *SSTR1* (Zhao et al. 2013a) were found to be hypermethylated in EBV-positive GCs (reviewed in Niller et al. 2009, 2014a). Further, hypermethylated promoters of *p16INK4A*, *FHIT*, *CRBP1*, *WWOX*, and *DLC1*, *HOXA11* have recently been reported or confirmed (Cui et al. 2015; He et al. 2015).

Candidate gene approaches were extended through genome wide screens. A remarkable genome-wide methylation analysis of 51 GC samples yielded three different GC epigenotypes, two low- and high-methylation epigenotypes in EBV-negative GCs, and one very high-methylation epigenotype of EBV-positive GCs. In the EBV-infected subtype, a surplus of 270 genes was methylated, the DNA repair gene *MLH1* was regularly unmethylated, and, contrary to the EBV-negative hypermethylated subtype, PRC target genes of embryonic stem cells were not enriched. Furthermore, EBV infection of an EBV-negative GC cell line led to an increase of DNA methylation which resembled the methylation patterns of the EBV-infected very high-methylation GC epigenotype. Thus, in addition to the methylation mechanisms of other GC subtypes which may mostly be due to long-lasting *Helicobacter pylori* infection, additional mechanisms of aberrant methylation are assumed to act in the EBV-infected epigenotype. Hypermethylation could so far not simply be attributed to one of the latent gene products (Matsusaka et al. 2011). Consistent with the results of Matsusaka et al. (2011), a comparison of cultured GC cells yielded 886 genes which became hypermethylated in EBV-infected subclones, but not in uninfected subclones. Many of those genes are involved in tumor suppression pathways (Zhao et al. 2013b).

Two recent genome-wide studies of genetic and epigenetic alterations, one on 98 GC samples of different subtypes in comparison with 31 normal tissue samples (Wang et al. 2014), and the other one on 295 primary gastric adenocarcinomas including 9 % of EBVaGC (Cancer Genome Atlas Research Network 2014), confirmed the very high genomic CIMP status of EBVaGC (Toyota et al. 1999; Matsusaka et al. 2011). EBVaGC turns out to exhibit the highest CIMP status of all tumor types studied so far by The Cancer Genome Atlas Research Network (TCGA, reviewed in Li et al. 2014b). A list of 526 genes epigenetically silenced in EBVaGC (Cancer Genome Atlas Research Network 2014) and a list of 90 genes epigenetically silenced in different subtypes of GC (Wang et al. 2014) is available in the Supplementary Material of the respective papers. Interestingly, there is an intersection of 36 genes between the two lists of TCGA and Wang et al. (2014), an intersection of eleven genes between TCGA and Matsusaka et al. (2011), and only one gene between Wang et al. (2014) and Matsusaka et al. (2011), but no single gene is present on all three lists.

The epigenetic analysis of **undifferentiated NPC** is somewhat lagging behind compared to that of EBVaGC. Like EBVaGC, also NPC belongs to the group of CIMP-cancers. In the cases of NPC, where LMP1 is expressed, LMP1 may induce the expression of all three DNMTs and therefore play a role in the frequent hypermethylation of tumor suppressor genes in the tumor tissue (Tsai et al. 2002, 2006), reviewed in (Niller et al. 2009, 2012, 2014a, c; Lo et al. 2012). A comparative analysis of the cellular methylation status and LMP1 expression between NPC tissues and normal nasopharyngeal tissue showed that LMP1 expression correlated with the degree of methylation of CGIs at ten pre-selected tumor suppressor genes (Challouf et al. 2012).

Candidate gene approaches have identified a number of genes frequently hypermethylated in NPC tissue in comparison with healthy nasopharyngeal mucosal tissue, e.g. *p16INK4A* (Lo et al. 1996), *CDH1* and *CTNNB1* (Zheng et al. 1999), *RASSF1A* (Lo et al. 2001), *EDNRB* (Lo et al. 2002), *TSLC1* (Hui et al. 2003), *ZMYND10*/*BLU* (Qiu et al. 2004), *CHFR* (Cheung et al. 2005), *GADD45G* (Ying et al. 2005), *PCDH10* (Ying et al. 2006), *LTF* (Yi et al. 2006; Zhou et al. 2008), *DLC1* (Peng et al. 2006), *RASAL1* (Jin et al. 2007a), *ADAMTS18* (Jin et al. 2007b), *CCNA1* (Yanatatsaneejit et al. 2008), *ADAMTS9* (Lung et al. 2008), *DLEC1* (Ayadi et al. 2008), *IRF8* (Lee et al. 2008), *OPCML* (Cui et al. 2008), *BRD7* (Liu et al. 2008), *ZNF382* (Cheng et al. 2010), *PCDH8* (He et al. 2012), *RRAD* (Mo et al. 2012). CGI hypermethylation may have practical consequences for diagnostics and therapy (reviewed in Niller et al. 2009, 2012, c; Li et al. 2011, 2014b; Lo et al. 2012). Tumor suppressor or marker genes frequently methylated in NPCs may allow the use of methylation sensitive PCR (MSP) for the early detection of NPCs in high

risk groups besides high levels of anti-EBV-IgA in serum samples, e.g. *RASSF1A*, *p16INK4A*, *WIF1*, *CHFR* and *RIZ1* (Hutajulu et al. 2011), *CACNA2D3* (Wong et al. 2013), *CDK10* (You et al. 2013), *ASS1* (Lan et al. 2014), *CYB5R2* (Xiao et al. 2014). In addition, epigenetic alterations acquired by tumor cells during chemotherapy may play a role in the development of drug resistance and may allow predictions on the prognosis of EBVaGC. Hypermethylation of *RARB2* (Kwong et al. 2002; Seo et al. 2008), *CRBP1* and *CRBP4* (Kwong et al. 2005b), and *RARRES/TIG1* (Sriuranpong et al. 2004; Kwong et al. 2005a) prevent retinoic acid-mediated growth inhibition. High levels of H3K27me3 (Cai et al. 2011) and EZH2 (Hwang et al. 2012) may indicate the overall level of tumor suppressor gene silencing. Hypermethylation at *DLC1*, *CHFR*, and *PEG10*, and hypomethylation at *ABCC5*, *ERBB2*, and *GSTP1* may predict taxol resistance of NPC tumors (Zhang et al. 2012).

Beyond the candidate gene analyses, genome-wide methylation analyses were performed on several NPC tumor samples and cell lines in comparison with normal tissue and non-transformed nasopharyngeal cell lines by chromatin immunoprecipitation combined with promoter microarray hybridization (MeDIP-chip) (Li et al. 2014a). In this study, an overall high methylation level of CGIs was found, and many previously reported genes were confirmed as methylated in NPC tumors and cell lines. Two newly found genes, *PAX5* and *SLIT2*, were selected and again confirmed as methylated in tumors and cell lines. Several genes from the Wnt pathway, i.e. *SFRP*, *DACT*, and *DKK* family members were selected and examined for their methylation status and tumor suppressor function in NPC tumors and cell lines. Several members of these gene groups were indeed more frequently hypermethylated in NPC derived material than in normal nasopharyngeal cells and exhibited a tumor suppressor function in a colony formation assay. Remarkably, while in NPC tumors, the top affected pathway was the Wnt signaling pathway, the MAPK signaling pathway was the mostly affected pathway of NPC cell lines (Li et al. 2014a). This raises a note of caution in dealing with cell lines instead of tumor samples, because their epigenotype may have been altered in the course of long-term *in vitro* culture. On the other hand, if not microdissected, tumor samples are composed of distinct tissue types, e.g. cancer cells and infiltrating immune cells which make their epigenotype a composite of distinct epigenotypes.

Until very recently there was only a surprisingly small subset of four known hypermethylated gene loci which EBVaGC and NPC had in common, i.e. *PTPRG*, *ASS1*, *GSTP1*, and *MIPOL1* (for a recent review and a detailed comparison of hypermethylated genes in EBVaGC versus NPC see (Niller et al. 2014a), and Table 3.3). However,

Table 3.3 List of genes hypermethylated both in EBV-associated gastric carcinoma and nasopharyngeal carcinoma

Hypermethylated in NPC and EBVaGC	Chromosomal location
PTPRG	03p14.2
SLC6A20	03p21.31
MARCH1	04q32.3
OXCT1	05p13.1
B3GALT4	06p21.32
TNXB	06p21.33
LY6G5C	06p21.33
PPP1R18	06p21.33
GNL1	06p21.33
TRIM31	06p21.3
GRB10	07p12.1
SCRN1	07p14.3
SGCE	07q21.3
ATP6V0E2	07q36.1
ASS1	09q34.11
NEUROG3	10q22.1
PRKCDBP	11p15.4
ZNF214	11p15.4
GSTP1	11q13.2
CHFR	12q24.33
MIPOL1	14q13.3
ZNF701	19q13.41
ZNF542P	19q13.43
GATA5	20q13.33

References for individual genes in the list are given in Matsusaka et al. (2011), Li et al. (2011, 2014a), Lo et al. (2013), Niller et al. (2014a), Wang et al. (2014), Cancer Genome Atlas Research Network (2014), and Dai et al. (2015)

a comprehensive 450K-chip methylome analysis of 60 NPC tumor samples in comparison with other tumor methylomes already established by The Cancer Genome Atlas (TCGA) Research consortium increased the overlap of methylated genes between EBVaGC and NPC (Dai et al. 2015). Dai et al. showed that also NPC belongs to the highest methylated of all cancers, described additional genes hypermethylated both in EBVaGC and NPC, confirmed a significant overlap of de novo methylated genes with bivalent chromatin markers H3K4me3 and H3K27me3 in all examined cancer types, and found a remarkably strong peak of hypermethylation at chromosomal position 6p21.3 for both EBVaGC and NPC, and a weaker, but significant methylation-peak at 6p21.3 also for prostate cancer (Dai et al. 2015). Chromosomal region 6p21.3 contains several genes apparently involved in tumorigenesis and importantly contains the human leukocyte antigen (HLA) genes which have earlier been shown to be strongly linked with NPC risk (Tse et al. 2009; Tang et al. 2012). The currently known intersection of hypermethylated genes in both EBVaGC and NPC is shown in Table 3.3. which illustrates the accent on chromosomal region 6p21.3. In-depth comparative analysis of complete genomic bisulfite sequence maps which have to be established yet for early-onset PTLD, NPC and EBVaGC, and the genome-wide analysis of histone marks may further reveal the overlaps and differences between EBV-associated tumors and thereby illuminate the specific role of EBV in the patho-epigenetics of both EBV-associated carcinomas. Furthermore, analysing the function of all newly described hypermethylated genes is of importance for understanding EBV-associated cancerogenesis.

3.8 Conclusion

The sequence-, promoter- or locus-specificity of the mechanisms targeting hypermethylation or hypomethylation to viral and cellular promoters in EBV-infected cells is most intriguing and needs further clarification. It should be sorted out which mechanisms are involved in the establishment of widespread hypomethylated blocks during the outgrowth of LCs, despite the expression of LMP1 and LMP2A which are capable of inducing cellular DNMTs. The mechanisms targeting hypermethylation at PRC target genes of embryonic stem cells in lymphomas, and targeting hypermethylation at non-PRC target genes in the highly methylated CIMP-cancer EBVaGC may clarify the reason for the apparently EBV-specific "epigenetic signature". Further in-depth analyses of the overlaps and differences in the methylomes of EBV-associated malignancies and functional analyses of the hypermethylated genes are required to sort out the specific contribution of EBV in each case. Epigenetic marks and genes differentially methylated in EBV-associated neoplasms may also have a diagnostic and prognostic value. Because epigenetic changes are principally reversible, drugs which affect the epigenotype of an EBV-associated tumor may be exploited for cancer treatment.

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Epigenetic Dysregulation in Virus-Associated Neoplasms

4

Janos Minarovits, Anett Demcsák, Ferenc Banati, and Hans Helmut Niller

Abstract

The oncoproteins of human tumor viruses regularly interact with the cellular epigenetic machinery. Such interactions alter the epigenome of the host cell and reprogram its gene expression pattern. Altered levels or redistribution of (cytosine-5)-DNA methyltransferases and changes in the cellular methylome were observed in Kaposi sarcoma-associated herpesvirus (KSHV), hepatitis B virus (HBV), hepatitis D virus (HDV), hepatitis C virus (HCV), and human papillomavirus (HPV) associated neoplasms and cell lines. Methylation-mediated silencing of cellular promoters was also noted in Merkel cell polyomavirus (MCPyV) positive Merkel cell carcinomas, and, as discussed elsewhere, in EBV-associated malignancies and adenovirus-induced rodent tumors as well. Promoter activation also occurred, either associated with DNA hypomethylation or with the induction of euchromatic histone modifications by viral oncoproteins. It is worthy to notice that HCV infection induced large, hypomethylated blocks of cellular chromatin, although the exact molecular mechanism remains to be elucidated. In hepatoma cells expressing HBx, the oncoprotein encoded by the HBV genome, demethylation of the repetitive satellite 2 sequences was observed, due to downregulation of the *de novo* DNA methyltransferase DNMT3B. Tax and HBZ, the oncoproteins of human T-cell lymphotropic virus type I (HTLV-I), can both activate and silence distinct cellular promoters by interacting with cellular enzymes involved in histone modification.

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J. Minarovits, H.H. Niller (eds.), *Patho-Epigenetics of Infectious Disease*, Advances in Experimental Medicine and Biology 879, DOI 10.1007/978-3-319-24738-0_4

Keywords

Viral oncoproteins • Epigenetic reprogramming • Relocation of chromatin binding proteins • Focal hypermethylation • CpG island shores • CpG shelves • Histone acetyltransferases

4.1 Introduction: Oncoprotein-Mediated Epigenetic Reprogramming of Host Cells and Host Cell-Dependent Viral Epigenotypes

For a long period of time, genetic theories dominated the field of cancer research (reviewed by Soto and Sonnenschein 2014). Carcinogenesis was typically viewed as a multistep process involving successive mutations in a cell that result in a clonal outgrowth in a changing microenvironment (Marjanovic et al. 2013). It became clear, however, that in addition to genetic changes including point mutations, deletions of tumor suppressor genes, amplifications of proto-onc genes, and chromosomal aneuploidy, all of which may confer a selective growth advantage to neoplastic cells, epigenetic reprogramming also plays an important role in tumorigenesis (Jones and Baylin 2007; Munoz et al. 2012). As a matter of fact, both genetic and epigenetic alterations may contribute to the plasticity and heterogeneity of cells residing in malignant tumors (Lindsey and Langhans 2014). In addition, the components of the tumor microenvironment that affect the phenotype and behavior of neoplastic cells may also interact with the epigenetic regulatory machinery (Marjanovic et al. 2013; Lindsey and Langhans 2014). By now it is well documented that all of the major classes of cancer-causing agents including chemical carcinogens, hormones, metals, radiation, viruses and bacteria elicit epigenetic alterations, and epigenetic changes occur both in preblastomatoses and in all subsequent stages of tumor progression (reviewed by Minarovits and Niller 2013).

Regarding viruses causing malignant tumors in multicellular organisms in general and human "tumor viruses" in particular, initial studies focused mainly on the interactions of the viral oncoproteins with cellular regulatory pathways and proteins that control the cell cycle. The effect of such "transforming proteins" on the integrity of the genome and apoptotic processes was also the subject of intense research. In addition, there were substantial efforts to establish how viral oncoproteins affect the gene expression pattern of host cells. Further studies revealed the influence of host cells on the usage of viral promoters. The host cell phenotype-dependent expression patterns of viral oncogenes were charted based on the analysis of tumor biopsies, tumor derived cell lines and transfected cells (Li and Minarovits 2003; Pagano et al. 2004; Javier and Butel 2008). By now it is also well documented, however, that oncoproteins encoded by human tumor viruses alter the epigenotype of cells (Ferrari et al. 2009; Minarovits 2009; Paschos and Allday 2010; Niller et al. 2012a, b).

The host cell phenotype-dependent expression of latent viral genomes that co-replicate with the cellular DNA is related to the establishment of viral epigenotypes, i.e. cell-type specific modifications of the viral chromatin. The existence of viral epigenotypes was revealed first during the study of latent Epstein-Barr virus genomes (reviewed by Minarovits 2006, see also Chap. 3). Further studies extended the concept of viral epigenotypes to other DNA tumor viruses as well (reviewed by Fernandez and Esteller 2010). Viral epigenotypes determine the activity of viral promoters controlling the expression of viral oncogenes that interact, in turn, with the cellular epigenetic machinery. In case of the latent, episomal genomes of oncogenic herpesviruses and the proviral genomes of RNA tumor viruses and other retroviruses, the switch between the latent and productive cycle of viral replication is also affected by the viral epigenotype (Ye et al. 2011; Ay et al. 2013; Chen et al. 2013a; Niller et al. 2014).

In this chapter we focus on epigenetic alterations induced in host cells by DNA tumor viruses infecting humans, except EBV which is discussed in Chap. 3 and human adenoviruses that are not linked to human neoplasms, although they induce malignant tumors and epigenetic alterations in rodents (reviewed by Doerfler 2012). We also describe how human T-cell lymphotropic virus type I (HTLV-I), a retrovirus replicating via a DNA intermediate, interacts with the cellular epigenetic machinery. Hepatitis delta virus (HDV) and hepatitis C virus (HCV) are RNA viruses associated with liver cancer in humans. In contrast to retroviruses, they are not known to synthesize a DNA copy of their genomes. They do not code for typical oncoproteins, either. They also elicit, however, epigenetic changes in their host cells, as outlined here.

4.2 Kaposi Sarcoma-Associated Herpesvirus (KSHV, Human Herpesvirus 8, HHV-8)

Kaposi sarcoma-associated herpesvirus (KSHV), also named human herpesvirus 8 (HHV-8), is the causative agent of Kaposi sarcoma (KS). KSHV, a double-stranded DNA virus belonging to the *Gammaherpesvirinae* subfamily of the family *Herpesviridae*, is also associated with two B lymphocyte disorders, primary effusion lymphoma (PEL, also called body-cavity based lymphoma, BCBL) and multicentric Castleman's disease (MCD) (reviewed by Ganem 2010). Both the classical, typically indolent form of KS, described originally by Moritz Kaposi in the nineteenth century, and the AIDS-related, more aggressive, apparently multicentric form of the disease occurring in patients with acquired immunodeficiency syndrome, are linked to KSHV infection. In KS lesions, spindle cells of endothelial origin carrying KSHV genomes proliferate and recruit inflammatory cells as well as new blood vessels by producing inflammatory cytokines and proangiogenic factors (Ganem 2010). In turn, the products of inflammatory cells and the blood supply provided by neovascularization favor the growth of the spindle cells. KSHV was capable to infect and transform primary rat embryonic mesenchymal precursor cells (MM cells) *in vitro* (Jones et al. 2012). The KSHV-transformed MM cells formed tumors in nude mice that resembled human KS lesions, with proliferating spindle cells, neoangiogenesis, and infiltrating inflammatory cells (Jones et al. 2012).

Using the MAPit method (methylation accessibility probing for individual templates), Darst et al. observed that there were diverse chromatin structures at three viral promoters in cells carrying latent KSHV episomes *in vitro*: accordingly, only a fraction of epigenetically distinct episomes reacted with lytic transcription to an inducer of the productive virus replication (Darst et al. 2013). Thus, there are distinct epigenotypes of latent KSHV episomes carried by the same cell type and the epigenotype apparently affects which episomes will give rise to progeny virus upon activation of the lytic cycle.

In latently infected cells, LANA, the latency associated nuclear antigen encoded by ORF73 of the viral genome is highly expressed (Table 4.1). LANA binds to the terminal repeats (TRs) within the viral episomes and recruits replication factors to the TRs where the latent origin of episomal DNA replication is located. LANA is associated with mitotic chromosomes as well and ensures the maintenance of latent KSHV genomes in replicating cells (reviewed by Ye et al. 2011). It is worthy to note that the genome of herpesvirus saimiri (HVS), a close relative of KSHV encodes a homolog of LANA. In HVS-transformed dividing T cell populations, HVS LANA is also involved in the maintenance of the viral episomes, in concert with the insulator protein CTCF (Zielke et al. 2012).

LANA acts both as a transcriptional activator and as a repressor of gene expression, targeting both viral and cellular promoters. In the latently infected body-cavity-based lymphoma cell line BCBL-1 and in long term-infected telomerase-immortalized human umbilical vein endothelial cells (TIVE-LTC) LANA not only bound to its two recognition sequences located to the TRs within the viral episomes, but its binding was detected at numerous lytic and latent promoters

Table 4.1 Epigenetic alterations induced by Kaposi sarcoma-associated herpesvirus (KSHV, human herpesvirus 8, HHV-8) in human cells

Virus	Viral protein	Epigenetic mechanism or regulator affected	Outcome or expected outcome
Family:			
Herpesviridae			
KSHV	LANA	DNMT1, DNMT3A, DNMT3B (recruitment to cellular promoters)	Promoter silencing
		MeCP2 (association)	Promoter silencing and activation
		HP1 (association)	Targeting
		Histone deacetylases (association)	Promoter silencing
		Brd2/RING3 (binding, induction, relocation)	Inhibits formation of heterochromatin
		Histone-lysine N-methyl-transferase hSET1 targeting histone H3K4 (association)	Promoter activation
		KDM3A, H3K9 histone demethylase (association)	Promoter activation
	LANA (?)	Polycomb group protein, EZH2 (upregulation)	?

as well (Hu et al. 2014). LANA also associated with the host cell genome, where its binding sites were typically enriched in the euchromatic histone mark histone H3K4me3 and they were co-occupied with the insulator-binding cellular protein CTCF (CCCTC-binding factor). Most but not all of the LANA-bound promoters were transcriptionally active, indicating that LANA binding alone was not necessarily sufficient for promoter activation. However, association of LANA with the histone-lysine N-methyltransferase hSET1 that targets histone H3K4 suggested a role for LANA in the induction of epigenetic dysregulation in lymphoid and endothelial cells (Hu et al. 2014). It is worthy to note, that LANA binding to the host genomes was cell type-specific: there were more LANA-bound promoters in the endothelial cells than in the lymphoma cell line (Hu et al. 2014).

In transfected cells expressing LANA, as well as in naturally KSHV-infected BCBL-1 cells, LANA formed a complex with the H3K9 histone demethylase KDM3A, recruiting it to distinct sites of the viral episome (Kim et al. 2013). The LANA-KDM3A interaction was mediated by the N-terminal region of KDM3A, whereas LANA also bound directly to histone H3K9me2. In latently infected cells triggered to reactivate the transcription of lytic genes, ablation of KDM3A expression inhibited the transcription of lytic genes. This observation suggested a regulatory role for LANA in the transcription of the KSHV genome by recruiting KDM3A that may prevent the deposition of or may erase a heterochromatic, repressive histone mark, H3K9me2. It remains to be established whether LANA also recruits the KDM3A histone demethylase to its cellular binding sites.

How epigenetic regulators are targeted to gene sets to be activated or silenced in virus transformed cells remains to be elucidated. It is interesting to note, however, that KSHV LANA, a pleiotropic regulator implicated in gene activation was also capable to recruit a *de novo* DNA methyltransferase to cellular promoters to be silenced (Shamay et al. 2006). Shamay et al. observed that LANA expression in telomerase-immortalized, microvascular endothelial cell lines transduced by a retrovirus (TIME-LANA cell lines) resulted in the downregulation of 80 cellular genes. The promoters of two silenced genes (*CCND2* and *CREG*, encoding cyclin D2

and cellular repressor of E1A-stimulated genes, respectively) were associated with LANA. In addition, the N-terminal region of LANA could bind three DNA methyltransferases, relocated the *de novo* MTase DNMT3A from the nuclear matrix to the chromatin, and recruited it to the *CCND2* promoter in transfected cells (Shamay et al. 2006). *De novo* methylation of the *CREG* promoter was also observed under similar experimental conditions. Shamay et al. concluded that LANA elicited promoter-specific epigenetic silencing via the recruitment of DNMT3A (Shamay et al. 2006).

In transfected cells, LANA interacted with the methylcytosine binding protein MeCP2 as well. Such an interaction may result either in gene activation or gene repression in a context dependent manner. LANA expression resulted in the dispersion of chromocenter-associated MeCP2 in the nuclei, in parallel with the release of DNA from heterochromatic areas and repositioning of chromosomal domains (Stuber et al. 2007). Stuber et al. suggested that LANA may function as an epigenetic modifier that generates locally altered chromatin states, contributing thereby to viral oncogenesis (Stuber et al. 2007).

It is also noteworthy that KSHV LANA binds to, induces, and relocates the chromatin binding protein Brd2/RING3 (Mattsson et al. 2002; Viejo-Borbolla et al. 2005). Such an interaction may contribute to the inhibition of heterochromatin formation in the neighborhood of cellular chromatin-associated KSHV episomes (reviewed by Niller et al. 2012a). KSHV infection was also accompanied by the relocalization of Sp100, a protein component of nuclear domain 10 (ND10) (Gunther et al. 2014). It was observed that a polycomb group protein, EZH2, was also upregulated in KSHV infected human endothelial cells, and contributed to the KSHV-induced angiogenesis (He et al. 2012).

4.3 Hepatitis B Virus (HBV)

Hepatitis B virus (HBV), one of the causative agents of hepatocellular carcinoma (HCC) belongs to the *Hepadnaviridae* family. Hepatocarcinogenesis is a multistep process and HBV may induce a series of pathological changes that may facilitate HCC development (reviewed by Tarocchi et al. 2014). These alterations include the integration of the viral genome into the host cell DNA, an early event during the development of HBV-associated liver cancer, and an increase in mutation frequency due to the generation of reactive oxygen species (ROS) and inhibition of DNA repair. The resulting chromosomal instability and alteration of gene expression may drive tumor progression. Aberrant chromosomal segregation and disturbances of centrosome replication caused by the viral HBx protein also contribute to the multiple chromosomal aberrations characteristic for HBV-positive HCC (reviewed by Hsieh et al. 2011). In parallel with genetic alterations, epigenetic changes also regularly occur in HBV induced liver cancers (Herceg and Paliwal 2011).

The HBV genome encodes a pleiotropic regulator protein, HBx, implicated in hepatocarcinogenesis. Expression of the *HBx* gene, regulated by its own enhancer, induced liver cancer in transgenic mice (Kim et al. 1991). Depending on the target promoter, HBx may either activate or repress gene expression. It is noteworthy that transient or stable transfection of Chang liver (CHL), HepG2 and Huh7 hepatocellular carcinoma cells by a HBx expression vector upregulated the DNA methyltransferases DNMT1, DNMT3A1 and DNMT3A2 (Park et al. 2007).

Park et al. observed that the promoter region of the *IGFBP-3*, a putative tumor suppressor gene encoding insulin-like growth factor-binding protein, was hypermethylated shortly after transfection of *HBx*, due to the activities of the *de novo* DNA methyltransferases DNMT3A1 and DNMT3A2 and the binding of the methyl CpG binding protein MECP2 (Park et al. 2007). They detected hypermethylation at the promoters of the tumor suppressor genes *Ras associated domain 1A* (*RASSF1A*), *glutathione S-transferase 1* (*GSTP1*), and *cyclin-dependent kinase inhibitor 2B* (*CDKN2B*), too (Park et al. 2007). It is worthy to notice, however, that the level of DNA methylation was not elevated at the promoters of

additional tumor suppressor genes carried by the very same cells. Thus, only a set of distinct target genes was silenced by promoter hypermethylation among the tumor suppressor genes analysed in HBx expressing hepatoma cell lines (Park et al. 2007).

The experiments of Park et al. demonstrated, that in parallel to the upregulation of DNMT1, DNMT3A1 and DNMT3A2, HBx downregulated DNMT3B, the enzyme involved in *de novo* methylation of satellite repeats (Park et al. 2007). Park et al. also found that HBx induced the demethylation of satellite 2 sequences in CHL cells both *in vitro* and *in vivo*, i.e. in *HBx*-transfected CHL cell-derived tumors growing in athymic nude mice (Park et al. 2007), reviewed by Niller et al. 2012a; Niller and Minarovits 2012, Table 4.2). Thus, prevention of repetitive DNA methylation by HBx may contribute to the establishment of a typical methylation pattern frequently observed in neoplasms of non-viral etiology: overall hypomethylation and focal hypermethylation of the tumor cell genome, compared to its normal counterpart (Jones and Baylin 2007).

Besides upregulation and recruitment of DNMTs, additional mechanisms may also contribute to HBx-induced gene silencing. In Hep3B hepatoma cells stably expressing HBx, repression of the *IGFBP3* promoter, where transcripts encoding the insulin-like growth factor-binding protein 3 are initiated, was apparently mediated by the formation of a HBx/HDAC1 complex and deacetylation of the transcription factor Sp1, which led to decreased binding of Sp1 to the *IGFBP3* promoter (Shon et al. 2009). It is interesting to note that Park et al. also observed a decreased Sp1 binding to hypermethylated *IGFBP3* promoter sequences (Park et al. 2007). These data suggest that multiple epigenetic mechanisms contribute to the silencing of the *IGFBP3* promoter in HBx expressing hepatoma cell lines.

Similarly to the dual effect of HBx on the expression of genes encoding DNA methyltransferases, Zheng et al. found that in Huh7 and HepG2 hepatoma cell lines HBx could both silence and activate cellular promoters (Zheng et al. 2009). Promoter silencing was due to the direct interaction of HBx with both DNMT3A and the histone deacetylase HDAC1. Zheng et al. argued that HBx recruited DNMT3A to the *interleukin 4 receptor (IL4R) and metallothionein 1F (MT1F)* promoters resulting in promoter silencing via *de novo* methylation (Zheng et al. 2009). At other genes, however, including *cadherin 6 (CDH6)* and *IGFBP3*, HBx removed DNMT3A from the promoters and thereby activated them. In HCC tissue biopsies and adjacent non-malignant tissue, gene expression levels and CpG-methylation status reflected the results from the cell culture experiments (Zheng et al. 2009). These data are at variance with the methylation-mediated inactivation of *IGFBP3* reported by others, see above (Park et al. 2007; Zheng et al. 2009).

Table 4.2 Epigenetic alterations induced by hepatitis B virus (HBV) in human cells

Virus	Viral protein	Epigenetic mechanism or regulator affected	Outcome or expected outcome
Family:			
Hepadnaviridae			
HBV	HBx	DNMT1, DNMT3A (upregulation)	Promoter silencing
		DNMT3A (interaction, displacement)	Promoter activation
		DNMT3B (downregulation)	Hypomethylation of repetitive sequences
		HDAC1 (complex formation)	Promoter silencing
	?	? (activated NK cells, IFNγ)	Aberrant methylation in hepatocytes

In HBx expressing HepG2 cells *ALDH1*, coding for aldehyde dehydrogenase, was upregulated by hypomethylation, whereas the genes for two calcium ion-binding proteins, S100A6 and S100A4, were hypermethylated and silenced (Tong et al. 2009).

The expression of *IGF2*, a gene encoding human insulin-like growth factor 2, is regulated by the tissue- and developmental stage-dependent activity of its four promoters. Tang et al. observed that in HBV-positive HCC and *HBx*-transduced HepG2 cells HBx induced demethylation and increased the activity of promoter P3, which is highly active in fetal liver but downregulated in adult liver (Tang et al. 2015). The mechanism of HBx-induced promoter-demethylation remains to be elucidated.

In HBV-associated HCC and HBx expressing HepG2 cells promoter methylation mediated silencing of the *retinoic acid receptor β 2 (RARβ2)* tumor suppressor gene was observed (Jung et al. 2010), whereas in HBV-positive HCC *ASPP1, ASPP2*, the genes encoding the apoptosis regulator ankyrin-repeat-containing, SH3-domain-containing, proline-rich-region-containing proteins 1 and 2, were downregulated by promoter methylation (Zhao et al. 2010). Zhao et al. suggested that *ASPP* methylation was dependent on HBx expression and appeared to be an early event in tumor progression (Zhao et al. 2010).

HBx also repressed *SFRP1* and *SFRP5*, the genes encoding secreted frizzled-related proteins that block the Wnt/β-catenin signaling pathway implicated in carcinogenesis (Xie et al. 2014). In HBx-transduced hepatoma cells, silencing of *SFRP1* and *SFRP5* was mediated by upregulation of DNMT1 DNMT3A and also HDAC1. DNMT1 and DNMT3A were recruited by HBx to the *SFRP1* promoter, resulting in promoter hypermethylation (Xie et al. 2014).

Recently, Hlady et al. demonstrated genome-wide epigenetic alterations in HBV positive cirrhotic liver samples, compared to normal liver (Hlady et al. 2014). They found 2,945 aberrantly methylated CpGs in HBV infected cirrhotic livers that are known to be at increased risk of developing liver cancer (Hlady et al. 2014).

Okamoto et al. raised the point that in addition to the direct interactions of HBV-encoded proteins, especially HBx, with the cellular epigenetic machinery, HBV (similarly to another hepatotropic virus, HCV, **see** Sect. 4.5**)** may affect the cellular epigenotype via an indirect mechanism, too (Okamoto et al. 2014). They argued that IFNγ produced by HBV activated NK cells may induce aberrant methylation in hepatocytes. We would like to emphasize, however, that expression of HBx in transgenic mice could induce hepatocellular carcinoma even in the absence of inflammatory changes (Kim et al. 1991).

4.4 Hepatitis Delta Virus (HDV)

Hepatitis delta virus (HDV) is a subviral satellite RNA virus that replicates only in the presence of a helper hepadnavirus which is HBV in humans (van Regenmortel et al. 2000; Huang and Lo 2014). For this reason, HDV has not been considered as an oncovirus, although it enhances the development of liver cirrhosis and hepatocellular carcinoma in HBV infected individuals. Benegiamo et al. (2013) found that the HDV encoded delta antigen upregulated DNMT3B in a human hepatoma cell line via the activation of the STAT3 pathway (Benegiamo et al. 2013, Table 4.3). Upregulation of DNMT3B was accompanied by an increased methylation of the *E2F1* promoter and G2/M cell cycle arrest. The effect of HDV delta antigen on the methylation of satellite repeats or other repetitive sequences was not

Table 4.3 Epigenetic alterations induced by hepatitis D virus (HDV, hepatitis delta virus) in human cells

Virus	Viral protein	Epigenetic mechanism or regulator affected	Outcome or expected outcome
Genus:			
Deltavirus			
HDV	Delta antigen	DNMT3B (upregulation)	Promoter silencing
		Histone acetylation (upregulation)	Promoter activation

investigated. Benegiamo et al. speculated that in HBV- and HDV-infected hepatocytes escape-mutations allow the cells to counteract the delta antigen-induced cell cycle arrest, resulting in cells with altered cell cycle regulation (Benegiamo et al. 2013).

The HDV genome encodes a single protein, HDV antigen (HDAg) that has two isoforms. Using the human hepatocellular carcinoma cell line Huh7, Liao et al. showed that expression of either the small form (S-HDAg) or the large form that contains an extra 19 amino acids (L-HDAg) upregulated the expression of *CLU*, a gene encoding clusterin, a chaperone-like secreted protein (Liao et al. 2009). Increased *CLU* transcription and increased clusterin protein levels were associated with increased acetylation of histone H3. Upregulation of clusterin expression decreases the sensitivity of Huh7 cells to adriamycin treatment (Liao et al. 2009). Liao et al. speculated that HDV epigenetically enhances the expression *CLU* and the increased level of clusterin may facilitate the survival of hepatocellular carcinoma cells *in vivo* (Liao et al. 2009).

4.5 Hepatitis C Virus (HCV)

Hepatitis C virus (HCV), an enveloped, positive-sense single-stranded RNA virus, is a member of the *Hepacivirus* genus within the family *Flaviviridae*. HCV, one of the causative agents of hepatocellular carcinoma, is a unique human oncovirus that replicates exclusively in the cytoplasm (Jeong et al. 2012). The single stranded RNA genome of HCV is not known to be reverse transcribed in human cells and unlike retroviral RNA genomes, it does not produce a DNA copy capable for integration into the host cell genome (reviewed by Wong and Walker 1998). The epigenetic signatures associated with HCV infection were reviewed recently (Herceg and Paliwal 2011; Rongrui et al. 2014). In the pioneering study of Hernandez-Vargas et al. it was clearly established that the DNA methylation pattern of selected CpG sites distinguished hepatocellular carcinoma from the surrounding liver tissue (Hernandez-Vargas et al. 2010). It was also revealed that unique methylation signatures, i.e. independent subsets of methylated CpG sites were characteristic for HBV-positive and HCV-positive hepatocellular carcinomas and for liver cancers associated with alcohol consumption (Hernandez-Vargas et al. 2010). It is noteworthy that hypermethylation of a set of 24 CpG sites appeared to be unique for advanced, poorly differentiated liver tumors (Hernandez-Vargas et al. 2010).

In a recent study Hlady et al. profiled the DNA methylome of normal and cirrhotic liver tissues and hepatocellular carcinomas (Hlady et al. 2014). They found that compared to normal liver, there were 28,558 aberrantly methylated CpG dinucleotides in HCV-positive cirrhotic liver samples; 18,515 of them were unique for the HCV-infected cirrhotic liver. There were aberrantly methylated CpGs in ethanol-exposed cirrhotic liver and HBV positive cirrhotic liver samples as well (10,162, and 2,945 aberrantly methylated CpGs, respectively). In HCV positive hepatocellular carcinomas there were 1,245 unique, aberrantly methylated CpGs, much less than in liver cancers associated with ethanol exposure (16,574 sites). Hlady et al. argued that ethanol exposure has a more significant impact on DNA methylation in hepatocellular carcinomas than HCV infection, and pointed out that hepatocarcinogenesis is characterized ba a massive epigenetic instability (Hlady et al. 2014). Similarly to Hernandez-Vargas et al. they also observed an increased number of methylation changes during tumor progression (Hlady et al. 2014). Whereas hypermethylated CpGs were located mainly to CpG islands, the also numerous hypomethylated CpGs were situated predominantly at CpG island "shores" and CpG shelves outside of CpG islands, in both HCV-positive and ethanol exposure-associated liver carcinomas. In contrast, a preferential hypermethylation of CpG island "shores" and CpG shelves was noticed in cirrhotic liver samples. These data suggested that hypermethylation of CpG island "shores" and CpG shelves may prime CpG islands for hypermethylation and transcriptional silencing during liver carcinogenesis (Hlady et al. 2014). The epigenetic alterations encompassed large domains in

cirrhotic liver and in hepatocellular carcinoma: hypermethylated and hypomethylated regions were observed, both in HCV-positive and in ethanol exposure-associated cases. It is worthy to note that among the differentially methylated regions (DMRs) there were significantly more hypomethylated regions than hypermethylated ones (Hlady et al. 2014). Thus, the alteration of the "epigenetic landscape" in HCV-infected cirrhotic liver and HCV-positive hepatocellular carcinoma is similar, in a way, to the EBV-induced methylation changes observed in lymphoblastoid cell lines, where large hypomethylated domains were identified (Hansen et al. 2014).

The exact molecular mechanism of HCV-induced hyper- and hypomethylation remains to be established. We would like to emphasize, however, that the HCV core protein was capable to upregulate DNMT1 and DNMT3B and induce hypermethylation of the E-cadherin promoter, resulting in a downregulation of E-cadherin level and increased invasion ability of the core protein expressing cells (Arora et al. 2008; Ripoli et al. 2011, Table 4.4). Ripoli et al. reported that in Huh-7 hepatoma cells expression of genotype 1b HCV core protein did not affect the methylation pattern of other cellular genes, including *GSTP1, APC, TIMP3, CNNTB1*, but induced SIRT1 (Ripoli et al. 2011). SIRT1 (sirtuin 1) functions as a NAD-dependent histone deacetylase, but it is capable to deacetylate nonhistone proteins as well (reviewed by Stunkel and Campbell 2011). One may speculate that SIRT1 contributed to the HCV core protein-induced silencing of the E-cadherin promoter because treatment with sirtinol, an inhibitor of SIRT1, increased E-cadherin mRNA expression and decreased E-cadherin promoter methylation (Ripoli et al. 2011).

The effect of HCV core protein on the host cell methylome may depend on the viral genotype. Similarly to other studies, Benegiamo et al. observed that expression of HCV core 1b protein upregulated the expression of DNMT1 and DNMT3B, both at the mRNA and protein level, in Huh-7 human hepatoma cell cultures (Benegiamo et al. 2012). Other HCV core proteins (genotypes 2a, 3a, 4h, 5a), however, did not influence *DNMT3B* transcription. The core proteins encoded by HCV subtypes 2a and 3a could increase the protein level of the *de novo* DNA methyltransferase DNMT3B in spite of the fact that they did not alter the level of DNMT3B mRNA (Benegiamo et al. 2012). One may speculate that expression of certain HCV core proteins may affect the stability of DNMT3B. HCV core induced upregulation of DNMT1 and DNMT3B mediated the hypermethylation and silencing of the *RARB2* promoter as well, where transcripts encoding the tumor suppressor protein retinoic acid receptor-β2 are initiated (Lee et al. 2013). Such a mechanism may block the growth-inhibitory effect of all-trans retinoic acid in HCV infected cells.

Epigenetic modifications elicited by the core protein of HCV may activate key signaling path-

Table 4.4 Epigenetic alterations induced by hepatitis C virus (HCV) in human cells

Virus	Viral protein	Epigenetic mechanism or regulator affected	Outcome or expected outcome
Family:			
Flaviviridae			
HCV	Core protein	DNMT1, DNMT3B (upregulation)	Promoter silencing
	Core protein (genotype 1b)	SIRT1 (induction)	Promoter silencing
	Core protein (?)	?	Induction of large hypomethylated chromatin domains
	?	? (activated NK cells, IFNγ)	Aberrant methylation in hepatocytes

ways that regulate the cellular phenotype and behaviour. Quan et al. found that there were hypermethylated CpG islands in the vicinity of the *SFRP1* promoter in HCV core-expressing Huh-7 cells (Quan et al. 2014). *SFRP1* codes for secreted frizzled-related protein (SFRP), a regulator of Wnt signaling. The HCV core protein downregulated SFRP expression, a phenomenon that could be reverted by the inhibition of DNA methylation. In addition, epithelial-mesenchymal transition (EMT) a phenotypic change induced by the HCV core protein in Huh-7 cells was also blocked by DNMT1 knockdown or SFRP1 overexpression (Quan et al. 2014). Because EMT facilitates migration and aggressive behaviour of tumor cells, these observations imply that an epigenetic change induced by the HCV core protein may contribute to the progression of HCV-positive liver cancer. HCV infection generated cancer stem-cell like signatures in tissue culture and stem cells were isolated from hepatocellular carcinomas that developed in an animal model based on HCV core transgenic mice or in HCV-positive and HCV-negative patients (Ali et al. 2011; Machida et al. 2012). The epigenetic changes associated with the establishment of cancer stem cell phenotype in HCV-infected cells and other cell types targeted and "reprogrammed" by various human tumor viruses remain to be explored (reviewed by Iacovides et al. 2013).

It is worthy to note that there were no reproducible alterations of host cell DNA methylation in HCV-infected Huh7.5.1 cells according to another study (Okamoto et al. 2014). Instead of a direct effect of HCV on the host cell epigenotype, the experiments of Okamoto et al. suggested an indirect mechanism for HCV-induced epigenetic dysregulation. Using chimeric immunodeficient mice in which up to 85 % of hepatocytes were replaced by human hepatocytes, they observed that HBV or HCV infection could induce genome-wide methylation changes. There were 149 common genes which showed an increased methylation both in HBV and in HCV infected mice. Because IFNγ expression was significantly elevated in HBV or HCV infected animals, but reduced by an antibody blocking NK cell function, Okamoto et al. speculated that natural killer cells activated by HBV or HCV infection may induce aberrant DNA methylation in human hepatocytes (Okamoto et al. 2014).

Hinrichsen et al. also found a common set of hypermethylated promoters in hepatocellular carcinomas of diverse etiologies (Hinrichsen et al. 2014). They observed that advanced-stage tumors associated with HBV, HCV or alcoholic liver disease carried hypermethylated mismatch repair genes, including *MLH1*, *MSH2* and *PMS2*. In addition, the promoter of the cell cycle regulator gene *p16* was also frequently inactivated by DNA methylation (Hinrichsen et al. 2014). Hinrichsen et al. argued that promoter methylation of mismatch repair gene promoters and *p16* is a frequent event during the progression of liver cancer, but it does not show a significant correlation to HCV infection (Hinrichsen et al. 2014).

CpG hypermethylation in nonpromoter regions was also detected in HCV-positive hepatocellular carcinomas. The first exon of *ZAR1*, – a gene coding for an ovary-specific maternal factor that has critical importance in the initiation of embryogenesis – showed a significantly higher methylation level in the tumorous versus the non-tumorous tissue, suggesting that it could be a marker of HCV-positive liver cancer (Takagi et al. 2013).

Besides hepatocellular carcinoma, HCV infection is also associated with the development of intrahepatic cholangiocarcinoma (ICC). Zeng et al. observed that expression of miR-124, a microRNA, was down-regulated in HCV-infected ICC cells, in parallel with an increase of cell migration (Zeng et al. 2012). This phenomenon was attributed to the upregulation of DNMT1 by the HCV core protein. Downregulation of miR-124 affected the level of its target, SMYD3 and – indirectly – the levels of c-Myc and MMP9 involved in migration and invasion (Zeng et al. 2012).

In addition to epigenetic silencing of genes during the process of hepatocarcinogenesis, HCV may also switch off cellular genes potentially blocking its replication. Chen et al. observed that efficient replication of HCV in Huh-7 subclones is associated with DNA methylation-mediated silencing of either *CREB3L1*, a gene encoding

cyclic AMP (cAMP) response element binding protein 3-like 1, or *MX1* (*myxovirus resistant 1*) (Chen et al. 2013b). Expression of both CREB3L1, a cellular transcription factor, and MX1, an antiviral protein up-regulated by interferon curbed HCV replication, suggesting that their downregulation may render a minority of HCV-infected cells permissive for virus replication (Chen et al. 2013b).

4.6 Human Papillomavirus (HPV)

High-risk human papillomaviruses are the causative agents of cervical carcinoma; they are also associated with other anogenital carcinomas and with carcinomas of the head and neck region, including oral carcinomas (zur Hausen 2002; Hoppe-Seyler and Hoppe-Seyler 2011). Human papillomaviruses belong to the *Papillomaviridae* family and according to recent phylogenetic studies the divergence of the human papillomaviruses preceded the emergence of *Homo sapiens* (reviewed by Van Doorslaer 2013).

The E6 and E7 oncoproteins of human papillomavirus (HPV) lack intrinsic enzymatic activity. E7 was initially characterized as a direct inhibitor of the tumor suppressor protein Rb, a regulator of the cell cycle (reviewed by Nead and McCance 1998). It was also demonstrated that E7, encoded by the HPV16 genome, induced Rb degradation via the ubiquitin-proteasome pathway (Boyer et al. 1996).

Holland et al. observed an epigenetic consequence of E7-Rb interaction. They found that E2F, the cellular binding partner of Rb, was released from its binding pocket in E7 protein expressing cells and activated *EZH2* (*enhancer of zeste homologue 2*), a gene coding for the histone methyltransferase EZH2 (Holland et al. 2008). Although the exact mechanism of action remains to be elucidated, EZH2 expression was indispensable for the proliferation of HPV-positive cancer cells *in vitro*, and contributed to their apoptosis-resistance as well (Holland et al. 2008, Table 4.5).

EZH2 is regularly associated with silent promoters enriched in the heterochromatic mark his-

Table 4.5 Epigenetic alterations induced by human papillomavirus (HPV) in human cells

Virus	Viral protein	Epigenetic mechanism or regulator affected	Outcome or expected outcome
Family:			
Papillomaviridae			
HPV	E7	DNMT1 (upregulation)	Promoter silencing
		Histone acetyltransferases (upregulation)	Promoteractivation
		H3K27me3 demethylases (induction)	Promoter activation
		HDAC	Promoter silencing
		KDM5B (JARID1B), histone demethylase targeting histone H3K4me3)	Promoter silencing
		EZH2, polycomb protein (induction via E2F release)	?
	E6, E7	Histone H3 acetylation (upregulation)	Promoter activation
	E6, E7	Lysine demethylase, KDM6A, targeting histone H3K27me3 (upregulation)	?
		PRC1 protein BMI1 (downregulation)	?

tone H3K27me3, due to the enzymatic activity of EZH2. Hyland et al. observed, however, that upregulation of the PRC2 component EZH2 did not increase the level of histone H3K27me3 in foreskin keratinocytes expressing the HPV16 oncoproteins E6 and E7 (Hyland et al. 2011). This unexpected finding was due to the fact that a lysine demethylase enzyme, KDM6A, removing the methyl groups from histone H3K27me3, was upregulated in parallel (Hyland et al. 2011). In addition, the PRC1 protein BMI1 was downregulated in the E6 and E7 expressing cells. These observations suggest that complex epigenetic changes may contribute to an increased proliferation capacity and apoptosis resistance of HPV positive cervical carcinoma cells (Holland et al. 2008; Hyland et al. 2011, reviewed by Szalmas and Konya 2009).

By comparing HPV positive and HPV negative head and neck squamous cell carcinoma (HNSCC) samples, Lechner et al. observed a widespread gain of methylation in HPV positive tumors: 87 % of the CpG sites at methylation variable positions showed increased methylation (Lechner et al. 2013a). Thus, the HPV positive HNSCCs apparently had a distinct epigenetic signature. It is interesting to note that a similar phenomenon, i.e. a virus-specific DNA methylation signature was observed earlier in EBV-associated gastric carcinomas as well (Matsusaka et al. 2011). Lechner et al. noticed that in HPV positive HNSCCs hypermethylation affected a series of PRC2 target genes, including cadherin genes (Lechner et al. 2013a). This is at variance with the EBV-specific epigenetic signature described by Matsusaka et al. because PRC2 targets were not enriched among the methylated genes in EBV-positive gastric carcinomas (Matsusaka et al. 2011). In addition to leaving an epigenetic signature on the host cell genome, HPV infection may associate with distinct genetic alterations as well: next-generation sequencing revealed that HPV-negative head and neck squamous cell carcinomas carried *TP53* mutations whereas HPV-positive HNSCCs frequently carried mutations or copy number alterations of PI3 kinase (phosphatidylinositol-3-kinase) pathway components (Lechner et al. 2013b). Amplification of PI3 kinase was also observed in the EBV-associated cancer nasopharyngeal carcinoma (see Chap. 3, Table 3.3, Or et al. 2005).

Ectopic expression of E6 and E7 proteins in a HNSCC cell line partly recapitulated the HPV-specific DNA methylation signature observed in HPV-associated neoplasms. Lechner et al. suggested that in the HNSCC cell line the main effector mediating the alteration of the host cell methylome was HPV E6 (Lechner et al. 2013a). Others, however, found that although the expression of HPV E6 repressed E-cadherin expression in human colon carcinoma cell lines, this effect was not mediated by DNA methylation (D'Costa et al. 2012). Expression of E7 in a spontaneously immortalized human keratinocyte cell line (NIKS, normal immortal keratinocytes) upregulated the cellular DNA methyltransferase (DNMT1) activity and suppressed the transcription of the E-cadherin gene, but did not change the methylation status of the E-cadherin promoter (Laurson et al. 2010). Laurson et al. suggested that E7-augmented DNMT1 activity is targeted to cellular genes encoding proteins that may affect the E-cadherin promoter indirectly (Laurson et al. 2010). E6 may repress E cadherin transcription in an indirect way, too.

Expression of HPV16 E6 and E7 in transduced primary human keratinocytes also resulted in DNA methylation alterations: using methylation-specific digital karyotyping, Steenbergen et al. observed increased methylation of 34 genes (Steenbergen et al. 2013). The expression of the very same genes was reduced in cervix carcinomas. In addition, 6 of the 34 genes frequently showed increased methylation in cervix carcinomas. Steenbergen et al. suggested that methylation analysis of the genes methylated with the highest frequencies (*FAM19A4, PHACTR3 and PRDM14*, coding for the proteins family with sequence similarity 19 (chemokine (CC-motif)-like) member A4, phosphatase and actin regulator 3, and PR domain-containing protein 14, respectively) may be a diagnostic tool to assess the presence of precancerous cervical lesions or cervix carcinoma in HPV positive women (Steenbergen et al. 2013). Other, newly identified methylated genes including *LHX1* and

NKX2 that encode LIM homeobox protein 1 and homeobox protein NKX2, respectively, may also play a role in the pathogenesis of cervical carcinoma (Steenbergen et al. 2013).

In a recent study, Schütze et al. transduced primary human foreskin keratinocytes using retroviral vectors expressing the E6/E7 open reading frames of HPV16, 18, 31, 33, 45, 66 and 70 (Schutze et al. 2015). They observed a progressive increase of methylation of selected host cell genes during successive stages of immortalization: *hTERT*, *miR124-2* and *PRDM14* were methylated already prior to immortalization, followed by *ROBO3*. *CYGB* and five additional genes were methylated at later stages of immortalization (Schutze et al. 2015). In spite of the fact that *hTERT* was activated in parallel with increased methylation of CpG dinucleotides located outside of its core promoter region, especially in cells transduced by less oncogenic HPV types, Schütze et al. suggested that increased methylation of CTCF binding sites did not contribute to the upregulation of the *hTERT* promoter (Schutze et al. 2015).

Wilson et al. used methylated DNA immunoprecipitation-sequencing (MeDIP-seq) for the whole-genome methylation analysis of 3 HPV-negative and 3 HPV-positive HNSCCs (Wilson et al. 2013). They confirmed the observation of Lechner et al. (2013a) as to the increased methylation of cadherin family genes regulating cell-cell adhesion processes in the HPV-positive tumors. In addition, Wilson et al. observed that the HPV-induced methylation changes also extended to predicted enhancer regions (Wilson et al. 2013). Comparison of methylated genes in cervical cancer, cervical intraepithelial neoplasia grade 3 (CIN3) and normal cervical tissue revealed that there were differentially methylated CpG sites in cancer tissue, especially on chromosomes 1, 6, and 19 (Farkas et al. 2013). The CpG sites hypomethylated in cervical cancer included genes involved in antigen processing and presentation, autoimmune thyroid disease and asthma, whereas the hypermetylated CpG sites were located to genes that play a role in cell development, cell differentiation and transcriptional regulation (Farkas et al. 2013).

HPV has a dual effect on the cellular epigenotype and on the activity of cellular promoters. Promoter silencing is possibly achieved by the upregulation of DNMT1, although by an indirect mechanism (Laurson et al. 2010; D'Costa et al. 2012, see above), or by the induction of the PcG protein EZH2 (Holland et al. 2008). Promoter activation may occur via histone acetyltransferases and histone H3K27me3 demethylases (Zhang et al. 2004; James et al. 2006; Hyland et al. 2011; McLaughlin-Drubin et al. 2011, reviewed by Niller et al. 2012a). Zhang et al. observed that HPV16 E7 upregulated histone H3 acetylation in human foreskin keratinocytes in general, and on the *CDC25A* and *E2F1* promoters in particular (Zhang et al. 2004). The other HPV oncoprotein, E6, increased acetylation of histone H3 at the promoter of the human telomerase reverse transcriptase gene (*hTERT*) (James et al. 2006). This may contribute to the activation of *hTERT* transcription by E6 in early passage human keratinocytes. hTERT expression plays an important role in the immortalization of human cells by preventing the shortening of telomeres and blocking the onset of telomere-controlled senescence (Lee et al. 2004).

In addition to transcriptional activation, E7 may be involved in promoter silencing as well. At the *TLR9* promoter, HPV16 E7 formed a transcriptional repressor complex by recruiting HDAC1 and KDM5B (JARID1B), a histone demethylase removing an activating histone modification (histone H3K4me3) (Hasan et al. 2013). This may contribute to the suppression of TLR9 (Toll-like receptor 9) expression in HPV16 positive cervical lesions and thereby may block the induction of type-I IFN responses as well as the apoptosis of virus-infected cells (Hasan et al. 2013).

In primary human epithelial cell cultures, HPV16 E7 induced the transcription of *KDM6A* and *KDM6B*, coding for lysine (K)-specific demethylases catalyzing the demethylation of the tri- or dimethylated lysine 27 residue of histone H3, removing thereby a heterochromatic histone mark involved in transcriptional silencing by polycomb repressor complexes (McLaughlin-Drubin et al. 2011). Although KDM6B activated

the tumor suppressor gene *CDKN2A* encoding the p16 protein involved in senescence, the cells continued to proliferate due to the inactivation of RB by E7. In parallel, the expression of homeobox (*HOX*) genes was upregulated in E7-expressing cells, possibly due to the decrease of the repressive histone H3K27me3 mark. McLaughlin-Drubin et al. speculated that dysregulated expression of HOX proteins may play a role in the reprogramming of gene expression patterns in HPV-infected epithelial cells, contributing to tumorigenesis (McLaughlin-Drubin et al. 2011). Derepression of *HOX* genes was also observed in HPV16 E6/E7 expressing human foreskin keratinocytes (HFKs) (Hyland et al. 2011). Hyland et al. argued that this phenomenon which occurred in HPV16-positive high-grade cervical intraepithelial lesions, too, could be attributed to a reduction of histone H3K27me3 level (Hyland et al. 2011).

4.7 Merkel Cell Polyomavirus (MCPyV)

Merkel cell carcinoma is a rare, aggressive, cutaneous neuroendocrine carcinoma associated with a recently discovered human polyoma virus, Merkel cell polyomavirus (MCPyV) (reviewed by Houben et al. 2009). MCPyV belongs to the family *Polyomaviridae* and its small, circular, double-stranded DNA genome encodes, in addition to structural proteins, two "tumor antigens" (large T and small t) as well (White et al. 2013). The viral genome is frequently integrated into the host cell DNA and expresses large T which is implicated in oncogenesis. Due to mutations, the large T antigen is truncated in Merkel cell carcinomas; it was observed, however, that MCPyV-truncated large T antigen effectively promoted the growth of human and mouse fibroblasts (Cheng et al. 2013) whereas MCPyV small t antigen proved to be oncogenic in transgenic mice (Verhaegen et al. 2015). Helmbold et al. detected MCPyV in 90 of 98 Merkel cell carcinomas and observed hypermethylation of the *RASSF1* promoter in approximately half of the neoplasms studied (Helmbold et al. 2009). They argued that frequent hypermethylation of *RASSF1* may have a pathogenetic significance (Table 4.6). It is important to notice that the large T antigen of SV40 polyomavirus, an oncoprotein related to the MCPyV large T antigen, could induce salivary gland cancer (Ewald et al. 1996) and prostate cancer (McCabe et al. 2006) in transgenic mice. Expression of the SV40 T antigen in prostatic intraepithelial neoplasia and advanced prostate cancer was associated with an elevated level of murine Dnmt1 that was required for malignant transformation, as demonstrated by experiments with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (McCabe et al. 2006). One may speculate that the MCPyV large T antigen may induce aberrant DNA methylation *via* a similar mechanism.

4.8 Human T-Cell Lymphotropic Virus Type I (HTLV-I)

Tax is a promiscuous transactivator oncoprotein encoded by a retrovirus, human T-cell lymphotropic virus type I (HTLV-I), the causative agent of adult T-cell leukemia/lymphoma (Newman and Rosenblatt 1998). Tax was characterized as an inhibitor of the tumor suppressor retinoblas-

Table 4.6 Epigenetic alterations induced by Merkel cell polyoma virus (MCPyV) in human cells

Virus	Viral protein	Epigenetic mechanism or regulator affected	Outcome or expected outcome
Family:			
Polyomaviridae			
MCPyV	T antigen (?)	?	*RASSF1A* hypermethylation (silencing)

toma protein (Rb), too. It associates with Rb and targets Rb for proteasomal degradation (Kehn et al. 2005).

Tax acts upon its recognition sequences located in the proviral LTR (long terminal repeat) and activates a series of cellular genes including *c-onc* genes and genes encoding growth factors via different transcriptional pathways (reviewed by Newman and Rosenblatt 1998; Nakachi et al. 2011; Cheng et al. 2012; Ishikawa et al. 2013). It is noteworthy that Tax binds both to histone acetyltransferases and to protein arginine methyltransferase 4 (PRMT4, also called CARM1) that act cooperatively in coactivator complexes (Table 4.7, reviewed by Jeong et al. 2009; Nyborg et al. 2010). At Tax-activated (HTLV-I) promoters, histone acetylation by cellular coactivators was followed by acetylation-dependent eviction of the entire histone octamer, a process mediated by the histone chaperone Nap1. Thus, a nucleosome-free promoter region, a signature of transcriptionally active genes was formed (Nyborg et al. 2010). Tax has a negative effect on the expression of a set of cellular genes. One of its targets is *SHP-1*, a gene coding for a candidate tumor suppressor protein, SH2-homology containing protein-tyrosine phosphatase 1 (Cheng et al. 2007). Tax recruits a histone deacetylase, HDAC1, to the *SHP-1* promoter resulting in the displacement of the transcription factor NF-κB.

Tax expression is frequently lost in aggressive forms of ATL due to hypermethylation of the HTLV-I promoter. HTLV-I bZIP factor (HBZ, a basic leucine zipper domain protein) encoded by the minus strand of the provirus is expressed, however, also in the late stages of ATL (reviewed by Zhao and Matsuoka 2012). Similarly to Tax, HBZ is involved both in activation and repression of transcription. HBZ formed a ternary complex with the coactivator p300 that also carries a histone acetyl transferase activity, and SMAD3, a mediator of TGF-β (transforming growth factor-β) signaling (Zhao and Matsuoka 2012). In mouse naive T cells HBZ enhanced the transcription of a battery of cellular genes including the gene for the pioneer transcription factor Foxp3. Foxp3 in mice and the corresponding FOXP3 in humans is a master regulator of regulatory T-cell (T_{REG} cell) development: it determines the cell type-specific epigenotype, gene expression pattern and cellular identity (reviewed by Ay et al. 2012). Zhao et al. (2011) speculated that in HTLV-I infected individuals HBZ-induced FOXP3 may convert the infected T cells into T_{REG} cells. Such a conversion may explain the suppressive effect exerted by ATL cells on bystander CD4+ cells (Zhao et al. 2011).

Tax and HBZ have frequently opposite effects on cellular gene expression. Tax repressed whereas HBZ activated transcription of *DKK1*, a gene coding for the Dickkopf-1 protein that inhibits osteoblast differentiation and facilitates bone resorption (Polakowski et al. 2010). Polakowski et al. (2010) suggested that during

Table 4.7 Epigenetic alterations induced by human T-lymphotropic virus type 1 (HTLV-I) in human cells

Virus	Viral protein	Epigenetic mechanism or regulator affected	Outcome or expected outcome
Family:			
Retroviridae			
HTLV-I	Tax	Arginine methyltransferase (binding)	Promoter activation
		Histone acetyltransferases (binding)	Promoter activation
		Nap1 (histone chaperone) (binding)	Nucleosome disassembly
		Histone deacetylase HDAC1 (recruitment)	Promoter silencing
	HBZ	Histone acetyltransferases (binding in a ternary complex)	Promoter activation
		Histone acetyltransferases (binding, direct inhibition)	Promoter silencing

late stages of ATL when Tax expression is switched off, HBZ may upregulate DKK1 contributing to the development of lytic bone lesions. Although the coactivator p300/CBP was indispensable for *DKK1* activation by HBZ, the very same research group reported direct inhibition of the p300/CBP associated acetyl transferase activity, too (Wurm et al. 2012). One may speculate that HTLV-I infection, via complex effects of HBZ, may reduce overall levels of acetylated histones in parallel with context dependent local increases of HAT activity.

4.9 Conclusion

We conclude that the oncoproteins encoded by human tumor viruses elicit complex epigenetic changes in their host cells during the initiation and progression of virus-associated malignant tumors. These epigenetic alterations may play a role in tumor progression as "epidrivers" or may regularly affect genes not involved directly in tumorigenesis. Because epigenetic changes are reversible, epigenetic drugs may alter the behaviour of malignant cells (epigenetic therapy). Detection of differentially methylated DNA sequences in body fluids may play a role both in early diagnosis of neoplastic diseases and in the monitoring of their therapy.

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Epigenetic Alterations Induced by Bacterial Lipopolysaccharides

5

Lorenzo Chiariotti, Lorena Coretti, Raffaela Pero, and Francesca Lembo

Abstract

Lipopolysaccharide (LPS) is one of the principal bacterial products known to elicit inflammation. Cells of myeloid lineage such as monocytes and macrophages, but also epithelial cells give rise to an inflammatory response upon LPS stimulation. This phenomenon implies reprogramming of cell specific gene expression that can occur through different mechanisms including epigenetic modifications. Given their intrinsic nature, epigenetic modifications may be involved both in the acute response to LPS and in the establishment of a preconditioned genomic state (epigenomic memory) that may potentially influence the host response to further contacts with microorganisms. Information has accumulated during the last years aimed at elucidating the epigenetic mechanisms which underlie the cellular LPS response. These findings, summarized in this chapter, will hopefully be a good basis for a definition of the complete cascade of LPS-induced epigenetic events and their biological significance in different cell types.

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J. Minarovits, H.H. Niller (eds.), *Patho-Epigenetics of Infectious Disease*, Advances in Experimental Medicine and Biology 879, DOI 10.1007/978-3-319-24738-0_5

Keywords

Inflammatory response • Epigenetic memory • Lipid A • Histone modifications • Innate immune cells • Histone deacetylases • High-throughput chromatin immunoprecipitation • Immunological imprinting

5.1 Lipopolysaccharide Triggers Pro-inflammatory Response in Innate Immune Cells and Other Cell Types: An Overview

Lipopolysaccharide (LPS) is the major component in the outer monolayer of the outer membrane of most Gram-negative bacteria. LPS structure consists of a hydrophobic lipid A domain, an oligosaccharide core, and the outermost O-antigen polysaccharide (Raetz and Whitfield 2002). LPS, through its lipid A portion, is able to trigger an inflammatory response in host cells. The best characterized LPS receptor is the Toll-like receptor 4 (TLR4), which is expressed by various cell types such as the myeloid lineage (i.e. monocytes, macrophages) and some non-immune cells like intestinal epithelial and endothelial cells (Medzhitov 2001). The pro-inflammatory action of LPS is crucial for the suppression of bacterial infections, and is aimed to protect the organism from bacterial invasion. However, an excessive and inappropriate host response to LPS leads to systemic inflammatory conditions.

Activation of the inflammatory response in LPS responsive target cells is a quite well described sequential process (Fig. 5.1, Akira 2006). In a classic scenario, the common structural pattern of LPS of diverse bacterial species is recognized by a cascade of LPS receptors, co-receptors including accessory proteins, such as LPS binding proteins, CD14 and the TLR4-MD2 complex. LPS-bound receptor complex triggers an intracellular signaling cascade through the adaptor proteins MyD88 and TRIF. The MyD88-dependent pathway induces the activation of MAPKs and NF-κB leading to the production of several pro-inflammatory cytokines. The TRIF-dependent pathway requires the adaptor TRAM and activates both pro- and anti-inflammatory responses (Fig. 5.1, Morris et al. 2015).

With regard to the dynamics of the response of innate immune cells to LPS via TLR4 signaling, it is well established that it depends on the amount and duration of LPS exposure. In acute infection, during the initial phases, LPS exposure consists in a rapid inflammatory response of innate immune cells that hinder the growth and dissemination of the infectious agent. This is realized through the induction of proinflammatory cytokines (IL-8, IL-6, IL-1β, IL-1, IL-12, IFNγ, TNFα) which are responsible for the recruitment of neutrophils, natural killer and antigen-presenting cells to the site of infection. In turn, the activation of the innate immune response stimulates the adaptive mechanisms against microbial pathogens. In a physiological process, the pro-inflammatory phase is then followed by a secondary anti-inflammatory phase where other cytokines (IL-10, TGFβ) combine with apoptosis of pro-inflammatory cells to resolve inflammation and restore homeostasis. In the case of LPS-induced chronic inflammation, instead, the pro-inflammatory phase fails to resolve, leading to a persistent low-grade inflammation and hence to a pathological state. LPS challenge is also known to induce a refractory state in cells subsequently exposed to endotoxin. This phenomenon is known as endotoxin tolerance and is characterized by suppression of expression of pro-inflammatory mediators to avoid excessive inflammation. In an opposite manner, pre-exposure of innate immune cells to low dose LPS (priming) can potentiate the pro-inflammatory response to subsequent endotoxin challenge.

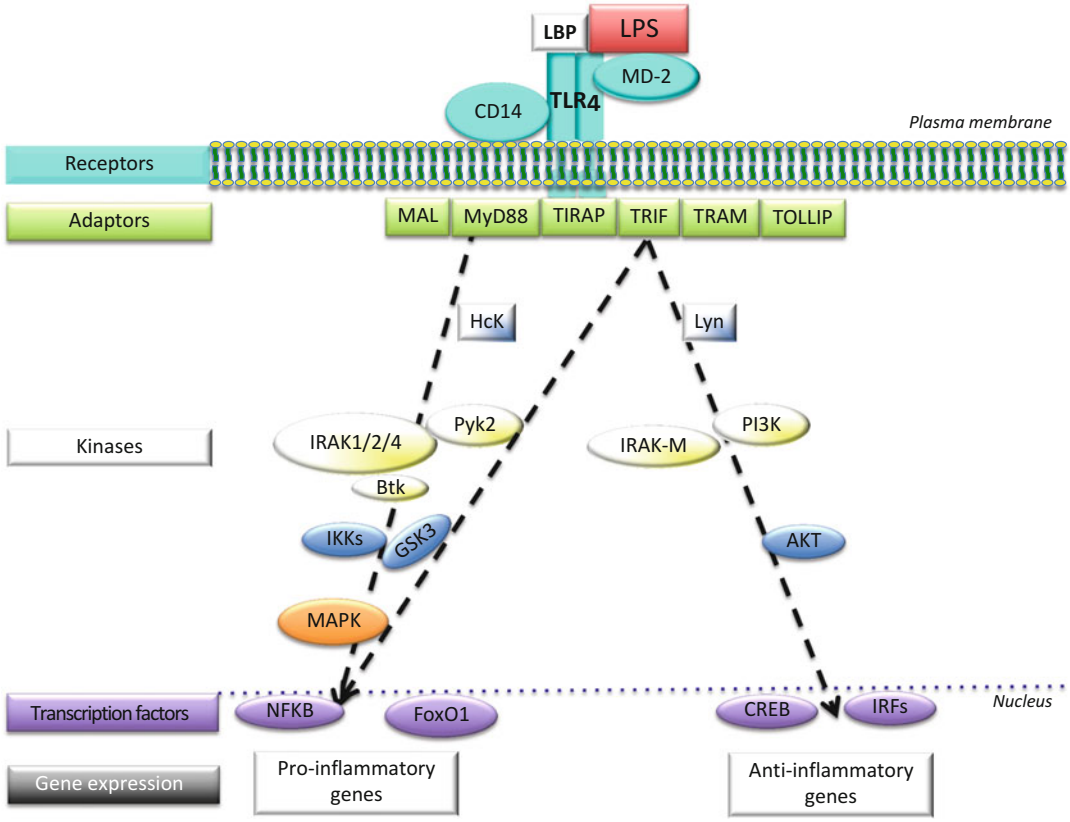

Fig. 5.1 Schematic representation of TLR4-mediated inflammatory response induced by LPS. LPS-bound receptor complex triggers an intracellular signaling cascade through two distinct pathways depending on the use of either adaptor protein MyD88 or TRIF. Signaling through MyD88 culminates in the activation of mitogen-activated protein kinases (MAPK) and nuclear translocation of NF-κB, with subsequent induction of pro-inflammatory genes (TNFα, IL1β, IL12 and IL6). The TRIF-dependent pathway activates both IRF3 to activate anti-inflammatory genes (IFNβ and related genes) and TRAF6 to activate pro-inflammatory genes (Adapted from Morris et al. 2015)

As already mentioned, not only innate immune cells but also non-immune cell types (i.e. epithelial cells) can respond in a TLR4-dependent manner to LPS, given their location at the interface with the environment and their direct contact with microorganisms. One level of regulation for these cell types to LPS exposure is the control of the TLRs cellular localization or expression levels, in order to mediate and modulate specific host response to commensal or pathogenic microorganisms. LPS recognition by TLR4, expressed on the surface of epithelial cells, determines both a rapid induction of anti-microbial peptides (i.e. defensins) and the production of inflammatory cytokines to recruit immune cells at infection sites. The pro- and anti-inflammatory cytokines most frequently produced by epithelial cells after LPS stimulation include those involved in immune regulation. Importantly, in epithelial cells a suppression of TLR-activation in response to commensal microbiota has been observed (McClure and Massari 2014).

The complexity of both the pathological and physiological inflammatory responses to LPS in the different cell types, requires the development of specific cell functions. In fact, prompt gene activation for immune response, antimicrobial defense, tissue remodelling and repair are necessary to counteract the LPS stimulus. In this scenario, it appears clear that a fine and dynamic regulation of gene expression programs is necessary for a harmonious and well coordinated

response. Increasing evidence indicates that epigenetic factors, in terms of histone modifications and DNA methylation play a central role in determining an appropriate cell context to LPS response.

5.2 Epigenetic Response to LPS during Inflammatory Process

Host exposure to bacterial LPS can affect epigenetic processes and lead to epigenetic modifications. Epigenetic changes induced by LPS have been studied in basic research, especially in the field of innate immune response, in order to assess how the host could adapt or protect itself against microbes. By this view, epigenetic control could represent a way for the host to compensate, minimize or memorize the contacts with both commensal or pathogenic bacteria.

In fact, LPS affects diverse sets of epigenetic factors such as histone modifications, DNA methylation, chromatin associated complexes, thus determining modifications of chromatin structure and gene expression programs. Although we are convinced that DNA methylation changes will deserve more attention, up until now histone modifications in response to LPS have been much more widely investigated.

In the following paragraphs we will focus on the mainly-reported LPS-induced epigenetic modifications in the body's first barrier systems against microbes (innate immune cells and epithelial cells) during inflammation processes.

5.2.1 LPS-Induced Histone Modifications in Innate Immune Cells

The correct timing of the innate immune response to LPS depends on the capacity of the involved cells (neutrophils, monocytes, macrophages, dendritic cells) to activate or repress signalling pathways in order to employ different sets of transcription factors to realize a specific cell program. Epigenetic modifications of histones such as methylation, acetylation and phosphorylation, represent a critical link that regulates the access of the transcription and chromatin remodeling factors to DNA sequences in the different immune cell types during inflammation processes. Although the whole story is far from being known, the main steps that have been investigated to date are: (i) signaling to the nucleus after LPS-stimulation (ii) changes in the levels of expression of chromatin factors (iii) their recruitment to specific gene promoters and (iv) final outcome in terms of expression or repression of specific sets of genes (Fig. 5.2).

A bulk of information about these molecular mechanisms came from the employment of mouse immune cells subjected to endotoxin stimulation in which epigenetic variations at inflammatory gene promoters were analyzed. Nevertheless, a complete drawing of the molecular mechanisms that define the specific cellular response to LPS is far from being depicted.

5.2.1.1 Histone Modifications at Inflammatory Genes after LPS Stimulation

The first evidence of a link between LPS stimulation and chromatin remodelling at inflammatory genes goes back to 1999 (Weinmann et al. 1999). In this study the authors demonstrated that, in murine primary activated macrophages, LPS stimulation induced IL-12 production by a rapid and specific nucleosome displacement at the IL-12 promoter region. Analysis of mutant mice revealed that nucleosome repositioning at the IL-12 promoter by LPS occurred in a TLR-4 dependent manner, and that it was dependent on histone H3 and H4 acetylation (Weinmann et al. 2001).

A key enzyme involved in the inflammatory reaction, that is rapidly induced in macrophages upon LPS stimulation, is cyclooxygenase-2 (COX-2). Changes of the chromatin structure at the COX-2 regulatory region were found in response to LPS (Park et al. 2004). Phosphorylation and acetylation of histone H3 in the COX-2 promoter contribute to COX-2 gene activation in macrophages. Accordingly,

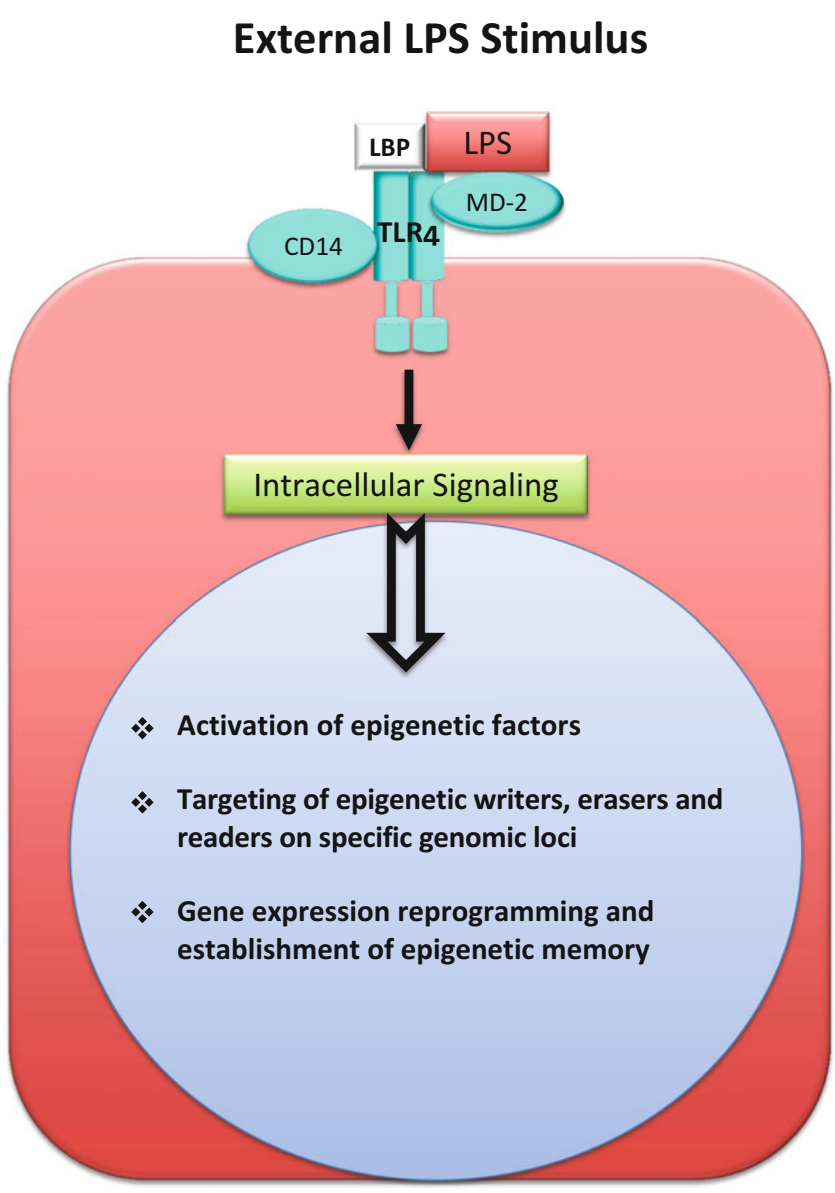

Fig. 5.2 Schematic cartoon of sequential steps to realize epigenetic-mediated gene expression reprogramming upon LPS stimulation. Ideally, the complete pathway should be elucidated in detail for each of the different cell contexts; to date, although some information has accumulated helping to draw a complete map of the events, it is clear that further efforts are needed in the next future

treatment with sodium butyrate (NaBT), an inhibitor of histone deacetylases (HDACs), enhanced H3 acetylation along with upregulation of COX-2 expression. Because MAP kinase inhibitors caused an alteration of H3 acetylation and phosphorylation in response to LPS stimulation, the authors concluded that NaBT accentuates LPS-induced COX-2 expression through a MAP kinase-dependent pathway (Park et al. 2004).

While histone acetylation is associated with an open chromatin conformation that facilitates transcription, histone lysine methylation can both promote or repress transcription depending on

which lysine residue is modified (Shilatifard 2006; Kouzarides 2007). Analyses of histone methylation revealed the importance of this epigenetic modification in activating inflammatory genes in response to LPS. In DCs, H3 lysine 9 (H3K9) methylation was found at a specific subset of promoters of inducible inflammatory genes, such as IL-12p40, ELC, and MDC mediating repression in unstimulated cells (Saccani and Natoli 2002). Upon LPS stimulation this repressive mark was rapidly removed to be then restored at a later time. This phenomenon was cell specific, because it was observed in DCs but not in monocytes (Saccani and Natoli 2002). Further experiments were aimed at elucidating the mechanism underlying chromatin remodeling and transcription factor accessibility at the IL-12 promoter (Saccani et al. 2002). In LPS stimulated primary DCs, the p38 MAPK pathway was involved in H3 phosphorylation (S10) and phosphoacetylation (S10/K14) at specific inflammatory gene promoters, including IL-12p40, thus increasing NF-κB recruitment and allowing transcription to occur.

Thus, yet in early studies it emerged that the response of innate immune cells to LPS was realized through histone modifications at specific inflammatory genes prompting further investigation to address the temporal cascade of epigenetic events and the effects of specific epigenetic inhibitors. A pivotal study (De Santa et al. 2009) addressed the role of histone demethylase Jmjd3, that specifically demethylates trimethylated lysine 27 (H3K27me3), a histone mark associated with transcriptional repression (Xiang et al. 2007). De Santa and colleagues demonstrated that Jmjd3 was rapidly induced by NF-κB upon LPS stimulation of macrophages. Although more than 70 % of LPS-induced genes recruited Jmjd3 at their promoter regions, only a subset of them, such as IL12b and Ccl5, showed a strong dependence on Jmjd3 demethylase activity for full activation (De Santa et al. 2009).

HDACs were also strongly involved in the LPS response and have been suggested to play a prominent role in the regulation of immunological pathways. LPS stimulation affects the expression of almost all classes of HDACs in macrophages, albeit at different magnitude and kinetics. For example, HDAC-6, -10 and -11 are gradually downregulated until up to 8 h after LPS stimulation with a slight recovery after 24 h (Aung et al. 2006). A similar expression pattern was observed for HDAC-4, -5 and -7 while HDAC-1 was gradually up-regulated until 8 h and stabilized by 24 h after stimulation (Aung et al. 2006). Hence, it is likely that sequential regulation of HDACs may control gene specific expression upon LPS stimulation. At the *Cox-2* gene it has been shown that LPS initially represses expression of HDAC-4, -5 and -7 and upregulates the acetyltransferase complex leading to *Cox-2* gene activation while, later on, increased levels of HDAC-1 caused a *Cox-2* switch down. The authors also demonstrated that HDAC-8 overexpression blocked the ability of LPS to induce Cox-2 mRNA. In particular, the acetylation state of H4K12 at the *Cox-2* gene was found to be related to the LPS response (Aung et al. 2006). A comparable mechanism has been described for IL-8 induction during *L. pneumophila* infection of lung epithelial cells. In this case, an initial decrease of HDAC-1 and -5 accompanied IL-8 induction while, at later time points, these HDACs were recruited back to the IL-8 promoter to terminate transcription (Schmeck et al. 2008).

An integrated genomic approach showed that LPS-stimulated *Hdac3*-deficient macrophages were unable to activate almost half of the inflammatory gene expression program (Chen et al. 2012). In particular, those genes involved in the second wave of induction, such as genes involved in microbial killing (i.e. *Nos2*), in antigen presentation (i.e. MHC class II genes), in T-cell costimulation (i.e. *CD86*) failed to be activated in LPS-stimulated $Hdac3^{-/-}$ cells. Specifically, the IFN-β-dependent branch of the LPS response was almost completely abrogated because of reduced basal and LPS-inducible Ifn-β expression. A further focus on the possible mechanisms of the altered LPS response in $Hdac3^{-/-}$ cells revealed an increased constitutive expression of Cox-1, due to the loss of HDAC3-mediated repression; interestingly, the use of Cox-1 inhibitors partially rescued Ifn-β levels indicating a causative role for Cox-1 overexpression in the

observed phenotype (Chen et al. 2012). Other studies indicated that both HDAC-6 and -7 are involved in proinflammatory gene expression in macrophages upon LPS-stimulation. In fact, a specific isoform of Hdac-7 (Hdac7-u) promoted the expression of a subset of TLR-inducible pro-inflammatory genes such as *Edn-1* (Shakespear et al. 2013) and suppression of HDAC-6 activity significantly restrains LPS-induced activation of macrophages and pro-inflammatory cytokines (Yan et al. 2014).

5.2.1.2 Use of HDAC Inhibitors to Study the LPS Response

The reported changes in HDAC expression in response to LPS have prompted the use of selective HDAC inhibitors (HDACIs), such as valproic acid (VPA), trichostatin A (TSA) and others. HDAC inhibition was found to either enhance or repress LPS-induced immune response depending on gene targets and cellular context. TSA displayed selective effects on a subclass of LPS-induced genes in macrophages, including *Cox-2*, *Cxcl2*, and *Ifit2*, by strongly enhancing their LPS-induced expression. Another class of LPS-inducible genes, which included *Ccl2*, *Ccl7*, and *Edn1*, was suppressed by TSA, an effect attributed to PU.1 degradation. The authors suggested that HDACs act as potent and selective negative regulators of proinflammatory gene expression and act to prevent excessive inflammatory responses in macrophages.

Halili et al. (2010) reported that HDACIs have either pro- or anti-inflammatory effects on LPS-stimulated macrophages *in vitro* (Halili et al. 2010). In fact, several broad-spectrum HDACIs including TSA, suppressed the LPS-induced mRNA expression of the pro-inflammatory mediators *Edn-1*, *Ccl-7*/*MCP-3*, and *IL12p40* while they amplified the expression of pro-atherogenic factors *Cox-2* and *Pai-1*/*serpine1* in bone marrow-derived macrophages. Moreover, the pro- and anti-inflammatory effects of TSA were separable over a concentration range, implying that individual HDACs have differential effects on macrophage inflammatory responses.

Importantly, treatment of macrophages with TSA downregulated the expression of secondary, but not primary genes induced by LPS. The secondary gene response implies the induction of genes that require new protein synthesis and chromatin remodeling at their promoters, and thus display a delayed response to LPS. NO synthase 2 (*NOS2*) is a representative secondary response gene whose expression is inhibited by TSA during LPS stimulation (Serrat et al. 2014). In this case, upon TSA and LPS treatment, the increased global acetylation level determined a strong binding of CDK8 at the *NOS2* promoter and the formation of a mediator complex submodule that has been characterized as a transcriptional repressor (Serrat et al. 2014).

It is clear that HDACs play a prominent role in coordinating gene expression during the inflammatory response especially in response to LPS in innate immune cells. The proved proinflammatory functions of certain HDACs suggested the use of HDACIs as anti-inflammatory agents. Very recently, a new generation of immunomodulatory drugs has been discovered that targets inflammatory gene expression by interfering with binding of bromodomain-containing BET proteins to acetylated histones (Nicodeme et al. 2010). These synthetic compounds (I-BET) disrupt chromatin complexes responsible for the expression of key inflammatory genes in activated macrophages, and confer protection against the LPS-induced inflammatory response (Nicodeme et al. 2010).

5.2.1.3 High Throughput Approach for Identification of LPS-Induced Epigenetic Marks

The development of genomic approaches such as high-throughput Chromatin Immunoprecipitation (HT-ChIP), has enabled the identification of the histone modifications that mark genomic regulatory elements upon LPS stimulation. In a model system of DCs stimulated with LPS at different time points, Garber et al. (2012) defined the dynamics of DNA binding by 25 transcription factors and 4 different chromatin marks. Two of the main permissive modifications, H3K4me3 and H3K36me3 were associated with active transcription at many loci involved in the inflammation response (Garber et al. 2012). Further

analyses on LPS-stimulated macrophages revealed a chromatin signature at responsive gene enhancers upon LPS stimulation (Ghisletti et al. 2010). The binding of the master regulator PU.1 to selected enhancers, together with recruitment of LPS-induced specific transcription factors, determined the deposition of H3K4me1 and the displacement of nucleosomes to determine accessible DNA sequences (Ghisletti et al. 2010). More recently Ostuni et al. (2013) reported that increased H3K4me1 levels at a subset of enhancers, named "latent enhancers", was conserved after LPS withdrawal, suggesting the establishment of an epigenomic memory able to condition the cellular response to subsequent stimuli (Ostuni et al. 2013).

5.2.2 LPS-Induced DNA Methylation Alterations in Innate Immune Cells

DNA methylation is generally related to transcriptional repression and is a more stable mark compared to histone modifications. The effect of LPS on the DNA methylation state of innate immune cells has been poorly investigated and mainly evaluating the effects of DNMT inhibitors on gene expression. Early studies of Kovacs et al. (1987) demonstrated that production of IL-1β in LPS-stimulated human monocytes could be regulated by DNA methylation. Pre-treatment of monocytes with a demethylating agent, 5-azadeoxycytidine (5-azadC), upregulated IL-1β mRNA levels upon LPS-stimulation (Kovacs et al. 1987). Accordingly, human monocytes treated with S-adenosylmethionine (SAM), the methyl donor in DNA methylation, showed inhibition of the inflammatory response following LPS stimulation (Pfalzer et al. 2014).

In monocytes, the chromatin architecture of the IL-1β promoter/enhancer has been well defined. The gene regulatory region was defined as a poised structure able to guarantee an efficient transcription upon LPS stimulation (Tsukada et al. 1994; Liang et al. 2006; Suzuki et al. 2006). Because LPS stimulation did not modify the chromatin structure of the IL-1β promoter, the role of DNA methylation was examined (Wessels et al. 2010). 5-azadC-induced DNA demethylation led to increased LPS-mediated induction of IL-1β both in promyeloid and mature monocytic cells. Poplutz et al. (2014) showed that treatment with 5-azadC and LPS of promyeloid HL-60 cells, undifferentiated and not-expressing IL-6, significantly enhanced IL-6 transcription and also resulted in increased IL-6 promoter accessibility, suggesting an important role for DNA methylation as epigenetic mechanism accompanying cell maturation (Poplutz et al. 2014).

A link between LPS stimulation and DNA methylation at the gene specific level has also been found for SOCS1, a negative regulator of cytokine-mediated signal (Davey et al. 2006; Cheng et al. 2014). Aberrant DNA methylation at specific CpG sites of the SOCS1 promoter correlated with loss of gene expression in LPS-activated macrophages (Cheng et al. 2014). Treatment with 5-azadC resulted in recovery of SOCS1 and in turn inhibition of cytokine expression. Further investigation on the mechanisms involving SOCS1 expression revealed that DNMT1 mediates gene hypermethylation during the inflammation processes (Cheng et al. 2014).

5.2.3 Epigenetic Response to LPS in Other Cell Types

Changes of the expression program upon LPS stimulation and the associated epigenetic mechanisms have been investigated in a variety of non-immune cell types. The intestinal epithelium (IE) plays a major role in the gastrointestinal tract as the first line of defence against pathogenic microorganisms and thus actively participate in the innate immune response by inducing pro-inflammatory gene expression (Cario et al. 2000; Suzuki et al. 2003). IE is also involved in maintaining intestinal homeostasis given its continuous exposition to commensal bacteria (Rakoff-Nahoum et al. 2004). Several pieces of

evidence showed that epigenetic modifications participate in the response of intestinal epithelial cells (IECs) to LPS, in particular at genes involved in cell signaling and at pro-inflammatory cytokine genes (Takahashi et al. 2009; Angrisano et al. 2010). Decreased expression of specific TLRs and accessory molecules has been reported as one of the mechanisms of hyporesponsiveness in IECs (Abreu et al. 2001; Melmed et al. 2003). Takahashi et al. (2009) reported epigenetically mediated TLR4 downregulation in LPS low-responder IECs. Upon *Escherichia coli* K12 LPS stimulation, histone deacetylation and DNA methylation at the 5′ region of the TLR4 gene was significantly higher in an LPS-low responder IEC than in an LPS-high responder IEC line. Chromatin immunoprecipitation (ChIP) experiments showed that TLR4 promoter deacetylation is, at least in part, dependent on the ZNF160 transcriptional repressor (Takahashi et al. 2009).

In LPS responsive human IECs, Angrisano et al. (2010) demonstrated that IL-8 gene activation is accompanied by both histone H3 acetylation and methylation changes. In particular, transient specific changes in H3 acetylation and H3K4, H3K9 and H3K27 methylation occurred at the IL-8 gene promoter during LPS stimulation. Changes of H3-acetyl, H3K4me2 and H3K9me2 levels occurred early, transiently and corresponded to transcriptional activity, while changes of H3K27me3 levels at the IL-8 gene occurred later and were long lasting (Angrisano et al. 2010). The authors suggested that a strong mark of gene repression, such as H3K27me3, could predispose to a more repressed state of the IL-8 gene and, thus, could render the gene less responsive to further LPS stimulation. Analysis of the methylation state of five CpG sites lying around the IL-8 gene transcription start site showed that all five CpGs were completely unmethylated (0–2 %) both in untreated and in LPS-treated HT-29 intestinal cells, and that CpG methylation at the IL-8 locus in normal colon mucosa displayed an almost unmethylated state (0–4 %), confirming that DNA methylation is likely not involved in IL-8 gene regulation. In T84 cells and HT-29/B6 cells, LPS induced the global Ac-H4 and the p-Ac-H3 at Ser- 10/Lys-14 in a time-dependent manner (Ghadimi et al. 2012).

In cultured mouse lung fibroblasts, dynamic changes of Thy-1 expression from 0 to 72 h after LPS stimulation, were described (He et al. 2013). In this study, the authors demonstrated the occurrence of deacetylation of H3 and H4 and inhibition of *Thy-1* gene transcription after LPS administration, indicating that LPS may induce *Thy-1* gene transcriptional silencing through histone deacetylation. In this study it is hypothesized that Thy-1-related lung fibroblast phenotype transformation contributes to the pathogenesis of LPS-induced lung fibroblast proliferation and pulmonary fibrosis (He et al. 2013).

Recent evidence demonstrated that LPS from *Porphyromonas gingivalis* induced changes in DNA methylation in infected human periodontal fibroblasts. Uehara et al. (2014) showed that LPS induces DNA hypermethylation of the Runt-Related Transcription Factor 2 gene (*RUNX2*) in periodontal fibroblasts. They also reported increased expression of DNMT1 suggesting that LPS inhibits osteoblastic differentiation of periodontal fibroblasts by DNA hypermethylation of target genes (Uehara et al. 2014).

In a study on cancer development in liver cells aimed at demonstrating a link between bacterial-induced chronic inflammation and genome instability, alteration of expression of epigenetic factors have been reported for naive host cells, that are not in direct contact with bacterial components. Liver cells from sacrificed mice that were provided with water supplemented with LPS showed increased levels of maintenance and *de novo* DNA methyltransferases (DNMT1, DNMT3A and 3B) and MeCP2 (Kovalchuk et al. 2013). DNA damage resulting from chronic exposure to LPS, may result in DNA repair as well as in loss of DNA methylation. The authors argued that increasing expression of methylases may serve to restore correct levels of DNA methylation.

Recent observations from Natarajan's group indicate that LPS treatment epigenetically regulates lung injury via a Sphingosine-1-Phosphate

(S1P)-mediated pathway in lung endothelial cells (Zhao et al. 2011). They observed that downregulation of Sphingosine-1-Phosphate-Lyase (S1PL) with siRNA blocked LPS-induced IL-6 secretion and partially blocked LPS-induced acetylation of histone H3 and Histone H4 with decreased HBO1 and IL-6 promoter activity and potentiated histone deacetylase (HDAC) 5 expression.

Recently, Das et al. (2013) reported the effect of LPS on neuroectodermal stem cell epigenetics, using an immortalized neuroectodermal stem cell line, NE-4C. They found that the histone demethylase, Jmjd2b is functional in long-term LPS treatment and regulates the histone demethylation of its target gene promoters suggesting that LPS has an inflammatory effect on NE-4C cells via epigenetic modulation (Das et al. 2013). Treatment of NE-4C cells with LPS significantly increased Jmjd2b expression and decreased the levels of H3K9me3. An induced presence of H3K9me3 has been observed at the promoters of the *Notch1*, *IL-1β*, and *IL-2* genes in Jmjd2b-kd (knockdown) NE-4C cells, suggesting that Jmjd2b can fine-tune the local chromatin state to enhance the transcription of these genes (Das et al. 2013).

A time-dependent increase in the levels of H3K36me3 was observed in the epididymis and testis challenged with LPS, suggesting multiple levels of epigenetic regulation in the male reproductive tract (Biswas and Yenugu 2013). Very recently it has been reported that dramatic epigenetic events occurred in a model of LPS-induced acute renal inflammatory diseases (Huang et al. 2015). One hour after LPS injection, significantly increased levels of total ac-K, H3K9ac, and a conspicuous raise of H3K18ac were found in the kidneys of LPS-injected mice when compared with PBS-injected mice (Huang et al. 2015). A number of epigenetic modifications were described in microglial cells exposed to LPS (Qin et al. 2007; Kannan et al. 2013; Singh et al. 2014; Schaafsma et al. 2015). All these studies pointed out that LPS-stimulated microglia may acquire an epigenetically regulated, immune-suppressed phenotype, possibly to prevent excessive damage to the central nervous system in case of recurrent inflammation.

5.3 Epigenetic Regulation in Endotoxin Tolerance

LPS- or endotoxin tolerance (ET) represents a refractory functional state of monocytes and monocyte-derived macrophages but also of DCs and neutrophils to LPS. The cells undergo a consistent gene reprogramming characterized by the incapacity to produce proinflammatory cytokines, upregulation of anti-inflammatory cytokines, increased phagocytosis, constant expression of antimicrobial genes and reduced ability of antigen presentation through decreased HLA-DR expression (Cavaillon et al. 2003). Clearly, the LPS-tolerant phenotype is the result of complex regulatory mechanisms extending from the cell membrane to cytosolic mediators to the nucleus. Signaling mechanisms in ET occur through TLR4 and have been associated with decreased TLR-4-MyD88 complex formation and with the end defects of activation of NF-κB (Fan and Cook 2004; Biswas and Tergaonkar 2007); therefore, the TRIF-IRF3 pathway seems to have an important role in mediating ET. Endotoxin-tolerant mouse embryonal fibroblasts possess a functional TRIF pathway and overexpress TRIF-induced genes such as *IFNβ* and *CCL5*, while *TRIF* and *IFNβ* knockout mice were resistant to endotoxin shock (Hacker et al. 2006; Biswas et al. 2007). Attempts to elucidate the role of signaling in silencing pro-inflammatory genes, such as *TNF-α* and *iNOS*, showed that in endotoxin tolerant murine lung macrophages, interleukin-1 receptor associated kinase-M (IRAK-M), a negative regulator of the TLR-4-MyD88 pathway, determined a decreased activation of epigenetic marks (Lyn-Kew et al. 2010).

ET often represents a complication to sustained exposure to LPS occurring in severe sepsis and is defined as Post Septic Immunosuppression (PSI). Inhibition of inflammatory genes in PSI helps to counteract the harmful effects of sepsis where high levels of LPS elicit an uncontrolled "cytokine storm" with subsequent tissue damage, vascular shock, and multiorgan failure. On the other hand, PSI defines a status of high susceptibility to successive infections for extended periods of time because of the inability of cells to

respond in a proper manner to further exposures to endotoxin. This phenomenon has been observed both *in vivo* and *in vitro* in animal models and humans. Furthermore, *ex vivo* challenge of blood monocytes from sepsis patients with LPS showed the characteristics of PSI (Medvedev et al. 2000; Dobrovolskaia and Vogel 2002). Compared to the acute response, in which we find transient gene expression and modification of epigenetic marks, prolonged exposure and subsequent challenge to LPS elicits a massive cell reprogramming with permanent gene repression. The state of "immunological imprinting" exhibited in ET led to hypothesize an important role of histone and DNA methylation to realize a specific functional cell program. Moreover, given the possibility to revert epigenetic marks, understanding the pathoepigenetic processes in ET is an important challenge for therapeutic applications.

5.3.1 Epigenetic Regulation of Pro-inflammatory Gene Loci in Endotoxin Tolerance

A prominent event occurring during ET is the downregulation of inflammatory cytokines and chemokines, like TNF-α, IL-6, IL-1β, IL-12, CCL3, CCL4 and CXCL10 (Mages et al. 2007; Draisma et al. 2009). Inflammatory cytokine expression has emerged to be controlled by epigenetic changes during ET; in particular, early studies reported that in macrophages following LPS tolerance induction two pro-inflammatory cytokines, critical for microbe clearance, IL-1β and TNF-α, showed increased levels of repressive histone marks at promoter regions. In human LPS-tolerant monocytes expressing low levels of IL-1β (LaRue and McCall 1994), reduced NF-κB p65 binding to the IL-1β promoter was accompanied by the reduction of phosphorylation of histone H3 at Ser10 and steady levels of H3K9me2. Thus, the remodeling of promoter nucleosomes, mainly obtained through increased phosphorylation of H3Ser10 and demethylation of H3K9, was disrupted during ET (Chan et al. 2005). Similar results were reported for the epigenetic regulation of TNF-α, whose reduction is an important mark for ET (El Gazzar et al. 2007). Further analysis to identify transcriptional factors and mediators recruited at silenced TNF-α and IL-1β proximal promoters in LPS-tolerant human monocytes unveiled the presence of high mobility group box 1 protein (HMGB1) and nucleosome linker histone H1 protein as factor able to help in heterochromatin formation and NF-κB factor RelB as transcriptional repressor (El Gazzar et al. 2007). Interestingly, siRNA experiments showed that removal of H1 and HMGB1 displaced RelB from the IL-1β and TNF-α promoters, also erasing H3K9 dimethylation (El Gazzar et al. 2009). These observation inspired the hypothesis that, as in the case of some acute pro-inflammatory genes (Yoza et al. 2006), also in ET, RelB could cooperate with other co-repressors to realize gene repression. In fact, it was demonstrated that RelB directly interacts with the histone H3K9 methyltransferase G9a at the IL-1β promoter by actively silencing the gene (Chen et al. 2009). Moreover, heterochromatin protein 1 (HP1) formed a complex with G9a dependent on RelB. Importantly, the connection between histones and DNA methylation during ET was demonstrated by the binding of HP1 to DNA methyltransferases Dnmt3a/b causing *de novo* methylation at the TNF-α promoter (El Gazzar et al. 2008). By this way, a stable epigenetic modification may mark the promoter in order to establish heritable and long lasting gene silencing.

As aforementioned, during ET a selective modulation of gene expression related to gene function is observed; in this way, inhibition of pro-inflammatory gene expression, upregulation of anti-inflammatory cytokines and constant expression of antimicrobial effectors can be achieved. Thus, a critical approach in ET is epigenetic profiling of ET associated genes. This point was well addressed by Foster et al. (2007). By expression microarray analysis in tolerant macrophages, Foster et al. identified two classes of genes, all dependent on TLR4 activation, named "tolerizeable" (T, genes not inducible in tolerant macrophages) and "non-tolerizeable" (NT, genes inducible in tolerant macrophages).

The main histone modifications at promoter regions of representative genes of the two classes were then analyzed. Chromatin immunoprecipitation data showed that H3K4 trimethylation was high at both classes of promoters, where NT promoters also exhibited increased levels of histone acetylation and accessibility. Interestingly, LPS stimulation of tolerant macrophages revealed that class T promoters remained deacetylated and inaccessible, whereas class NT promoters became more acetylated and accessible (Foster et al. 2007). These findings indicated that gene-specific chromatin modifications may coordinate the dynamics of gene expression programs during ET. More recently Saeed et al. (2014) reported the epigenomic profiling of histone marks performed on purified circulating macrophages from human volunteers primed with β-glucan and then exposed to LPS to generate PSI (Saeed et al. 2014). Their finding uncovered the epigenetic and transcriptional program of LPS-tolerant differentiated macrophages and provided an important tool to further understand and manipulate innate immune tolerance to identify potential pharmacological targets.

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Patho-epigenetics of Infectious Diseases Caused by Intracellular Bacteria

6

Hans Helmut Niller and Janos Minarovits

Abstract

In multicellular eukaryotes including plants, animals and humans, epigenetic reprogramming may play a role in the pathogenesis of a wide variety of diseases. Recent studies revealed that in addition to viruses, pathogenic bacteria are also capable to dysregulate the epigenetic machinery of their target cells. In this chapter we focus on epigenetic alterations induced by bacteria infecting humans. Most of them are obligate or facultative intracellular bacteria that produce either bacterial toxins and surface proteins targeting the host cell membrane, or synthesise effector proteins entering the host cell nucleus. These bacterial products typically elicit histone modifications, i.e. alter the "histone code". Bacterial pathogens are capable to induce alterations of host cell DNA methylation patterns, too. Such changes in the host cell epigenotype and gene expression pattern may hinder the antibacterial immune response and create favourable conditions for bacterial colonization, growth, or spread. Epigenetic dysregulation mediated by bacterial products may also facilitate the production of inflammatory cytokines and other inflammatory mediators affecting the epigenotype of their target cells. Such indirect epigenetic changes as well as direct interference with the epigenetic machinery of the host cells may contribute to the initiation and progression of malignant tumors associated with distinct bacterial infections.

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J. Minarovits, H.H. Niller (eds.), *Patho-Epigenetics of Infectious Disease*,
Advances in Experimental Medicine and Biology 879, DOI 10.1007/978-3-319-24738-0_6

Keywords

Intracellular bacteria • Histone code • Cholesterol-dependent cytolysin • Short-chain fatty acids • Nuclear effector • SET-domain protein • Inflammatory cytokines • Downregulation of imprinted genes • Nucleomodulin protein

6.1 Introduction: Epigenetic Alterations Induced by Bacteria in Host Cells

Epigenetic alterations induced by micro- and macroparasite infections in multicellular organisms became a fascinating research area in recent years (Minarovits 2009). The short or long term changes of host cell epigenotype elicited by pathogens may play a role in disease initiation and progression. An increasing number of bacterial pathogens may also elicit chromatin modifications in various host species. In this review we wish to focus on bacteria that cause epigenetic dysregulation in human cells. Bacteria belonging to as diverse phyla as *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Fusobacteria*, and *Proteobacteria* encode proteins or possess cell wall constituents that may interact with the epigenetic machinery of eukaryotic host cells. These interactions frequently result in a reprogramming of the host cell epigenotype and gene expression pattern and associate with pathological changes. This field has been comprehensively reviewed (Bierne et al. 2012; Niller et al. 2012a, b). The quickly accumulating new data, however, necessitate a novel overview and synthesis of bacterial patho-epigenetics.

Pathogenic bacteria may interfere with the epigenetic regulatory machinery of the host cells not only directly, by introducing bacterial products into the host tissue, but also in an indirect way, by eliciting a chronic inflammatory response. Certain bacterial epigenetic effectors may target the cell membrane of host cells and activate signal transduction pathways that mediate their action. Others are injected into the target cells or may be produced intracellularly and translocate to the nucleus where they induce localized chromatin alterations, resulting in dysregulation of gene expression, e.g. in silencing of specific immune defense or of tumor suppressor genes or activation of pro-inflammatory cytokine genes. Sustained epigenetic changes may cause chronic inflammation and malignant transformation of the affected tissue. A typical example is *Helicobacter pylori* associated gastric carcinoma where the bacteria play a role both in eliciting a local inflammation and in the establishment of an epigenetic field for cancerization at the initial phase of carcinogenesis (Ushijima 2007).

Pathogenic bacteria may grow and reproduce either outside the cells of their host (extracellular bacteria) or they may rely so much on intracellular resources that they can't reproduce themselves outside their host cell (obligate intracellular bacteria, Table 6.1.). Certain bacteria are capable to survive and grow both inside and outside cells (facultative intracellular bacteria, Table 6.2.) (Silva 2012). In addition to obligate intracellular bacteria, other microparasites also replicate exclusively inside host cells; these obligate intracellular pathogens include viruses, certain protozoa that belong to the Apicomplexans (*Plasmodium* spp., *Toxoplasma gondii*, *Cryptosporidium parvum*), Trypanosomatids (*Leishmania* spp. and *Trypanosoma cruzi*), and certain fungi (*Pneumocystis jirovecii*) (Table 6.1.). Other fungi are facultative intracellular pathogens (*Histoplasma capsulatum*, *Cryptococcus neoformans*, *Candida glabrata*, *Candida parapsilosis*, *Candida albicans*) (Seider et al. 2011; Toth et al. 2014) (Table 6.2.). It is important to note, that typical extracellular pathogens frequently hinder phagocytosis by host cells whereas intracellular pathogens facilitate entry into macrophages or other target cells (Silva 2012). In addition, there are numerous examples of pathogens, including pathogens that induce epigenetic alterations in their hosts, which

Table 6.1 Obligate intracellular pathogens infecting humans

Viruses
All of the pathogenic viruses replicate intracellularly
Bacteria
Chlamydia trachomatis,
Chlamydophila pneumoniae
Chlamydia psittaci
Anaplasma phagocytophilum (*Rickettsia*)
Ehrlichia chaffeensis (*Rickettsia*)
Coxiella burnetii
Mycobacterium leprae
Protozoa
Apicomplexans (*Plasmodium* spp., *Toxoplasma gondii* and *Cryptosporidium parvum*)
Trypanosomatids (*Leishmania* spp., *Trypanosoma cruzi*)

Table 6.2 Typical facultative intracellular bacteria and fungi infecting humans

Bacteria
Francisella tularensis
Listeria monocytogenes
Salmonella typhi
Brucella
Legionella
Mycobacterium tuberculosis
Nocardia
Rhodococcus equi
Yersinia
Neisseria meningitidis
Fungi
Histoplasma capsulatum
Cryptococcus neoformans
Candida glabrata
Candida parapsilosis
Candida albicans

do enter distinct target cells and replicate within their host cells in spite of the fact that they are traditionally categorized as extracellular (Silva 2012, see also the following paragraphs).

Epigenetic regulation based on DNA adenine methylation plays an important role in the control of transcription in several pathogenic bacteria including *Escherichia coli*, *Salmonella*, *Vibrio*, *Yersinia*, *Haemophilus*, and *Brucella* (Casadesus and Low 2006; Heusipp et al. 2007). Thus, adenine methyltransferases expressed within the host cells of facultative intracellular bacteria may, in principle, methylate the DNA of eukaryotic hosts as well. Expression of the *E. coli* Dam DNA methyltransferase in eukaryote (yeast) cells resulted in methylation of adenines located within GATC recognition sites of the linker and adjacent 21 bp of nucleosome-associated DNA sequences (Kladde and Simpson 1994). In addition, active promoter regions in a 140-kb human genome locus were highly accessible to methylation by Dam methylase (Bulanenkova et al. 2011). Because the presence of N-6-methyl adenine in DNA affects protein-DNA interactions (Wion and Casadesus 2006), one may speculate that bacterial adenine methyltransferases, when transported or released from intracellular bacteria may reach and modify the genome of the eukaryotic host, resulting in altered protein-DNA interactions that may dysregulate gene expression patterns.

Lysogenic conversion, i.e. integration of bacteriophage genomes into the DNA of their bacterial hosts plays a major role in the evolution of pathogenic bacteria (reviewed by Fortier and Sekulovic 2013). Expression of extracellular bacterial toxins and enzymes, adhesion and mitogenic factors as well as superantigens or other virulence factors encoded by the prophage genome affect the lifestyle, fitness and disease spectrum of lysogenic bacteria. It is worthy to note, however, that genes coding for DNA methyltransferases are also frequently transmitted by bacteriophages, resulting in altered modification of the bacterial genome. Fang et al. observed that the lambdoid phage ΦStx104 – integrated into the genome of hemolytic uremic syndrome (HUS)-linked *Escherichia coli* O104:H4 serotype outbreak strain C227-11 – not only encoded Shiga toxin that caused the symptoms of HUS, but also M.EcoGIII, an adenine-specific methyltransferase that altered the bacterial epigenome (Fang et al. 2012). In parallel with M.EcoGIII expression and m6A modification of the CTGCAG recognition sequence, increased transcription of 1.155 genes and decreased transcription of 796 genes could be detected when compared with a derivative of C227-11 lacking

the genes for M.EcoGIII and the corresponding restriction endonuclease. Although there was no significant correlation between altered adenine methylation events and differential gene expression, Fang et al. argued that the M.EcoGIII methylase not only functions as a component of a restriction-modification system, but may also cause physiologically meaningful changes in the expression of the bacterial genome (Fang et al. 2012).

It is worthy to note, that certain bacterial proteins possess a nuclear localisation signal, and HsdM, a *Klebsiella pneumoniae* encoded adenine-specific DNA methyltransferase exhibited nuclear localisation when transiently expressed in an African green monkey kidney fibroblast-like cell line and could methylate eukaryotic DNA in an *in vitro* assay (Lee et al. 2009). Although *K. pneumoniae* is viewed as an extracellular bacterium, it is capable to invade epithelial cells (Cortes et al. 2002). Thus, in principle, *K. pneumoniae* HsdM may reach the genome of the invaded host cell, although there are no data as to its role in pathogenesis. The genome of the opportunistic pathogen *Acinetobacter baumannii* also encodes a protein possessing a nuclear localisation signal (Moon et al. 2012). Similarly to *K. pneumoniae*, *A. baumannii* typically replicates at extracellular sites, and it is capable to invade epithelial cells (Choi et al. 2008). A transposase (Tnp) of *A. baumannii* ATCC 17978 fused with green fluorescent protein (GFP) could enter the nuclei of COS-7 cells and as judged by methylation-specific PCR, it induced CpG methylation at the *E-cadherin* promoter (Moon et al. 2012). The significance of these findings regarding *A. baumannii* pathogenesis remains to be established.

Mycoplasma penetrans, an obligate intracellular pathogen that causes persistent infection in humans and contributes to the deterioration of the immune system in AIDS patients (Sasaki et al. 2002) encodes a CpG specific DNA methyltransferase, M.MpeI (Wojciechowski et al. 2013). M.MpeI is constitutively active in *Mycoplasma penetrans* infected cells, methylating its target sequences in a locus-nonselective manner. CpG methylation followed by deamination of 5-methylcytosine, a phenomenon observed in eukaryote genomes, may also account for the underrepresentation of CpG dinucleotides in the *Mycoplasma penetrans* genome (Wojciechowski et al. 2013). It remains to be established whether M.MpeI gets access to the host cell nuclei and whether it alters the host cell methylome. Although Spiroplasma spp. is regarded as an obligate intracellular bacterium that usually colonizes plants and insects, recently a human case of systemic infection by *Spiroplasma turonicum* was recorded (Aquilino et al. 2014). Because the genome of the Spiroplasma strain MQ1 encodes a CpG specific DNA methyltransferase (M.SssI) (Renbaum and Razin 1992), one can't exclude the possibility that rare Spiroplasma infections in humans may also have patho-epigenetic consequences.

Analysis of the whole genome sequence of various *Helicobacter pylori* strains revealed that they encode a large number of adenine- and cytosine-specific DNA methyltransferases (Sitaraman 2014). Because H. *pylori* can invade epithelial cells and persist in macrophages and neutrophil granulocytes (see Sect. 6.11), there is a chance that its DNA methyltransferases enter the host cell nucleus in spite of the fact that they are not known to be transmitted by bacterial membrane vesicles or translocated by the type IV secretion system of the bacterium (Sitaraman 2014). At present, however, there are no data regarding the alteration of the host cell genome by bacterial DNA methyltransferases during *H. pylori* infection. We conclude that modification of eukaryotic host genomes by bacterial methyltransferases may occur under experimental circumstances. The available evidence suggests, however, that during natural infections other mechanisms mediate the patho-epigenetic alterations elicited by bacteria in their eukaryotic hosts (Table 6.3).

6.2 *Listeria monocytogenes*

One of the best-studied examples for bacterial interference with the cellular epigenetic machinery is *Listeria monocytogenes*, a Gram-positive

facultative intracellular bacterium that has a 2.9 Mb long low G+C content genome (Buchrieser et al. 2003). *Listeria monocytogenes* can adapt to various environments as well as to intracellular life, even to the phagosomal environment of macrophages and granulocytes. Food-borne infections can have serious consequences, especially in immunosuppressed or immunodeficient individuals (meningitis, encephalitis, septicemia). In pregnant women, untreated listeriosis may result in abortion; it may cause stillbirth or premature birth, too.

Table 6.3 Induction of epigenetic alterations by human pathogenic bacteria

Phylum and strain	Epigenetic alteration
Firmicutes	
Listeria monocytogenes	Histone modifications
Bacillus anthracis	Histone modification
Actinobacteria	
Mycobacterium tuberculosis	Histone modification, CpG methylation?
Mycobacterium leprae	CpG methylation
Bacteroidetes	
Porphyromonas gingivalis	Histone modification, CpG methylation
Chlamydiae	
Chlamydia trachomatis	Histone modification?
Chlamydophila pneumoniae	Histone modification?
Chlamydophila psittaci	CpG methylation
Fusobacteria	
Fusobacterium nucleatum	Histone modification? CpG methylation?
Proteobacteria	
Helicobacter pylori	Histone modifications, CpG methylation
Campylobacter rectus	CpG methylation
Anaplasma phagocytophilum	Histone modification
Ehrlichia chaffeensis	PCG protein redistribution?
Bordetella bronchiseptica	Histone modification?
Burkholderia thailandensis	Histone modification?
Legionella pneumophila	Histone modifications
Aeromonas hydrophila	Histone modification
Shigella flexneri	Histone modification
Escheria coli	CpG methylation

The epigenetic effects of *L. monocytogenes* infection, i.e. the alteration of the "histone code" (see Chap. 1.) by the bacterial toxin listeriolysin O (LLO) were described in the pioneering work of Schmeck et al. and Hamon et al. (Schmeck et al. 2005; Hamon et al. 2007). Recently, however, it was revealed that additional virulence factors encoded by the genome of *Listeria monocytogenes* also affect the epigenetic machinery of the host cell. The InlB (internalin B) protein is expressed on the surface of *Listeria monocytogenes* whereas LntA (Listeria nuclear targeted protein A) is a secreted protein. InlB facilitates entry into nonphagocytic cells by binding to c-Met, a tyrosine kinase receptor located to the plasma membrane of various human cell types. Interaction of LntA with c-Met activates a signaling pathway and – indirectly – induces histone deacetylation at a battery of host cell promoters causing gene silencing (Eskandarian et al. 2013). In contrast, the secreted bacterial protein LntA enters the host cell nucleus and activates a set of host genes (Lebreton et al. 2011, 2014).

6.2.1 Listeriolysin O

Listeriolysin O (LLO) produced by *L. monocytogenes* and a group of related bacterial toxins produced by *Streptococcus pneumoniae* (pneumolysin), *Clostridium perfringens* (perfringolysin), *and Aeromonas hydrophila* (aerolysin) belong to a class of secreted, cholesterol-dependent and pore-forming cytolysins (reviewed by Hamon et al. 2006, Table 6.4.). At the initial phase of infection secreted LLO downregulated the immune response through histone H3 serine 10 dephosphorylation, i.e. through modification of the histone code, at the promoters of a specific set of immune genes coding for the pro-inflammatory chemokine CXCL2, the phosphatase DUSP4 and interferon regulatory factor 3 (IRF3) (Hamon et al. 2007). At the *CXCL2* and *DUSP4* promoter histone H3 dephosphorylation was accompanied by deacetylation of histone H4 (Hamon et al. 2007). The efflux of K^+ through membrane pore formation seems to play a major signaling role for the transcriptional reprogram-

Table 6.4 Epigenetic alterations induced by *Listeria monocytogenes*

Bacterial protein	Epigenetic change and consequence
Listeriolysin O (LLO),	Histone H3 dephosphorylation,
	gene silencing
	Histone H4 deacetylation,
	gene silencing
Internalin B (InlB)	Histone H3 K18 deacetylation,
	gene silencing
Listeria nuclear targeted protein A (LntA)	Interaction with the silencing factor BAHD1,
	gene activation

ming of the immune genes (Hamon and Cossart 2011).

After entering the host cell, however, LLO enables Listeria to degrade and evade the phagosome which permits bacterial entry into the cytosol where cellular nucleotide-binding oligomerization domain (NOD)-like receptors recognise conserved peptidoglycan motifs of the bacterial cell wall and induce a proinflammatory response (Schmeck et al. 2005; Opitz et al. 2006; Corr and O'Neill 2009). In human umbilical vein endothelial cells (HUVEC) infected with pathogenic (wild type, invasive) *Listeria monocytogenes*, upregulation of interleukin 8 (IL-8) expression was associated with histone H4 acetylation and histone H3 phosphorylation and acetylation both globally and at the *IL-8* promoter (Schmeck et al. 2005). Signaling *via* the protein kinases p38 MAPK and MEK1 contributed to IL-8 induction (Schmeck et al. 2005; Opitz et al. 2006). Thus, LLO may contribute both to silencing and activation of host genes at different stages of infection.

We notice here that *Streptococcus pneumoniae* (phylum: *Firmicutes*), an extracellular Gram-positive bacterium, produces a neurotoxin, PLY (pneumococcal pneumolysin) that is classified – similarly to LLO – as a cholesterol-dependent, cytolysin. Like LLO, PLY also induced K^+ efflux in HeLa cells that acted as a signal for dephosphorylation of histone H3 (Hamon et al. 2007; Hamon and Cossart 2011). In addition, perfringolysin O (PFO), an exotoxin produced by *Clostridium perfringens* (phylum: *Firmicutes*), a Gram positive spore-forming, extracellular bacterium, also induced histone H3 dephosphorylation (Hamon et al. 2007; Hamon and Cossart 2011).

6.2.2 Virulence Factor InlB (Internalin B)

The virulence factor InlB mimicks the action of hepatocyte growth factor (HGF), the natural ligand of the c-Met tyrosine kinase receptor. C-Met is expressed at high level not only on the surface of hepatocytes but also in other epithelial cell types and microglial cells. Binding of HGF or InlB to c-Met activates a signaling cascade involving phosphatidylinositol 3-kinase (PI3K) and Akt, a pathway apparently indispensable to the translocation of the histone deacetylase SIRT2 from the cytoplasm to the nucleus (Eskandarian et al. 2013) (Table 6.4). In the nucleus SIRT2 is targeted to a subset of promoters resulting in histone H3K18 deacetylation and silencing of distinct genes coding for transcription factors (SMAD1, FOXM, IRF2) and members of chromatin remodeling complexes (SMARCA2, SAP130). Immune response regulators and the chemokine Cxcl12 are downregulated as well (Eskandarian et al. 2013).

6.2.3 Virulence Factor LntA (Listeria Nuclear Targeted Protein A)

The virulence factor LntA is a 205 amino acid long basic protein secreted by *Listeria monocytogenes*. LntA is a protein highly conseved in all *Listeria monocytogenes* strains studied (Lebreton et al. 2011, 2014) (Table 6.4). It accumulates in the host cell nuclei and targets the silencing factor BAHD1 (bromo adjacent homology domain-containing protein 1), a regulator of heterochromatin formation (Bierne et al. 2009; Lebreton et al. 2011). Direct interaction of LntA and BAHD involves a dilysine motif in the elbow region of LntA and a proline-rich region in BAHD1 (Lebreton et al. 2014). BAHD1 is

involved in the repression of interferon stimulated genes (ISGs) in epithelial cells. During the listerial infection cycle LntA translocates to the host cell nuclei and induces the transcription of ISGs through the de-repression of BAHD1-containing silencing complexes. This listeria-controlled expression of ISGs may create a transient, localized pro-bacterial condition favouring listerial colonization of the host (Bierne et al. 2012; Dussurget et al. 2014). The timing of type I interferon production is delayed in *Listeria monocytogenes* infected mice compared to *E. coli* infected animals affecting the outcome of the infection (Pontiroli et al. 2012).

6.3 *Bacillus anthracis* Effector Protein *Ba*SET, a Histone Lysine Methyltransferase Targeting Histone H1

Bacillus anthracis, a Gram-positive endospore-forming bacterium typically found in soil environment is the causative agent of anthrax (Zink and Burns 2005). The infecting spores are ingested by macrophages. Within the macrophage the spores are either killed or germinate, and the vegetative bacilli replicate before rupturing the host cell (Ruthel et al. 2004). In addition to the exotoxins responsible for the lethality of *B. anthracis* infection and edema induction, the bacterium also expresses an effector protein called *Ba*SET (*B. anthracis* SET protein) that enters the nuclei of the infected macrophages (Mujtaba et al. 2013). SET domain proteins resemble **S**uppressor of variegation, **E**nhancer of zeste and **T**rithorax proteins that can methylate lysines at specific locations on histone tails (histone lysine methyltransferases, see Chap. 1). Mujbata et al. observed an elevated methylation of histone H1 in nuclear extracts of *B. anthracis* infected cells and demonstrated that *Ba*SET acts as a histone H1 methyltransferase trimethylating lysine residues, although the exact position of the modified lysine residues remains to be clarified (Mujtaba et al. 2013). They also demonstrated that expression of *Ba*SET repressed the activity of NF-κB targeted IL-6 and IL-8 promoters which were transfected as plasmid constructs into TNFα treated 293T cells and downregulated the levels of cellular cytokine gene transcripts in TNFα treated HeLa cells. *Ba*SET proved to be an indispensable virulence determinant in a murine bacteriemia model and also affected the growth kinetics and septum formation of the bacteria (Mujtaba et al. 2013). It is worthy to note that the genome of other bacteria, including human pathogens, also code for SET domain proteins (Alvarez-Venegas et al. 2007; Mujtaba et al. 2013), see also the paragraphs below). The SET domain protein of the Gram-positive spore-forming bacterium *Bacillus cereus* (*Firmicutes*) that shows a significant amino acid sequence homology to *Ba*SET remains to be characterized (Mujtaba et al. 2013).

6.4 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is a slow growing, aerobic, facultative intracellular bacterium with a 4.4 Mb G+C-rich genome (Cole et al. 1998). *M. tuberculosis* is carried by approximately one third of the human population. It is the causative agent of tuberculosis, a disease developing in 5–10 % of the infected individuals. Tuberculosis typically damages the lungs but all other organs may be affected. Immune control of *M. tuberculosis* is mostly cell-mediated with a requirement for IFNγ secretion to activate macrophages, because only activated macrophages are able to kill off intracellular mycobacteria (Prezzemolo et al. 2014; Xu et al. 2014). In a co-culture experiment with THP-1 cells differentiated to achieve a macrophage-like phenotype, *M. tuberculosis* blocked IFNγ-mediated induction of MHC class II genes HLA-DRα and HLA-DRβ *via* TLR2 signaling that resulted in impaired histone acetylation (Wang et al. 2005). HDAC inhibitors could rescue HLA-DR expression. It was observed that mycobacterial infection upregulated mSin3A, a corepressor that associated with the HLA-DRα promoter (Wang et al. 2005). The expression of CIITA, a regulator of class II major histocompatibility complex gene transcription was also downregulated by *M. tuberculosis* (Wang et al.

2005; Pennini et al. 2006). Pennini et al. demonstrated that this effect was due to the hypoacetylation of the IFNγ-regulated pIV promoter of *CIITA*, the gene coding for CIITA, and identified the *M. tuberculosis* 19K lipoprotein (LpqH) as the mycobacterial component that elicited the repressive chromatin alteration *via* TLR2 and MAPK signaling (Pennini et al. 2006, 2007).

Comparing the methylation statuses of CpG islands in the vitamin D receptor gene and a CpG dinucleotide located at the *IL18R1* gene has uncovered variations at several CpG-dinucleotides between different ethnic groups and between *M. tuberculosis*-infected and -uninfected persons (Andraos et al. 2011; Zhang et al. 2014). These data suggested that the CpG-methylation profile of an individual may be predictive for *M. tuberculosis*-susceptibility. Alternatively, *M. tuberculosis* infection could play a role in the induction of different CpG-methylation profiles (reviewed by Esterhuyse et al. 2012). However, as a note of caution, one of the studies did methylation analyses using EBV-transformed lymphoblastoid cell lines (LCLs) obtained from the respective donors (Andraos et al. 2011). EBV-infection by itself contributes to an altered methylation profile in the host cell (Niller et al. 2009, see Chap. 3).

6.5 *Mycobacterium leprae*

Leprosy or Hansen's Disease which was existent already in biblical times is caused by the obligate intracellular pathogen *Mycobacterium leprae*, another family member of the mycobacteria. Fortunately, the world-wide incidence rates are decreasing. Intriguingly, *M. leprae*, upon infection of Schwann cells which were derived from adult mouse peripheral nerves led to a fundamental reprogramming of those cells. Reprogramming resembled epithelial-mesenchymal transition (EMT) and involved the epigenetic downregulation of differentiation-associated genes, upregulation of mesoderm-developmental genes and imparted plasticity, migratory and immune modulatory behaviour to the cells. The new progenitor/stem-like cell (pSLC) phenotype promoted the dissemination of the infection. A comprehensive set of mesodermal and differentiation genes was compared between Schwann cells and pSLC for their methylation status and showed significant differences according to the cellular phenotype (Masaki et al. 2013).

6.6 *Porphyromonas gingivalis*

Porphyromonas gingivalis is a Gram-negative anaerobic bacterium residing in the oral cavity. Although typically a component of subgingival biofilms, *Porphyromonas gingivalis* can invade human gingival epithelial cells, i.e. it acts like a facultative intracellular bacterium, and plays an important role in the pathogenesis of periodontal disease (Lamont et al. 1995; Yilmaz et al. 2003; Bao et al. 2014). *Porphyromonas gingivalis* owns a series of virulence factors that have pleiotropic functions. Gingipains are secreted arginine-specific or lysine-specific cysteine proteinases that provide nutrients for the bacterium, mediate adhesion to oral surfaces and to other bacteria, facilitate invasion and impair host immune responses (Bostanci and Belibasakis 2012). Another virulence factor, the capsular polysaccharide, also plays a role in the evasion of host defence, whereas fimbriae are involved in adhesion, invasion, and biofilm generation (Brunner et al. 2010; Enersen et al. 2013).

Porphyromonas gingivalis interferes with the human epigenetic machinery through the secretion of a short-chain fatty acid, butyrate, an HDAC inhibitor that may affect the expression of 2 % of genes in mammalian cells (Davie 2003). Inhibition of HDAC activity causes histone hyperacetylation and transcriptional activation of genes associated with butyrate response elements. Such butyrate-induced gene expression alterations in various host cells may contribute to the pathogenesis of periodontal disease and they were implicated in facilitating adverse pregnancy outcome, too (Darveau 2009).

The epigenetic effects of lipopolysaccharide (LPS) are discussed in Chap. 5. Here we notice only that in human periodontal ligament fibroblast cell culture, *P. gingivalis* lipopolysaccha-

ride up-regulated DNMT1, resulting in hypermethylation of RUNX2, a gene encoding runt-related transcription factor 2 (Uehara et al. 2012). Because RUNX2 is a master regulator of bone formation (Camilleri and McDonald 2006), these data suggest that interference of *P. gingivalis* LPS with the cellular epigenetic machinery may block osteoblastic differentiation in the periodontium. The effect of *P. gingivalis* LPS, however, appears to be cell type-specific, because in cultured keratinocytes exposed to LPS a down-regulation of DNMT1, DNMT3A and the histone demethylase JMJD3 (Jumonji domain containing 3) mRNAS was observed whereas LPS did not affect the level of these transcripts in gingival fibroblasts (de Camargo Pereira et al. 2013). In HIV-infected individuals with periodontal disease, *P. gingivalis* infection may contribute to the progression of AIDS (acquired immunodeficiency syndrome) because under anaerobic condition the bacterial metabolite butyric acid is produced in significant amounts in the periodontal pockets. Butyrate may reactivate silent HIV proviruses by derepressing inactive HIV promoters (Imai et al. 2009; Imai and Ochiai 2011). Thus, prevention and early treatment of *Porphyromonas gingivalis*-associated periodontal disease may slow the dissemination of HIV and curb the clinical progression of AIDS (Imai et al. 2012c).

Short-chain fatty acids produced as metabolic by-products by the periodontal pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* activate the lytic (productive) replication of Kaposi's sarcoma-associated herpesvirus (KSHV) (Yu et al. 2014). Increased KSHV replication induced by the products of oral bacteria may explain the fact that in 20 % of Kaposi sarcoma patients the first lesions develop in the oral cavity. Short-chain fatty acids not only inhibit the activity of HDACs but also decrease the level of the NAD-dependent protein deacetylase SIRT1 (sirtuin-1, silent information regulator-1) as well as the histone N-lysine methyltransferases EZH2 (enhancer of zeste homolog 2) and SUV39H1 (suppressor of variegation 3–9 homolog 1) (Yu et al. 2014). EZH2 and SUV39H1 deposit the heterochromatin histone marks H3K27me3 and H3K9me3 to the chromatin, respectively. It is worthy to note that the level of butyric acid, isobutyric acid and propionic acid was significantly elevated in the saliva of patients with severe periodontal disease (PD), compared to healthy controls and the relative level of *F. nucleatum* and *P. gingivalis* was also higher in saliva samples of PD patients. Among the short-chain fatty acids studied, butyric acid was the most potent inducer of the KSHV lytic cycle. It increased the level of the activating epigenetic mark H4K12Ac, but decreased the level of the repressive marks H3K27me3 and H3K9me3 (Yu et al. 2014). Butyric acid produced by *P. gingivalis* induced lytic (productive) replication of Epstein-Barr virus (EBV) as well by activating the promoter of the EBV immediate-early (IE) gene *BZLF1* (Imai et al. 2012a, b).

6.7 *Chlamydia trachomatis*

Chlamydia trachomatis is an obligate intracellular Gram-negative bacterium that can cause a wide variety of diseases including trachoma (eye infection), inflammation of the urethra and pelvis, ectopic pregnancy, neonatal infections and lymphogranuloma venereum, a sexually transmitted disease. NUE (nuclear effector), a protein secreted by *Chlamydia trachomatis* is translocated to the host cell nuclei (Pennini et al. 2010). NUE has a histone methyltransferase activity targeting histone H2B, H3 and H4. Its role in *Chlamydia trachomatis*-induced pathological alterations remains to be established.

6.8 *Chlamydophila pneumoniae*

Chlamydophila pneumoniae, an obligate intracellular Gram-negative bacterium, causes pharyngitis, bronchitis and atypical pneumonia in humans. Similarly to the *B. anthracis*-encoded SET-domain protein *Ba*SET, and the *C. trachomatis*-encoded NUE, the genome of *Chlamydophila pneumoniae* also encodes a SET-domain protein that functions as a protein methyltransferase: cpnSET methylated murine histone

H3 and Hc1 (Murata et al. 2007). It remains to be established, however, whether cpnSET targets histones in infected human cells or modifies chlamydial histone H1-like proteins.

6.9 *Chlamydophila psittaci*

Chlamydophila psittaci infects birds, mammals and humans and it is the causative agent of psittacosis (also called ornithosis, parrot fever), a fulminant, life-threatening disease of both birds and humans. *Chlamydophila psittaci* can also establish latent and persistent infections (Knittler et al. 2014). In distinct geographical areas it is associated with ocular adnexal marginal zone B-cell lymphoma (OAMZL) in humans (Dolcetti et al. 2010). OAMZL may regress in response to the eradication of bacteria through antibiotic therapy.

Choung et al. observed aberrant CpG island methylation in OAMZL. Hypermethylation of the E-cadherin (CDH1) gene was a characteristic of *C. psittaci* infection-associated tumors (Choung et al. 2012). A genome-wide DNA methylation study identified a CpG site within the *DUSP22* gene encoding dual specificity protein phosphatase 22 as an epigenetic signature of *C. psittaci* infection (Lee et al. 2014). The mechanism of *C. psittaci*-induced methylation changes remains to be elucidated. In addition to *C. psittaci*, another bacterium, *Helicobacter pylori*, is also associated with lymphomagenesis (see Sect. 6.11).

6.10 *Fusobacterium nucleatum*

Fusobacterium nucleatum, a Gram-negative anaerobic oral bacterium involved in the development of periodontal diseases can invade various cell types present in the oral cavity, including gingival epithelial cells, gingival fibroblasts and periodontal ligament fibroblasts (Gursoy et al. 2008; Dabija-Wolter et al. 2009). *Fusobacterium nucleatum* not only invaded HaCaT keratinocyte cultures *in vitro*, but also multiplied intracellularly (Gursoy et al. 2008). This suggests that they are facultative intracellular bacteria capable to survive within the host cells under aerobic conditions. *Fusobacterium nucleatum* is capable to attach to the cells of other bacterial genera and the co-adhering cells form biofilms consisting of multiple bacterial species (Zilm and Rogers 2007). By producing short-chain fatty acids, *Fusobacterium nucleatum* may contribute – similarly to *P. gingivalis* – to the activation of latent herpesvirus (KSHV, EBV) and HIV genomes in the oral cavity (see Sect. 6.6.) (reviewed by Doolittle and Webster-Cyriaque 2014). In addition, gingival epithelial cells treated *in vitro* with *Fusobacterium nucleatum* responded with a decrease of DNMT1, HDAC1 and HDAC2 mRNA expression and an increase of human β-defensin 2 and CC chemokine ligand 20 (CCL20) levels, suggesting that epigenetic mechanisms may control short-term innate immune responses to *F. nucleatum* (Yin and Chung 2011).

6.11 *Helicobacter pylori*

Helicobacter pylori (*H. pylori*) is a spiral-shaped, flagellated, Gram-negative, microaerophilic bacterium. Although *H. pylori* is traditionally regarded as an extracellular bacterium, its capacity to invade gastric epithelial cells, preneoplastic lesions and gastric carcinoma cells is well documented (Tsuji et al. 2003; Necchi et al. 2007; Sepulveda 2013). In addition, internalized bacteria can also survive in mononuclear phagocytes and neutrophils by disrupting phagosome maturation (Allen 1999). Thus, there are intracellular niches occupied by *H. pylori*, suggesting a role for intracellular virulence factors in *H. pylori*-associated carcinogenesis and lymphomagenesis (Terebiznik et al. 2006; Dubois 2007; Necchi et al. 2007; Kuo and Cheng 2013).

6.11.1 Secreted *H. pylori* Virulence Factors CagA and VacA

Helicobacter pylori infection is regarded as one of the causative agents of gastric carcinoma and the *H. pylori*-elicited pathological changes

including chronic inflammation, gastric atrophy, metaplasia and dysplasia may contribute to neoplastic development. The *H. pylori* virulence factors cytotoxicity-associated antigen (CagA) and vacuolating cytotoxin (VacA) appear to be important determinants of pathogenesis: CagA expression is capable to induce gastrointestinal and hematopoietic neoplasms in transgenic mice and VacA is a strong marker of gastric cancer associated *H. pylori* strains (Ohnishi et al. 2008; Polk and Peek 2010; Memon et al. 2014). Although CagA, encoded on the *cag* pathogenicity island (*cag*PAI; Alm and Trust 1999), is regarded as a bacterial oncoprotein that has pleiotropic effects, its direct role in reprogramming the host cell epigenome remains to be established (reviewed by Stein et al. 2013). VacA also has multiple effects: in addition to the induction of vacuole formation and apoptosis, VacA secretion is also involved in the blockade of T cell activation and proliferation (reviewed by Polk and Peek 2010). A direct effect of VacA on the host cell epigenome also needs further studies, although infection with a cagA positive, vacA s1m1 *H. pylori* strain was associated with hypermethylation of distinct cellular genes in the gastric mucosa and the presence of the *H. pylori* vacA s1 genotype correlated with hypermethylation of the *COX-2* and *HMLH1* genes in gastric cancer (Alves et al. 2011; Schneider et al. 2013).

6.11.2 Secreted *H. pylori* Peptidyl Prolyl Cis-, Trans-Isomerase (PPIase), HP0175

In addition to CagA and VacA, other proteins secreted by *H. pylori* also play an important role in the pathological alterations caused by the bacterium. HP0175, a peptidyl prolyl cis-, trans-isomerase (PPIase) targets macrophages by interacting with TLR4 (Toll-like receptor 4) and may contribute to the increased local chemokine and cytokine production observed at the margin of gastric ulcer as well as in early gastric carcinomas associated with *H. pylori* infection (Pathak et al. 2006). It was observed that cocultivation of *H. pylori* with human THP-1 cells that were differentiated to macrophage-like cells *in vitro* led HP0175-TLR4 interaction and induction of IL-6 expression *via* NF-κB and MAPK signaling (Pathak et al. 2006). It turned out that HP0175 upregulated the activity of the *IL-6* promoter by eliciting phosphorylation of MSK1, by MAP kinases, in the nucleus. MSK1 activation resulted in an activating chromatin modification, i.e. phosphorylation of histone H3 serine 10. In addition, activated MSK1 also phosphorylated the p65 subunit of NF-κB, recruiting it to the IL-6 promoter. Thus, the *H. pylori*-secreted PPIase HP0175 that may reach mucosal macrophages *in vivo* after the junctions between epithelial cells are disrupted, may induce an epigenetic alteration favouring *IL-6* transcription. We notice that *H. pylori* may elicit not only increased histone H3 phosphorylation, which is an activating chromatin mark, but repressive histone modification as well (Ding et al. 2010). In gastric epithelial cells *H. pylori* induced cagA-, vacA-independent dephosphorylation of H3S10 and decreased acetylation of H3K23 (Ding et al. 2010).

In addition to the activation of the *IL-6* promoter in macrophages, the interaction of HP0175 with gastric epithelial cells may also have potential pathogenic consequences. It was observed that HP0175 induced production of VEGF (vascular endothelial growth factor, a tumor angiogenesis factor) in the gastric epithelial cell line AGS expressing TLR4 (Basu et al. 2008). HP0175 binding resulted in TLR4 translocation into lipid rafts followed by TLR4 phosphorylation and activation of EGFR (epithelial growth factor receptor), a tyrosin kinase controlling VEGF production (Basu et al. 2008). It is worthy to note that EGF stimulation could increase histone H3 phosphorylation in another human epithelial cell line (Choi et al. 2005).

6.11.3 *H. pylori* Protein JHP0290

H. pylori encoded effector proteins may induce epigenetic alterations not only directly, but also indirectly, by inducing the production or release of inflammatory cytokines that elicit epigenetic changes in their target cells. JHP0290, a homolog

of the secreted *H. pylori* protein HP0305 could bind to a series of human cell types including macrophages, gastric epithelial cell lines, monocyte-derived dendritic cells and neutrophils. It was observed that JHP0290 induced macrophage apoptosis and, in parallel, TNF release from macrophages *via* a signaling pathway involving the Src family of tyrosine kinases as well as ERK and MAPK (Pathak et al. 2013). Expression of TNFα and other inflammatory proteins correlated with increased DNA methylation in an animal model of *H. pylori*-induced gastric cancer (Niwa et al. 2010). The effect of TNFα on the host cell methylome may be more complex, however, because TNFα could also induce site specific demethylation in a human keratinocyte cell line (Ling et al. 2013).

6.11.4 *H. pylori* Protein HP986

HP986 is an approximately 29 kDa protein that induced TNFα and IL-8 in human macrophage cultures (Alvi et al. 2011). Enhanced cytokine production was associated with NF-κB signaling. Similarly to JHP0290, HP986 also induced apoptosis of macrophages *in vitro*, through a Fas mediated pathway (Alvi et al. 2011). It is worthy to note that TNFα, similarly to IL-1β, another inflammatory cytokine upregulated in *H. pylori*-associated gastritis, is a potent inhibitor of acid secretion in the stomach (reviewed by Wroblewski et al. 2010).

6.11.5 *H. pylori* Co-chaperonin GroES

GroES was identified as a gastric cancer-associated virulence factor in an immunoproteomics study (Lin et al. 2006). In PBMCs, GroES stimulated the production of a series of inflammatory mediators including IL-8, IL-6, GMCSF, IL-1β, TNF, COX2, and PGE2 (prostaglandin E2). It also affected the gene expression pattern of the KATO-III human gastric carcinoma cell line *in vitro*; it upregulated IL-8 production and the transcription of cell cycle regulatory genes encoding *c-jun* and *c-fos*. In addition, it increased the protein level of cyclin D1, but downregulated that of $p27^{Kip1}$ (CDKN2B, cyclin-dependent kinase inhibitor 2B, a tumor suppressor protein) (Lin et al. 2006). These data suggested that – unexpectedly – the *H. pylori*-encoded GroES co-chaperone may play a role in the initiation and promotion of tumorigenesis in the stomach due to its pro-inflammatory and cell proliferation-stimulating effects. Indirectly, GroES may elicit patho-epigenetic changes as well, because the increased local production of PGE2 may alter the methylome of neighbouring cells carrying PGE2 receptors. In human colorectal carcinoma cell lines PGE2 upregulated the expression of DNMT1 and DNMT3B resulting in increased promoter methylation of a set of selected genes including *CDKN2B*, *MLH1*, *CNR1* (cannabinoid receptor 1), *MGMT* (O-6-methylguanine-DNA methyltransferase), *BAX*, *CHEK2*, *NOTCH1*, *CAV1*, *NHS*, *MYOD1*, and *TMEFF2* (Xia et al. 2012). PGE2 may also facilitate tumorigenesis by the recruitment of tumor-promoting macrophages and by the upregulation of VEGF expression (Oshima et al. 2011; Liu et al. 2014a).

6.11.6 Epigenetic Alterations in *H. pylori*-Associated Gastric Carcinoma

Direct interaction of *H. pylori* with various host cells and the chronic inflammation process elicited by *H. pylori* infection is accompanied by alterations of the histone code and by extensive alterations of DNA methylation (Ding et al. 2010; Liu et al. 2014b; Matsusaka et al. 2014). Global hypomethylation and local hypermethylation at CpG islands of tumor suppressor gene loci is strongly associated with the presence of *H. pylori* in chronic gastritis and gastric adenocarcinoma (reviewed by Niller et al. 2012a, b; Chiariotti et al. 2013). *H. pylori*–associated gastric carcinoma is developing through multiple steps and epigenetic alterations may occur already at the initial stages (Ushijima 2007; Correa and Piazuelo 2012). Cheng et al. observed that in *H. pylori* infected mice there were 317 hypermeth-

ylated promoters in the gastritis-affected tissues compared to the stomach tissues of control mice (Cheng et al. 2013). They also found a difference between gastric cancer samples from short term and long term survivors of the disease: there were 24 hypermethylated genes associated with shorter survival. Comparison of the hypermethylated genes identified in the mouse model and in short term survivors resulted in a single common gene, *FOXD3*, a forkhead box (Fox) gene involved in transcriptional regulation. *FOXD3* proved to be a tumor suppressor gene in gastric cancer, a function mediated by *CYFIP2* (encoding cytoplasmic FMR1-interacting protein 2) and *RARB* (coding for retinoic acid receptor beta), the direct transcriptional target genes of the FOXD3 protein. The *FOXD3* promoter was increasingly methylated and thereby silenced during the progression of gastric carcinoma in humans, and the expression of FOXD3 protein was downregulated in 83 % of stomach cancers (Cheng et al. 2013). In addition, in tumor samples with *FOXD3* promoter methylation the transcription of FOXD3-dependent genes coding for the proapoptotic proteins CYFIP2 and RARB was decreased (Cheng et al. 2013).

In an animal model, treatment of *H. pylori*-infected Mongolian gerbils with 5-aza-dC, a DNA-demethylating agent could prevent the development of gastric carcinoma (Niwa et al. 2013). Thus, epigenetic dysregulation induced by *H. pylori* plays an important role in the initiation and progression of gastric carcinoma (Niwa et al. 2013). However, the contribution of chronic inflammation to *H. pylori*-induced carcinogenesis remains to be elucidated. In Mongolian gerbils gastric inflammation resulted in increasing DNA methylation at ten selected CpG islands, whereas. *H. pylori*-eradication or immune suppression with cyclosporin A led to a recovery of the normal methylation status, suggesting that epigenetic disruption is mostly due to the chronic inflammation, but not to infection itself (Niwa et al. 2010). Other data, however, support a direct role for *H. pylori* in the epigenetic reprogramming of gene expression (Chiariotti et al. 2013).

6.11.7 Recruitment of Bone Marrow-Derived Stem Cells and Induction of Mesenchymal Stem Cell Differentiation in Experimental Mouse Models of *Helicobacter pylori*-Induced Gastric Carcinoma: Additional Sources of Epigenetic Diversity

In mouse models of gastric carcinogenesis, *H. pylori* infection leads to the development of chronic inflammation in the stomach, followed by the pathologic changes also observed in *H. pylori*-infected humans, including hyperplasia, metaplasia, and dysplasia. It was observed, however, that in mice bone marrow-derived stem cells (BMDCs) repopulate the *H. pylori*-infected stomach. Thus, *H. pylori* may recruit BMDC to the gastric epithelial mucosa where BMDCs may fuse with local gastric epithelial cells or undergo phenotypic changes and contribute to the development of dysplastic lesions (Houghton et al. 2004; Varon et al. 2012; Donnelly et al. 2013; reviewed by Bessede et al. 2014). The *H. pylori*-induced BMDC recruitment may alter, indirectly, the epigenotype of gastric epithelial cells, because it is well documented that cell fusion and phenotypic alterations including epithelial-mesenchymal transition are associated with profound epigenetic changes. In addition, *H.pylori* infection may also induce the differentiation of mesenchymal stem cells into cytokine producing cancer-associated fibroblast (CAF)-like cells, generating thereby an additional source of epigenetic diversity in the affected region of the stomach (Zhang et al. 2013).

6.12 *Campylobacter rectus*

Campylobacter rectus was characterized as an exclusively oral Gram-negative anaerobic bacterium involved in the pathogenesis of adult periodontitis and rapidly progressive periodontitis (Wang et al. 2000). However, *Campylobacter rectus* was also implicated in intrauterine growth

restriction and adverse pregnancy outcomes, because during pregnancy it may disseminate systemically, and it is capable to invade human trophoblast cells *in vitro* (Arce et al. 2010). Thus, similarly to the related *Campylobacter jejuni*, *Campylobacter rectus* can be regarded as a facultative intracellular bacterium, too.

Invasion of human trophoblasts and upregulation of cytokine expression in the infected cells, possibly mediated *via* TLR4 (Toll-like receptor 4), may play a role in *C. rectus* associated intrauterine growth restriction (Arce et al. 2010, 2012). Furthermore, a pioneering study of Bobetsis et al. revealed that in a murine model of intrauterine growth restriction *C. rectus* induced epigenetic alteration in the placenta (Bobetsis et al. 2007). They found that the P0 promoter of the *Igf2* gene encoding insulin-like growth factor 2 was silenced by DNA hypermethylation in the placenta of *C. rectus*-infected mice. Because Igf2 promotes growth during gestation, downregulation of its expression may contribute to intrauterine growth restriction. During *C. rectus* infection-mediated growth restriction 9 genes were upregulated and 65 genes were downregulated in murine placentas (Bobetsis et al. 2010). The downregulated genes included 26 imprinted genes associated with placental development and fetal growth, like *Igf2*. The role of *C. rectus*-induced epigenetic alterations in human diseases remains to be elucidated.

6.13 *Anaplasma phagocytophilum*

Infection with *Anaplasma phagocytophilum*, a tick-transmitted, Gram negative, LPS-negative, obligate intracellular rickettsial pathogen results in human granulocytic anaplasmosis, a flu-like disease associated with multiple peripheral blood cytopenias (Johns et al. 2009). The tick vectors are *Ixodes scapularis*, *Ixodes pacificus*, and *Dermacentor variabilis* whereas in the USA the major mammalian reservoir of *Anaplasma phagocytophilum* is the white-footed mouse, *Peromyscus leucopus*. *Anaplasma phagocytophilum* is capable to survive in hostile intracellular environments, i.e. inside granulocytes and monocytes. This is partly accomplished through the inhibition of key cellular defence genes through the bacterial effector AnkA. AnkA was characterized as a "nucleomodulin" protein that alters the gene expression pattern of host cells. AnkA is translocated to the nucleus where it increases the level of HDAC1 (histone deacetylase 1) which is targeted to several defence gene promoters repressed in *Anaplasma phagocytophilum* infected cells (Garcia-Garcia et al. 2009a, b; reviewed by Rennoll-Bankert and Dumler 2012; Sinclair et al. 2014). The gene expression alterations and their phenotypic consequences are quite complex, however, following *A. phagocytophilum* infection: although the respiratory burst and phagocytic activity is decreased and apoptosis is delayed, chemokine production is increased (reviewed by Sinclair et al. 2014). Whereas the downregulation of 19 defense genes including *CYBB*, *RAC2*, *MPO* (*myeloid peroxidase*), *BPI*, *EPX* (*eosinophil peroxidase*), *AZU1*, *ELA2*, *PRTN* and most of the defensin genes could possibly be connected to HDAC1 mediated deacetylation at their promoters, most of the differentially expressed genes were upregulated after *A. phagocytophilum* infection (Borjesson et al. 2005; Garcia-Garcia et al. 2009a, b; Sinclair et al. 2014). This complex pattern affecting genes and gene clusters on all chromosomes could be a result of reprogramming elicited by bacterial effectors including AnkA, or could be a manifestation of cellular responses to bacterial infection, or both (Sinclair et al. 2014).

6.14 *Ehrlichia chaffeensis*

Ehrlichia chaffeensis is an obligate intracellular rickettsial pathogen infecting mononuclear phagocytes. *E. chaffeensis* is transmitted by ticks and it is the causative agent of human monocytotropic ehrlichiosis, a zoonotic disease. The primary reservoir of *E. chaffeensis* is the white tailed deer (*Odocoileus virginianus*) (reviewed by Dunphy et al. 2013). TRP47 and TRP120, the tandem repeat containing effector proteins of *E. chaffeensis* interact with a wide variety of host

proteins, i.e. they are "moonlighting proteins" (pleiotropic regulators) (Wakeel et al. 2009; Luo et al. 2011; Dunphy et al. 2013). TRP120 is SUMOylated in human cells (Dunphy et al. 2014) and interacts, *via* its tandem repeat, with PCGF5 (polycomb group ring finger protein 5), an epigenetic regulator (see Chap. 1). This interaction results in the redistribution of PCGF5, a member of the PRC1 complex, from the nucleus to the cytoplasm, i.e. to the ehrlichial inclusion bodies (reviewed by Dunphy et al. 2013). The effect of PCGF5 redistribution to the gene expression pattern of the host cell remains to be determined. SUMOylation of TRP120 is critical for PCGF5 recruitment to the inclusion bodies and to replication and intracellular survival of *E. chaffeensis* (Dunphy et al. 2014). The effector protein TRP47 also interacts with PCGF5 (Wakeel et al. 2009).

Although devoid of a typical nuclear localization signal, the secreted TRP120 protein is translocated from the cytoplasm to the nucleus of host cells and binds, *via* its tandem repeat region, to a GC-rich motif (Zhu et al. 2011). In THP-1 cells, high affinity TRP120 binding was detected within or around approximately 2,000 cellular genes and resulted in increased expression of a set of inflammatory chemokine genes (*CCL2*, *CCL20*, and *CXCL11*) (Zhu et al. 2011). Another effector of *E. chaffeensis*, the ankyrin repeat protein Ank200 is also translocated to the host cell nucleus. Ank200 contributes to the reprogramming of host gene expression by binding to an adenine-rich motif (5′-AAAATACAAAA-3′) located at a series of promoters and intronic Alu sequences (Zhu et al. 2009). Among the 456 host genes associated with Ank200 binding sites are the genes coding for TNFα, STAT1 and CD48 that are known to be upregulated in *E. chaffeensis* infected cells.

6.15 *Bordetella bronchiseptica*

Bordetella bronchiseptica is a Gram-negative bacterium that can cause infectious bronchitis in humans, but more more frequently infects animals and birds (Forde et al. 1998). *B. bronchiseptica* can infect murine macrophages and resist intracellular killing in macrophages and persist up to 4 days, without affecting the viability of the host cell (Forde et al. 1998). Thus, *Bordetella bronchiseptica* is a facultative intracellular bacterium. The *B. bronchiseptica* genome encodes a putative histone methyltransferase, BbSET (Li et al. 2013). Ectopic expression of BbSET in HeLa cells *in vitro* revealed that it entered the nucleolus (Li et al. 2013). Thus, similarly to the SET-domain proteins of *Burkholderia thailandensis* and *Legionella pneumophila* (see below, Sects. 6.16 and 6.17), BbSET may play a role in the epigenetic dysregulation of ribosomal RNA transcription in *B. bronchiseptica-infected* cells.

6.16 *Burkholderia thailandensis*

The *Burkholderia thailandensis* genome encodes a histone methyltransferase, BtSET, that targets histone H3K4 (Li et al. 2013). Ectopic expression of BtSET in HeLa cells *in vitro* revealed that it entered the nucleolus and associated with the rDNA promoter (Li et al. 2013). Thus, BtSET may potentially introduce an euchromatin histone mark upregulating the transcription of ribosomal RNA.

6.17 *Legionella pneumophila*

Legionella pneumophila is the causative agent of Legionnnaire's disease, a life-threatening pneumonia of humans with a mortality rate of 8–10 %. Smoking, alcohol consumption and higher age increase the risk to acquire the disease when exposed to bacteria-containing aerosols, typically generated by using a shower whose water pipes are bacterially contaminated. The sources can also be air conditioning systems and cooling towers where the bacteria thrive. *Legionella pneumophila* an aerobic, Gram-negative flagellated bacterium, infects and replicates within free-living amoebae and human alveolar macrophages. Although the numerous free living amoeba species are considered to be the major reservoir of *L. pneumophila*, the bacterium also

infects ciliated protozoa, and in addition to lung alveolar macrophages it may target mammalian epithelial cells, too (Escoll et al. 2013). It was suggested that the interaction of *L. pneumophila* with amoeba species living in aquatic environments and soil led to the acquisition of virulence factors that permit its replication in mammalian macrophages encountered accidentally by the bacteria (Al-Quadan et al. 2012). In contrast to other respiratory pathogens, transmission of *L. pneumophila* between humans has not been reported.

After phagocytosis of *L. pneumophila* either by amoeba or by macrophages a special vacuole (LCV, *Legionella* containing vacuole) is formed within the cytoplasm of the host cell. LCV is not fused with lysosomes and provides a suitable environment for intracellular replication of the bacterium (Escoll et al. 2013). *L. pneumophila* acquired, by horizontal gene transfer from one of its eukaryotic (protozoan) hosts, approximately 300 genes coding for effector proteins containing eukaryotic domains. These effectors allow the exploitation of multiple protist and mammalian hosts (Franco et al. 2009).

6.17.1 Effector Protein LegAS4: A Histone Lysine Methyltransferase

L. pneumophila contains a type IV secretion system called Dot/Icm (defect in organelle trafficking/intracellular multiplication) that allows rapid translocation of the bacterial effectors through the LCV membrane. A series of effectors are anchored to the cytosolic face of the LCV membrane and remodel the phagosome. A unique effector protein, called LegAS4, however, enters the host cell nucleus where it affects the function of the nucleolus (Li et al. 2013). LegAS4 isa SET-domain protein named after drosophila **S**u(var)3-9, **e**nhancer of zeste [E(z)], and **t**rithorax [trx], (see Chap. 1). LegAS4 has histone lysine methyltransferase (HKMTase) activity, up-regulating the transcription of the ribosomal DNA (rDNA) (Li et al. 2013). LegAS4 forms a direct contact with heterochromatin proteins (HP) 1α and -γ and methylates histone 3 at lysine 4, generating H3K4me marks. It was observed that the genomes of other pathogenic bacteria, *Bordetella bronchiseptica* and *Burkholderia thailandensis*, encode similar SET-domain proteins; like LegAS4, these effectors are also targeted to the host nucleolus and may activate rDNA transcription (see above, Sects. 6.15 and 6.16) (Li et al. 2013). Li et al. suggested that an increased rDNA transcription may be a general bacterial virulence strategy which helps to survive in the harsh intracellular environment (Li et al. 2013).

6.17.2 Effector Protein RomA: A Histone Methyltransferase with a Novel Target

Another type IV secreted SET-domain protein of *L. pneumophila*, RomA, encoded by the gene *lpp1683* of the Paris strain, was also characterized. Unlike LegAS4, RomA had a different lysine target: it generates H3K14me3 marks and decreases, in parallel, H3K14 acetylation. These alterations result in the repression of 4.870 H3K14 methylated promoter regions, including innate immune genes (Rolando et al. 2013). RomA was required for efficient intracellular replication of the bacterium in *Acanthamoeba castellani* cells, i.e. in a natural protozoan host of *L. pneumophila*, as well as in human lung epithelial cells and human macrophages (Rolando et al.2013). H3K14 is usually not methylated in mammalian cells. It is interesting to note that the *L. pneumophila* effectors LegAS4 and RomA were identified in different *L. pneumophila* strains using different methods. A comparative study of their functions and activities could be informative.

6.17.3 Flagellin: A Virulence Factor and Epigenetic Regulator of *Legionella pneumophila*

In *L. pneumophila*-infected cultured lung epithelial cells, flagellin, a major component of the bacterial flagellum that is essential for the motility of

Legionella, activated the p38 mitogen-activated protein kinase- and nuclear factor-kappaB/RelA signal transduction pathway to induce a series of pro-inflammatory cytokines. It upregulated the expression of the chemoattractant IL8 as well, *via* H4 acetylation, H3 acetylation and H3-phosphorylation (Schmeck et al. 2008; reviewed by Bierne et al. 2012).

6.18 *Aeromonas hydrophila*

Aeromonas hydrophila is a Gram negative bacterium associated with gastroenteritis in humans and various diseases in freshwater fish, frogs and lizards. Under experimental conditions it can infect the protozoan *Tetrahymena thermophila* and express its virulence factor genes coding for aerolysin and serine protease (Li et al. 2011). Survival of the virulent bacteria within the protozoan may indicate that *A. hydrophila* interferes with phagocytosis. Aerolysin, a pore forming toxin secreted by *A. hydrophila* differs from the cholesterol-dependent cytolysins (CDCs) produced by Gram positive bacteria (see above), and its inactive precursor is activated by proteolytic cleavage (Hamon and Cossart 2011). However, similarly to CDCs, aerolysin also permits K^+ efflux into the cells through the pores formed by its heptamers and induces dephosphorylation of H3S10 in target cell nuclei (Hamon and Cossart 2011). The epigenetic alterations induced by bacterial toxins are summarized in Table 6.5.

6.19 *Shigella flexneri*

Shigella flexneri, a Gram-negative bacterium causing ulcerative dysentery produces an effector protein, OspF, that enters the target epithelial cells and translocates to the host cell nuclei (Arbibe et al. 2007). OspF blocks phosphorylation of H3S10 at the promoters of NF-κB regulated genes including *IL8*, interfering therebywith the recruitment of neutrophil granulocytes (Arbibe et al. 2007).

6.20 Uropathogenic *Escherichia coli* (UPEC)

Uropathogenic *Escherichia coli* (UPEC) strains are well adapted to the colonization of uroepithelia (Hunstad and Justice 2010). *E. coli*, a Gram-negative, facultatively anaerobic, rod-shaped bacterium is a member of the normal gut flora. Although *E. coli* is typically present as an extracellular bacterium in the gut, UPEC strains causing ascending urinary tract infections not only attach *via* their P fimbriae (pyelonephritis-associated pili) to the luminal surface of the bladder, but a minor population also invades the superficial umbrella cells. Thus, UPEC strains are facultative intracellular bacteria forming bacterial communities within the cytoplasm of their host cells (Hunstad and Justice 2010). Within the host cell they are protected from host defense and frequently establish persistent infection (recurrent cystitis).

Colonization of the uroepithelium is facilitated through the upregulation of siderophore expression. Siderophores scavenge iron to support bacterial survival and growth, and it was observed that in *E. coli* isolates from the urine of patients with recurrent urinary tract infection there was an increased production of two siderophores, yersiniabactin and salmochelin, as com-

Table 6.5 Epigenetic alterations induced by bacterial toxins

Toxin and toxin producing bacterium	Epigenetic change and consequence
Listeriolysin O (LLO),	Histone H3 dephosphorylation,
Listeria monocytogenes	gene silencing
	Histone H4 deacetylation,
	gene silencing
Pneumococcal pneumolysin (PLY),	Histone H3 dephosphorylation,
Streptococcus pneumoniae	?
Perfringolysin O (PFO),	Histone H3 dephosphorylation,
Clostridium perfringens	?
Aerolysin,	Histone H3 dephosphorylation
Aeromonas hydrophila	?

pared to rectal strains from the same patients (Henderson et al. 2009). Henderson et al. argued that because there were no detectable genetic differences between the isolates, qualitative and quantitative epigenetic optimization occurred in the *E. coli* secondary metabolome among human uropathogens. The epigenetic regulatory mechanism is unknown at present (Henderson et al. 2009). The phenomenon resembles, however, the quick epigenetic alteration observed in *M. avium* spp. *paratuberculosis* isolates upon their change of habitat which was possibly mediated by the action of a DNA methyltransferase (O'Shea et al. 2011).

Whether the putative epigenetic mechanism controlling siderophore expression in the urine contributes to the epigenetic changes elicited by UPEC in human target cells is unknown at present. It was demonstrated, however, that UPEC-infected uroepithelial cells internalized bacterial colonies *in vitro* and strongly upregulated the maintenance methyltransferase DNMT1 in their host cells (Tolg et al. 2011). In parallel, the expression of the tumor suppressor gene *CDKN2A* and the DNA repair gene *MGMT* was downregulated while that of four other cellular genes was unaffected. Methylation analysis of *CDKN2A* and *MGMT* CpG islands revealed increased methylation in *CDKN2A* exon 1 whereas the methylation pattern of *MGMT* was unaffected. Internalization of bacteria was dependent on the bacterial *FimH* gene encoding the fimbrial protein adhesin (Tolg et al. 2011; reviewed by Schulz 2011; Tolg and Bagli 2012). Although the exact mechanism of action remains to be established, this study indicated that internalization of UPEC by human uroepithelial cells may trigger changes in the expression of distinct host cell genes. An epigenetic change, i.e. increased methylation of *CDKN2A* exon 1, presumably mediated by the upregulation of DNMT1, possibly contributed to silencing of *CDKN2A* in UPEC-infected cells, because hypermethylation of exon 1 sequences may be tightly linked to transcriptional silencing (Brenet et al. 2011). In addition, the interplay of UPEC with other epigenetic mechanisms could play a role in the downregulation of *MGMT* expression.

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J. Minarovits, H.H. Niller (eds.), *Patho-Epigenetics of Infectious Disease*,
Advances in Experimental Medicine and Biology 879, DOI 10.1007/978-3-319-24738-0

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