THESIS / THÈSE

DOCTOR OF SCIENCES

Role of DNA methylation and CpG sites in the viral telomerase RNA promoter during Gallid herpesvirus type 2 induced lymphomagenesis

Pejaković, Srđan

Award date:
2020

Awarding institution:
University of Namur

Université de Namur
Faculté des Sciences
Département de Médecine Vétérinaire
Unité de Recherche Vétérinaire Intégrée

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Srđan Pejaković

Promoteur
Prof. Benoît Muylkens

Thèse présentée en vue de l'obtention du grade de
Docteur en Sciences Biologiques

Septembre 2020

Thesis examining board:

Prof. Thierry Arnould, Université de Namur

Dr. Luca D. Bertzbach, Freie Universität Berlin

Prof. Olivier De Backer, Université de Namur

Dr. Anabelle Decottignies, Université catholique de Louvain

Prof. Jean-Pierre Gillet, Université de Namur

Prof. Damien Hermand, Université de Namur

University of Namur
Faculty of Sciences
Department of Veterinary Medecine
Integrated Veterinary Research Unit

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Srđan Pejaković

Supervisor:
Prof. Benoît Muylkens

The thesis presented to fulfil the requirements to obtain the grade of
PhD in Biological Sciences.

September 2020

"Imagination will often carry us to worlds that never were. But without it we go nowhere."

Carl Sagan

Acknowledgements

First and foremost, I would like to thank my supervisor and mentor, Benoît Muylkens. Dear Benoît, I am so ever grateful to you. Since the first day we met, you welcomed me with arms wide open in URVI. Who knew that six years could bring so many memories? Through good times and hard ones, I could always count on your support. I thank you for all what you have thought me in research, but more importantly, outside of it. Thank you for believing in me, pushing me to give my best and never to give up, especially during the last year of the thesis when everything seemed to work against me. And most importantly, thank you for always being there when I needed someone to talk to, even when you were swamped with work, and for all of your understanding. I will never be able to thank you enough for the opportunity you gave me.

Also, I would like to express my gratitude to prof. Benedikt Kaufer and prof. Jean-Pierre Gillet, who supported me as members of my thesis committee through my PhD. All the ideas you brought over the years and your expertise allowed me to steer my research and experiments in the best direction.

I am thankful to all Members of the thesis jury, prof. Thierry Arnould, dr. Luca Bertzbach, prof. Olivier De Backer, dr. Anabelle Decottignies, prof. Jean-Pierre Gillet and prof. Damien Hermand, for a comprehensive discussion, comments and suggestions that improved this manuscript.

I am grateful to James Mason for his restless scientific spirit that gave me an opportunity to meet Benoit. What a story of pure chance our meeting was. Jim, thank you enormously for bothering me on my summer job with your data that needed to be analysed and plotted on the graphs. You curiosity open new doors for me to explore endless opportunities, and I am so grateful for all of your support throughout the years. I am so proud that our initial contact resulted in publication and friendship. You coming to Belgium with Gertie during my interviews meant so much to me. Thank you both for everything.

I would also like to thank Natalie Kirschvink. It was a pleasure working with you on the student courses that broadened my knowledge even more. Thank you for supporting me during these six years, and thank you for inviting me to each Christmas dinner with your lovely family when you knew I could not go back home.

I thank Nicolas Gillet for making the work environment so pleasant and bringing a new vision to practical courses. It was great working with you on developing a new approach for Microbiology practical courses, and the student's feedback we received after speaks to itself.

When talking about the practical courses, I need to say enormous thank you to Kévin Willemart and Wiebke Jansen. Kevin, thank you for all the help during the practicals and all the impeccable preparations you did for each course. It was a pleasure to work with you, we made a great team, and for sure had some good times with the students. Wiebke, what a fun time we had during our courses. I will never forget all the faintings or food preferences of each student. We did great, and moreover "saved" some lives. I will cherish that forever. Thank you both for being great colleagues and great friends.

I am so grateful to Damien Coupeau. Dam, thank you for all the help during my thesis. From small things, all the way to the times I was utterly lost in the beginning. Any lab would be lucky to have you as a post-doc and a scientist, always curious and ready to help. Thank you for making the lab such a nice place to work, and thank you for all the fun we had outside the lab.

I would like to express my gratitude to Laëtitia Wiggers. Thank you for sharing your tiny office with me and making it a fantastic place to work in. I will never forget all the times you helped me with endless experiments or always being there when I had questions or needed any help. The lab was always running smoothly, thanks to you. And most importantly, thank you for being such a sincere and kind friend during my stay in Namur.

I am thankful to all the URVI PhD students I shared time in the lab with, Nicolas Burton, Florian Poulain, Noémie Lejeune, Astrid Petit and Laura Bonil. Keep URVI a fun place to be and a great lab to work in. I wish you all successful finish of your theses and best of luck in your careers.

I am grateful to our amazing secretary Chantal Ippersiel. You made this little department so warm and welcoming. Thank you for your masterful organisation and preparation of all events, exams and documentation, making work so smooth and easy. Also, I would like to thank all the members of URVI, Claire Diederich, Jean-Michel Vandeweerd, Marianne Raes, Carole Cambier, Fanny Hontoir, Gaston Amzati, Yves De Raeve and Vincent Simon for their kindness, support and help during my thesis.

I especially thank my dear friends Vicky, Devdutt, Bush and Maxime. You made the years we shared together in Namur great fun. Thank you for your endless support, inexhaustible discussions, never-ending beer degustations, all the travels and parties. You made the life outside the lab full of joy and new experiences.

I am immensely grateful to Isabelle Gennart. Isa, my dear friend, you were the first to welcome me when I arrived in Belgium, and since that first day, you were there whenever I needed help in and outside the lab. The times we shared the office were unforgettable. Thank you for the endless laughter and fun, of course, when we were not seriously working on our research.

Endless thanks to Morgane. Thank you for your love, support and your faith in me. You raised me a million times when I was down. Your courage, strength and unbreakable spirit are my best inspirations. You are my best friend, my companion, and I cannot wait to experience all future adventures awaiting, with you by my side. I love you.

Finally, I am forever grateful to my family, my mom and dad, Branka and Ranko, and my sister Andrea. Neizrecivo vam hvala za sve ove godine, vašu bezgraničnu ljubav i podršku. Mama i tata, hvala vam za sva odricanja i što ste nas uvijek poticali da izaberemo svoj put. Seko, hvala ti što si uvijek bila na mojoj strani sve ove godine i što si uvijek kroz smijeh riješavala sve prepreke koje su nam bile na putu. Bez vas ne bih uspio i bio tu. Volim vas.

Summary

Gallid herpesvirus 2 (GaHV-2) is an avian alphaherpesvirus that causes highly malignant T-cell lymphoma considered to be the most prevalent cancer in the animal kingdom. In susceptible chickens, the ultimate consequence of the host-virus interactions is the transformation of the $CD4^+$ T-cells, which eventually proliferate to form visceral lymphomas, resulting in high mortality.

The GaHV-2 genome belongs to a class E genome with a size of 175 to 180 kilobase pairs. GaHV-2 genome consists of a unique long (U_L), and a unique short (U_S) segments bracketed by inverted repeats known as terminal and internal repeats long (TR_L and IR_L) and terminal and internal repeats short (TR_S and IR_S). GaHV-2 genes, similar to those of other herpesviruses, also belong to three kinetic classes of immediate-early, early and late genes based on the requirements for viral protein synthesis and DNA replication.

During GaHV-2 infection, several viral factors, proteins and diverse RNAs, including the major oncoprotein Meq, the viral interleukin-8 and GaHV-2-encoded miRNAs, contribute to lymphomagenesis. In addition, GaHV-2 encodes two copies of viral telomerase RNA subunit (vTR), which is expressed both during productive infection and in virus-transformed T-cell lines. vTR, a non-coding RNA, shares 88% sequence homology with chicken TR (chTR) and it was likely acquired from the chicken genome during virus-host co-evolution. vTR interacts with the chicken telomerase reverse transcriptase subunit (TERT) enhancing telomerase activity and contributing to the efficient and rapid onset of lymphoma. Furthermore, vTR re-localises ribosomal protein L22 that plays an essential role in T-cell development and transformation. Moreover, vTR functions independent of the telomerase complex are responsible for tumour progression and dissemination. It is the most abundant viral transcript detected in GaHV-2-induced tumour cells with higher expression than chTR in infected cells, consequence likely due to differences in their promoters. The vTR promoter has additional AP-1 sites, c-Myc transcription response elements (namely E-box 1, E-box 2 and E-box 3) and EBS transcription factor binding sites. However, it was demonstrated that E-box 1 was not functional. It was shown that the c-Myc oncoprotein is involved in the regulation of vTR during GaHV-2-induced lymphomagenesis and that increased expression of vTR is essential for the oncogenic function of the virus.

During the viral life cycle, transcriptional modifications and epigenetic changes, together with post-transcriptional and post-translational modifications, regulate expression of cellular and viral genes. Altogether, they allow GaHV-2 to switch between the productive and latent phases, and to induce infected cell transformation.

The focus of this study was on the epigenetic mechanisms involved in the switch between the productive and latent phase of GaHV-2 life cycle and on the importance of functional c-Myc response elements during virus-induced lymphomagenesis.

We established DNA methylation/hydroxymethylation patterns of vTR promoter *in vitro* and *in vivo* and measured the impact of methylation on the telomerase activity and c-Myc response elements (c-Myc REs) of the vTR promoter. Furthermore, to study the importance of the c-Myc binding sites in virus-induced tumorigenesis, a recombinant virus bearing mutations in functional c-Myc REs, as well as revertant, were produced using the bacterial artificial chromosome of a highly oncogenic strain (pRB-1BΔIRL) by two-step Red-mediated mutagenesis. Susceptible ($B^{13}B^{13}$) chickens were infected with the recombinant viruses to assess the impact of c-Myc REs mutations. To investigate GaHV-2 replication and telomerase activity during infection, blood and feather follicle epithelium were collected at specific time points from infected chicken. Animals were daily monitored for the clinical symptoms of the disease and euthanised 55 days-post infections to assess the number of tumours developed in visceral organs.

We demonstrated that telomerase activity was considerably increased following viral reactivation. Furthermore, CpG sites within functional c-Myc REs showed specific changes in methylation after *in vitro* reactivation and in infected animals over time. Promoter reporter assays indicated that c-Myc RE, located two nucleotides downstream of the transcription start site, is involved in regulating vTR transcription and that methylation strongly influenced vTR promoter activity.

To study the importance of the CpG sites found in c-Myc REs in virus-induced tumorigenesis, we generated a recombinant virus containing mutations in both CpG sites of c-Myc REs as well as revertant. Introduced mutation in vTR promoter did not affect the replication properties of the recombinant viruses *in vitro*. In contrast, replication of the mutant virus in infected animals was severely impaired and tumour formation completely abrogated. Our data provide a more in-depth characterisation of c-Myc oncoprotein REs and DNA methylation involvement in transcriptional regulation of vTR.

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List of abbreviations

μg -microgram

2-OG - 2-oxoglutarate

5caC - 5-carboxylcytosine

5fC - 5-formylcytosine

5hmC - 5-hydroxymethylcytosine

5hmU - 5-hydroxymethyluracil

5mC - 5-methylcytosine

Ac- acetylation

AID - activation-induced cytidine deaminase

AP-1 - activator protein 1

APOBEC - apolipoprotein B mRNA-editing enzyme complex

A^{vy} - yellow agouti mouse

Bcl-2 - B-cell lymphoma 2

BER - base excision repair

BGSA - bisulfite genomic sequencing assay

BPA - bisphenol A

BRLF - EBV immediate-early proteins

BZLF1 - EBV immediate-early proteins

cAMP - cyclic adenosine monophosphate

CEF - chicken embryo fibroblasts

CGI - CpG island

CH_3 - methyl

ChIP - chromatin immunoprecipitation

Chl - chloramphenicol

chTR - chicken telomerase RNA subunit

CIP - Calf Intestinal Phosphatase

circRNA - circular RNA

CoA - coenzyme A

CS - chicken serum

Cyt - cytosine

DF-1 - avian fibroblast cell line

DMEM - Dulbecco's modified eagle medium

DNA - Deoxyribonucleic acid

DNMT - DNA methyltransferase enzyme

dpi - day post-infection

DSBH - double-stranded β-helix

dsDNA – double-stranded DNA

E - early

EBER - Epstein-Barr virus-encoded small RNA

EBNA - Epstein–Barr nuclear antigen

EBV- Epstein-Barr virus

EMEM - Eagle's Minimum Essential medium

ESC - embryonic stem cell

ESCDL-1 - embryonic stem cell-derived line-1

FBS - foetal bovine serum

FFE - feather follicle epithelium

For - forward

GaHV-2 - Gallid herpesvirus 2

GR - glucocorticoid receptor

H1 - histone linker

H2A - histone 2A

H2B - histone 2B

H3 - histone 3

H4 - histone 4

HAT- histone acetyltransferases

HBV - hepatitis B virus

HCV - hepatitis C virus

HDAC - histone deacetylases

HDM - histone demethylase

HMT - histone methyltransferases

HP1 - heterochromatin protein 1

HPA - hypothalamic-pituitary-adrenal axis

HPC - human papillomavirus

HTLV - Human T-cell leukemia virus

HTLV - human T-cell lymphotropic virus

IE - immediate-early

IR_L - long internal repeat

IR_S - short internal repeat

ITAS - internal telomeric amplification standard

JBP - J-binding protein

kbp - kilobase pair

kDa - kilo Dalton

KSHV - Kaposi's sarcoma herpesvirus

L - late

LANA - latency-associated nuclear antigen

LAT - latency-associated transcripts

LB - Luria-Bertani medium

LEM-2 - nuclear membrane protein

LG-ABN - licking/grooming and arched-back nursing

LMH - chicken liver carcinoma epithelial cells

LMP - latent membrane protein

lncRNA- long non-coding RNA

M - moderate

M.SssI - methyltransferase

m1A - N1- methyladenosine

m5C - 5-methylcytidine

m6A - N6-methyladenosine

m7G - 7-methylguanosine

MBD - methyl-binding proteins

MCPyV - Merkel cell polyomavirus

MCS - multiple cloning site

MD - Marek's disease

MeCP2 - methyl CpG binding protein 2

Meq - Marek's EcoRI-Q-encoded protein

MERE - Meq responsive element

miRNA - microRNA

mL -millilitre

mtDNA - mitochondrial DNA

mut - mutant

NaButy - sodium butyrate

ncRNA – non-coding RNA

ND2 - NADH dehydrogenase 2

NEB - New England Biolabs

NGFI-A - nerve growth factor inducible protein A

NH_2 - amine

nt - nucleotides

NuRD - histone deacetylase

O/N - overnight

OD_{600} - optical density at 600 nm

p300 - histone acetyltransferases

PBL - peripheral blood leukocytes

PCAF78 - histone acetyltransferases

PCR - polymerase chain reaction

PFU - plaque-forming unit

piRNA - piwiRNA (piRNA), and long non-coding RNA

PKD - pseudoknot domain

PKMT - protein lysine methyltransferases

Pol II - RNA polymerase 2

PRMT - protein arginine methyltransferases

pSTAT3 - phosphorylated signal transducer and activator of transcription 3

PTL - peripheral tumour leukocytes

PTM - post-translational modification

qPCR - quantitative polymerase chain reaction

RB1 - retinoblastoma tumour-suppressor gene

REs - response elements

Rev - reverse

rev -revertant

RNA - ribonucleic acid

RNAi - RNA interference

RpL22 - ribosomal protein L22

rpm- revolution per minute

RPMI - Roswell Park Memorial Institute medium

SAM - S-adenosyl-L-methionine

siRNA - short interfering RNA

SMUG1 - single-strand-selective monofunctional uracil-DNA glycosylase 1

TDG - thymine DNA glycosylase

TERT - reverse transcriptase enzymatic subunit

TET - ten-eleven translocation enzyme

TF - transcription factor

TR - telomerase RNA subunit

TRAP - telomeric repeat amplification protocol

TR_L - terminal long repeat

TR_S - terminal short repeat

TSS - transcription start site

v - virulent

vIL-8 - viral interleukin-8

vTR- viral telomerase RNA subunit

vv - very virulent

vv+ - very virulent +

wt - wild-type

Zta - EBV virus transcription factor

βGT - β-glucosyltransferase

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CHAPTER 1. INTRODUCTION

1.1. Epigenetics and epigenetic modifications

1.1.1. General overview

For nearly eight decades since Conrad Waddington introduced the term epigenetics and defined it as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" [1], researchers were trying to untangle the clues that suggested gene function could be altered by more than just changes in the DNA sequence. With the rapid expansion of genetics over the following years, the original meaning of this definition, that referred to all molecular pathways modulating the expression of a genotype into a particular phenotype, has gradually evolved [2]. Today is generally accepted that epigenetics is "the study of changes in gene function that are mitotically and meiotically heritable and which do not entail a change in the DNA sequence" [3].

Epigenetic programming and regulation have crucial roles in animal and plant development as well as adult life, and stable inheritance of epigenetic settings is essential for the maintenance of tissue- and cell-specific functions through stable expression or repression of genes [4]. To date, many examples of epigenetic gene repression or activation were described [5]. These include the inactivation of one of the two X chromosomes in mammalian female somatic cells [6], the allelic silencing at imprinted genes, the control of lineage-specific maintenance of gene expression at different loci and the heritable repression of repeat elements of viral or retroviral origin [7]. In addition, epigenetic imprints have a crucial role in maintaining genomic stability. Indeed, the silencing of centromeres and telomeres ensures the correct attachment of microtubules to centromeres and reduces excessive recombination between repetitive elements, respectively [8,9]. Furthermore, epigenetic regulation of transposable elements prevents their transposition and possible insertional mutagenesis [10].

Epigenetic processes that generate the epigenome involve non-coding RNA-associated silencing, post-translational histone modification and DNA methylation [11]. These processes, which alter gene expression and can affect cell fate and phenotype plasticity as well as behaviour [12], will be presented further below.

Phenotypic plasticity is an essential mechanism for organisms to buffer their population from environmental changes [13]. It was long ago established that natural populations possess genetic variation in the extent of plasticity; however, such difference in phenotypic variance cannot

be attributed only to single-gene effects. A recent rise in the evidence suggests that other sources that include multigene effects, environmental influences and importantly epigenetic effects, could also play a significant role in the shaping of phenotypic responses [13].

Furthermore, the long-lasting influences of the environment on phenotypic characteristics without apparent underlying genetic change were repeatedly demonstrated [14]. For example, in honeybees (*Apis mellifera*), fertile queens and sterile workers are alternative forms of the adult bee that develop from genetically identical larvae following differential feeding with royal jelly (Figure 1.0.a). This feeding results in differential DNA methylation that is a vital component of an epigenetic network controlling a most crucial aspect of eusociality, the reproductive division of labour and the differential expression of many genes between queen and worker larvae [15].

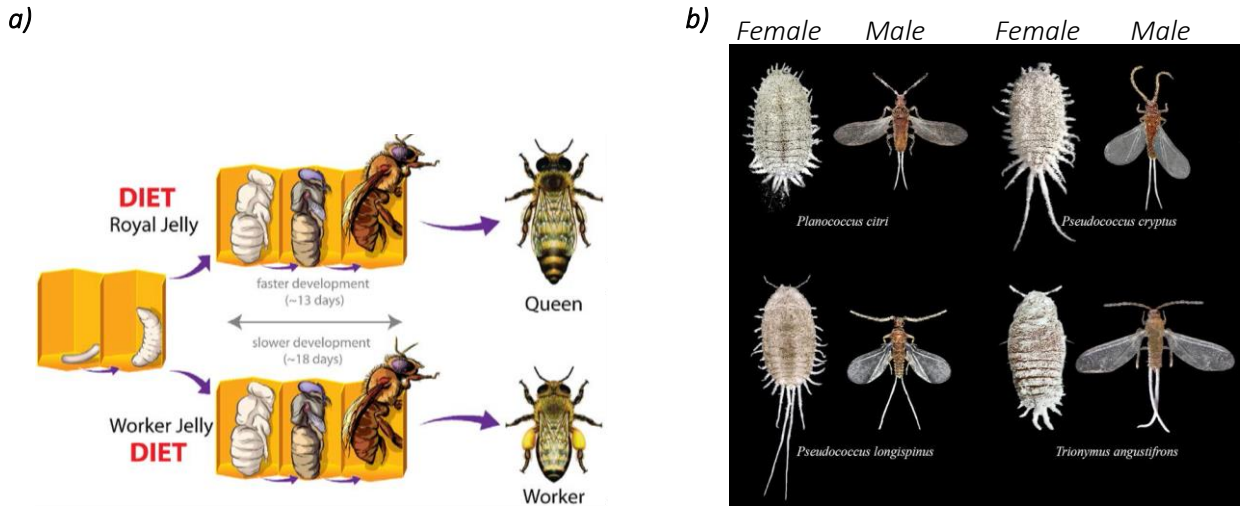

FIGURE 1.0. EFFECT OF THE ENVIRONMENTAL FACTORS ON PHENOTYPIC CHARACTERISTICS OF DIFFERENT SPECIES.

a) Honeybee biology depends on a polyphenism that produces different female castes. During larval development, female larvae that were fed royal jelly develop faster, producing queen bees. Female larvae that were fed with worker jelly have slower development and result in worker bees [16]. **b)** In mealybugs, temperature change during early life cycle results in highly stable heterochromatin that induces development of males, with distinct phenotypic differences compared to females [17].

Moreover, in some groups of insects, like mealybugs (*Pseudococcidae*), sex determination is triggered by temperature changes during gametogenesis. Male and female mealybugs are morphologically highly divergent, although genetically identical. In the mealybugs, the formation of stable heterochromatin and the silencing of the entire paternally inherited genome induce male sex during early development (Figure 1.0.b) [17].

Furthermore, complex animal models have shown that mothering style, nutrition and different environmental exposures during development can lead to locus-specific changes in the epigenome [18,19]. For mice, *in utero* or neonatal exposure to bisphenol A (BPA), a chemical used in the manufacture of polycarbonate plastic, is associated with higher body weight, increased breast and prostate cancer occurrence and altered reproductive function. This endocrine-active compound shifts the coat colour distribution of viable yellow agouti (A^{vy}) mouse offspring toward yellow by decreasing DNA methylation in a retrotransposon upstream of the Agouti gene (Figure 1.1.a and 1.1.b). Moreover, maternal dietary supplementation with methyl donors reversed DNA hypomethylating effect of BPA. This indicated that early developmental exposure to BPA could change offspring phenotype by stably altering the epigenome and are counteracted by maternal dietary supplements [18].

In addition, rat models showed that in times of increased environmental stress, there is less time for maternal care in the form of postnatal maternal licking/grooming and arched-back nursing (LG-ABN). Low levels of LG-ABN in the first week after birth cause offspring to be more fearful, contrasting the offspring of high LG-ABN mothers. These behavioural characteristics will persist into adulthood, where a female usually displays the same behaviour as her mother [20]. Interestingly, epigenetic modifications of the regulatory elements of relevant stress response genes were detected. The stress response in mammals is mediated through the hypothalamic-pituitary-adrenal (HPA) axis and involves glucocorticoid hormones. The reduced fearfulness of high LG-ABN rats is the result of an increase in the number of glucocorticoid receptors in the hippocampus. High LG-ABN mothering results in a high serotonergic tone in the hippocampus of the pups, leading to activation of cAMP and increased expression of the nerve growth factor inducible protein A (NGFI-A). Increased binding of NGFI-A to the promoter of the glucocorticoid receptor (GR) gene is associated with DNA hypomethylation, histone acetylation, and increased expression of GR. This increase in expression, in turn, results in more glucocorticoid receptors in the hippocampus. The epigenetic marks appear to maintain the GR expression state for the rest of the rat's life (Figure 1.1.c) and act as the molecular memory that confers persistence of the phenotype into adulthood [21,22].

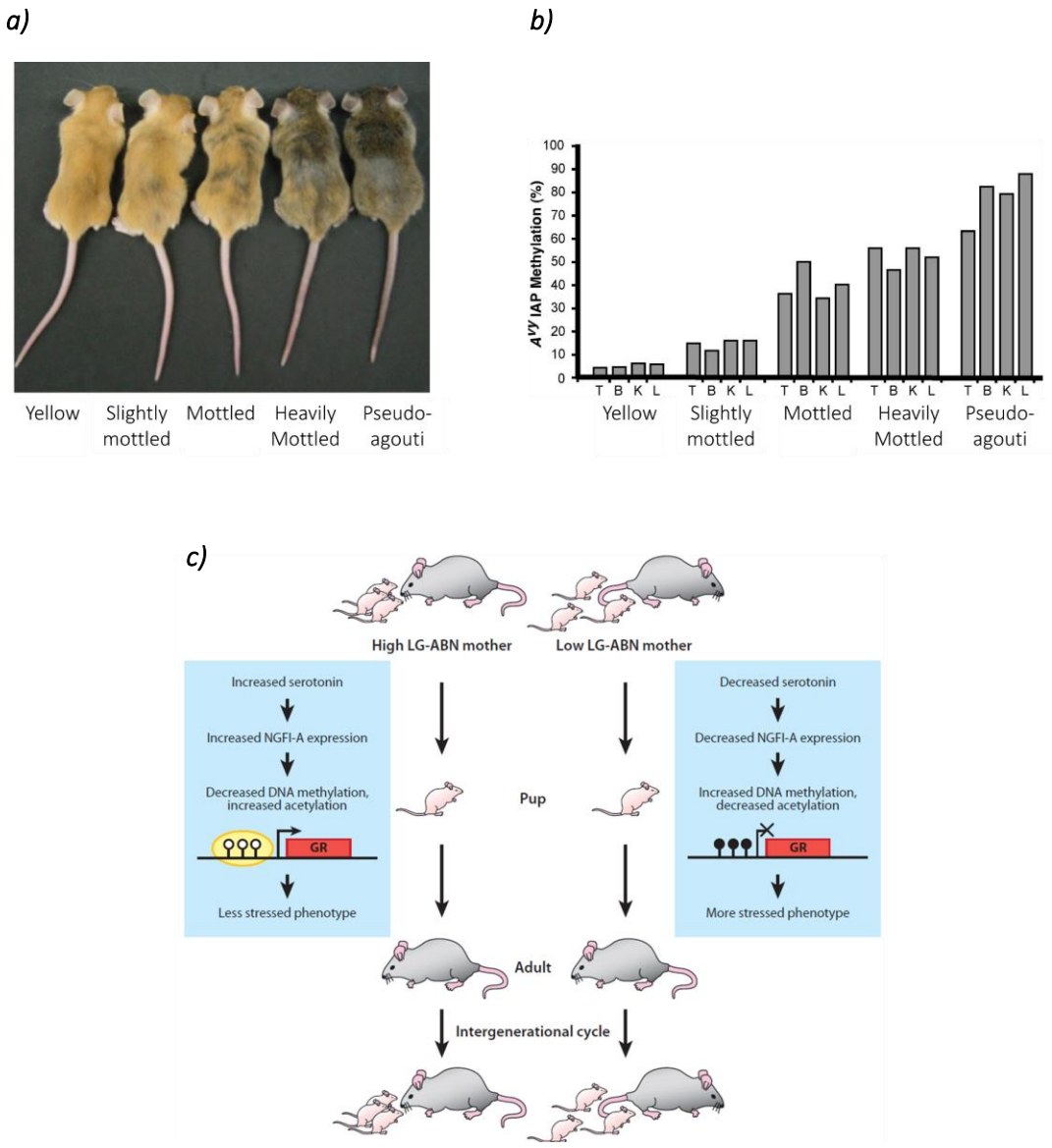

FIGURE 1.1. EXAMPLES OF RELATIONSHIPS BETWEEN EPIGENETIC MODIFICATIONS AND ENVIRONMENTAL FACTORS.

a) Maternal bisphenol A (BPA) exposure shifts offspring coat colour distribution toward yellow. Genetically identical A^{vy}/a offspring representing the five coat colour phenotypes. **b)** Average methylation measured in ectodermal (brain (B) and tail (T)), mesodermal (kidney (K)), and endodermal (liver (L)) tissues from BPA-exposed A^{vy}/a offspring is positively correlated to the five coat colour phenotypes (adapted from [18]). **c)** Mothering style in the rat, characterised by licking/grooming (LG) and arched-back nursing (ABN), is preserved across generations by molecular events marking the first week of pup's life. High LG-ABN mothering results in a high serotonergic tone in the hippocampus of the pups, leading to increased expression of the nerve growth factor inducible protein A (NGFI-A). NGFI-A binds to the promoter of the glucocorticoid receptor (GR) gene and stimulates DNA hypomethylation, histone acetylation and increases GR expression. Higher glucocorticoid receptor numbers in the hippocampus are associated with reduced stress levels. The epigenetic signatures maintain the GR expression into adulthood and determine the level of LG-ABN mothering (adapted from [21,22]).

Importantly, the relationship between epigenetics and specific phenotype appears more evident in the states of the disease. For instance, divergent epigenetic signatures were identified in atherosclerosis [23], osteoarthritis [24], lupus erythematosus [25], imprinting disorders [26], neuropsychiatric disorders [27] and improper gene inactivation in cancer [28]. Furthermore, epigenome abnormalities related to developmental disorders and late-onset adult diseases such as metabolic and mental disorders were reported [29].

1.1.2. Non-coding RNA-associated gene silencing

The idea of RNA-mediated gene regulation originated at the inception days of molecular biology with the proposition that sequence-specific non-coding RNA (ncRNA) might interact with promoters to regulate gene expression [30]. In recent years, accumulating knowledge has revealed that ncRNAs dominate the transcriptional output of mammals and other complex organisms [31], with only 2–3% of the human genome constituting for protein-coding genes [32] and that ncRNAs regulate many levels of gene expression during development [33].

This RNA machinery expresses vast repertoires of regulatory ncRNAs [25,34] that act through RNA interference (RNAi) pathways. ncRNAs are central figures in the genetic and epigenetic processes that coordinate precise patterns of gene expression during the maturation of multicellular organisms [35]. This regulation can occur at the essential levels of genome function, including RNA processing, chromatin structure, RNA stability, chromosome segregation, transcription and translation [36,37].

Regulatory ncRNAs are mainly divided into two categories based on their size, short-chain ncRNAs (<200 nt) that include microRNA (miRNA), short interfering RNA (siRNA) and piwiRNA (piRNA), and long ncRNA (lncRNAs) and circular RNA (circRNA) (Table 1.1) [8,38,39].

TABLE 1.1. MAIN NON-CODING RNAS INVOLVED IN THE EPIGENETIC REGULATION.

Name	Size (nt)	Source precursor	Examples of the main functions
siRNA	19-24	double-stranded RNA	gene transcription silencing
miRNA	19-24	pri-miRNA	gene transcription silencing
piRNA	26-31	long single-chain precursor	transposon repression DNA methylation
lncRNA	> 200	multiple precursors	genomic imprinting X-chromosome inactivation
circRNA	100-4000	pre-mRNA	miRNA sponge RNA binding protein sponge/decoy gene transcription/translation

(adapted from [8,37,38])

1.1.2.1. The field of RNA epigenetics

Recent technological advances and novel mechanistic approaches have discovered diverse chemical modifications of cellular RNAs, including N6-methyladenosine (m6A) [40], N1-methyladenosine (m1A) [41], 5-methylcytidine (m5C) [42], 7-methylguanosine (m7G) [43] and pseudouridylation (Ψ) on lncRNA [44]. Similar to conventional epigenetic modification of DNA and histone modification, which will be discussed later, these RNA epigenetics have emerged as pivotal regulators of gene expression [45]. The discovery of these novel epigenetic marks expanded the knowledge of the chemical and topological properties of four basic nucleotides that, in the end, affect the structure and the function of RNA [46]. m6A, as the most frequent internal modification of RNA in eukaryotic cells, heads the research in the field of molecular biology regarding the functional importance in biological processes of the mechanical components, which install, remove and recognise the m6A residues [47,48]. These insights form new levels of the post-transcriptional regulatory landscape. Moreover, it is becoming evident that the alterations of the RNA modification machinery can have damaging effects during human diseases, especially in cancer [49].

1.1.3. Histone post-translational modifications

DNA in eukaryotic cells is compacted and packaged into a macromolecular complex termed chromatin. The fundamental unit of chromatin is the nucleosome, composed of an octamer of the four core histones (H3, H4, H2A, H2B), around which 147 base pairs or approximately 1.75 turns of DNA are wrapped [12,50]. In addition, linker DNA connecting nucleosomes associates with the linker histone H1 [2]. The carboxyl (C)-domain of core histones are predominantly globular except for their amino (N)-terminal tail endings, which are unstructured. Moreover, chromatin is further organised into two different levels of general structure, silent heterochromatin and active euchromatin. Heterochromatic domains are in general inaccessible to DNA binding factors and are transcriptionally silent. Euchromatin domains, in contrast, define more accessible and transcriptionally active portions of the genome [51].

In the early 1960s, it was first indicated that histones could be post-translationally modified [52]. However, the more profound understanding of how these modifications could affect chromatin structure came in 1997 from solving the high-resolution X-ray structure of the nucleosome. It was demonstrated that histone N-terminal tails could protrude from their nucleosome and make contact with adjacent nucleosomes, indicating that modification of these tails would affect inter-nucleosomal interactions and thus affect the overall chromatin structure [53]. Up to date, a large number of different histone post-translational modifications (PTMs) are described, and these modifications have the potential to encode epigenetic information.

Histone modifications include methylation of arginine (R), methylation, acetylation, ubiquitination and sumoylation of lysines (K), phosphorylation of serine (S) and threonine (T) (Table 1.2), and are deposited or removed from histones by specific enzymes [50]. The extra complexity of histone modifications comes from the fact that methylation may be in one of three different forms: mono-, di-, or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines. Furthermore, not all of these modifications will happen on the same histone at the same time, and the timing will depend on the signalling conditions within the cell [50].

These modifications form a histone code that regulates chromatin function by changing the nucleosome structural dynamics and dictates gene expression patterns by exposing genes to the transcription machinery. In addition, histone tail modifications prevent accessibility of the genome to other types of cell machinery, such as DNA repair, replication and chromosomal segregation [54].

TABLE 1.2. DIFFERENT CLASSES OF HISTONE MODIFICATION AND THEIR FUNCTIONS.

Chromatin modifications	Modified residues	Regulated function
Acetylation	K-ac	transcription, repair, replication
Methylation of lysines	K-me, K-me2, K-me3	transcription, repair
Methylation of arginines	R-me1, R-me2a, R-me2s	transcription
Phosphorylation	S-ph, T-ph	transcription, repair, condensation
Ubiquitination	K-ub	transcription, repair
Sumoylation	K-su	transcription

(adapted from [50])

To date, the best-characterised histone modifications include acetylation and methylation of lysine residues on histones H3 and H4. It was demonstrated that the acetylation of all lysine residues on H3 and H4 is associated with transcriptional activation [55,56]. However, the methylation of lysine residues may be either associated with transcriptional repression or activation depending on which amino acid and to what extent (mono-methylation, di-methylation, or tri-methylation) the residue is modified [57].

1.1.3.1. Switch between repressive and permissive chromatin by histone acetylation

Since the discovery of histone acetylation in 1964 [52], it was established that this modification of lysines is highly dynamic and is regulated by the opposing action of two families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs) [58]. This modification is an N-acetylation, which corresponds to the replacement of a hydrogen atom in a reactive amino group by an acetyl moiety ($CO–CH_3$) (Figure 1.2.a). N-acetylation occurs either on the N-terminal α-amine of proteins (α-N-acetylation) or the ε-amino group of the lateral chain of lysines (ε-N-acetylation). Lysine ε-N-acetylation is a major histone PTM involved in transcription, chromatin structure and DNA repair.

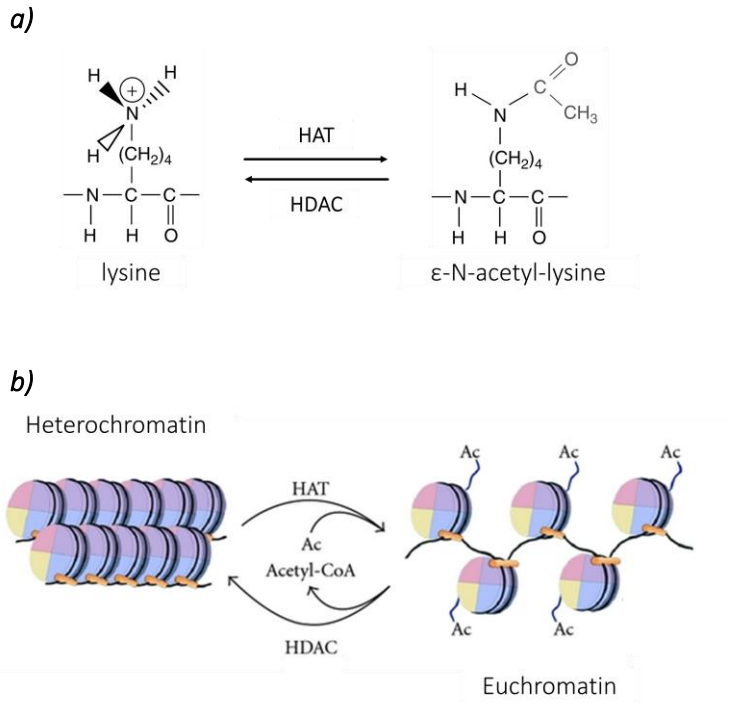

FIGURE 1.2. HISTONE MODIFICATION BY LYSINE ACETYLATION.

a) Acetylation of histone lysine amino acids is performed by histone acetyltransferases (HATs) and reversed by histone deacetylases (HDACs). **b)** Histones are highly basic proteins due to their enrichment of positively charged residues (lysines and arginines) that increase their affinity for DNA, which is negatively charged. Acetylation (Ac) neutralises the positive lysine charge, resulting in the weakening of the interaction of histones with DNA and formation of euchromatin (adapted from [59]).

The HAT enzymes utilise acetyl CoA as a co-factor and catalyse the transfer of an acetyl group to the ε-amino group of lysine side chains [58]. However, acetylation is highly reversible by HDACs (Figure 1.2.b) [60].

Furthermore, it was revealed that there are additional sites of acetylation present within the globular histone core, H3K56 for example, that is acetylated in humans. The H3K56 side chain points towards the DNA major groove, suggesting that acetylation would affect histone/DNA interaction, similar to the effects of acetylating the histone N-terminal tail lysines [61].

1.1.3.2. The regulation and function of histone methylation

Histone methylation is one of the most critical and complicated covalent modifications of the histone N-terminal region. Methylation usually occurs on arginine (R) and lysine (K) residues of H3 and H4 [62]. Unlike acetylation and phosphorylation, histone methylation does not alter the charge of the histone protein. Furthermore, methylated lysines can be found in either mono-, di or tri-methylated state (Figure 1.3.a), whereas arginines can be either mono- or di-methylated (which can be asymmetric or symmetric) (Figure 1.3.b) [63].

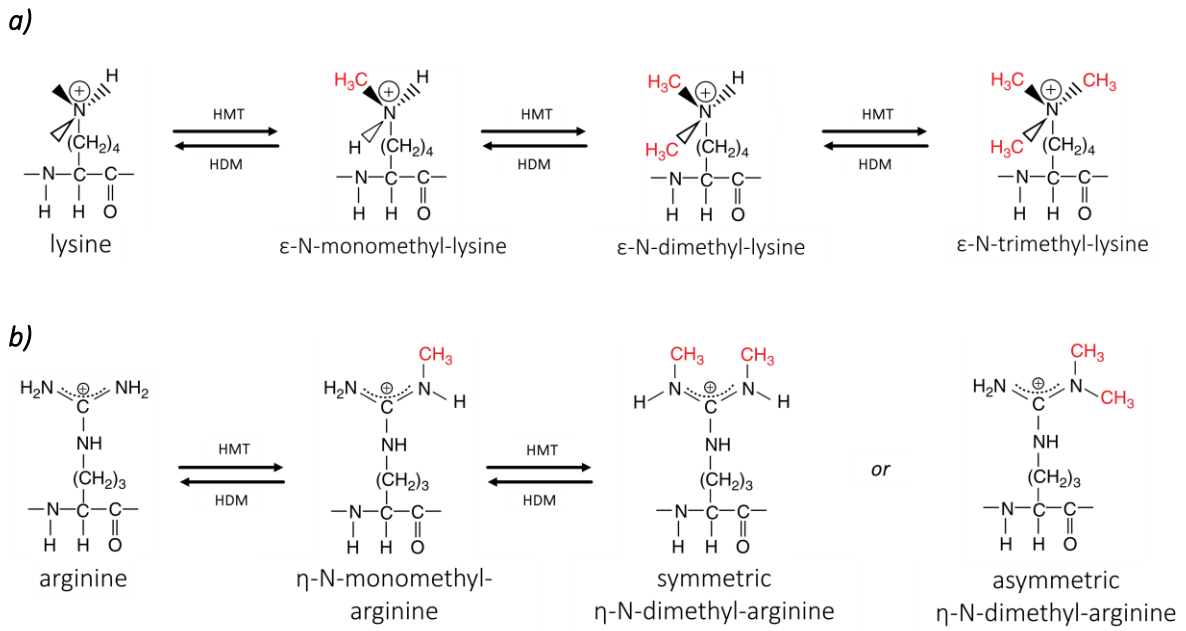

FIGURE 1.3. HISTONE METHYLATION OCCURS ON ARGININE AND LYSINE RESIDUES OF H3 AND H4 BY THE ACTION OF HISTONE METHYLTRANSFERASES (HMTS) AND DEMETHYLASES (HDMS).

a) Chemical structures of lysine and its methylated derivatives. Lysines may be mono-, di- or tri-methylated. **b)** Chemical structures of arginine and its methylated derivatives. The two forms in which di-methyl-arginine can be found are symmetric or asymmetric (adapted from [59]).

Histone methylation is regulated by two families of proteins: histone methyltransferases (HMTs) and histone demethylases (HDMs) [64]. Methylation occurs at both lysine and arginine side chains, which are catalysed by protein lysine methyltransferases (PKMTs) and protein arginine methyltransferases (PRMTs). The methyl group is provided by the S-Adenosyl methionine (SAM).

Methylation modifications usually occur on lysines 4 (H3K4), 9 (H3K9), 27 (H3K27), 36 (H3K36), 79 (H3K79) of H3 and lysines 20 (H4K20) of H4, with monomethylation, dimethylation and trimethylation for each site. The methylation of H3K4, H3K36 and H3K79 is often associated with euchromatin in the regions of transcriptionally active genes, while the methylation of H3K9, H3K27 and H4K20 is associated with heterochromatic regions of the genome [65].

For example, studies in yeast and animals demonstrated that H3K4me3 mainly occurs in the promoter and transcription start site (TSS) regions and had a high degree of overlap with the binding sites of Pol II in the genome [66]. On the other hand, in nematodes, it was verified that H3K9me3 interacts with nuclear membrane protein LEM-2, combined with heterochromatin and nuclear membrane to maintain the structure of heterochromatin and suppress transcription [67].

Furthermore, the main characterised sites of arginine methylation are H3R2, H3R8, H3R17, H3R26, and H4R3. The methylation of H3R17 and H3R26 is associated with specific active genes, while methylation of H3R8 and H4R3 was proven to occur concurrently with gene repression [68]. Unfortunately, relatively little is known concerning the distribution and precise functions of arginine-methylated histones, and further studies will be needed to deepen the knowledge on the histone modification. Even more so, because of the complex crosstalk between different histone modifications, which presumably helps to fine-tune the overall control. This crosstalk can occur *via* multiple mechanisms [50]. For example, *trans*-regulation in *Saccharomyces cerevisiae* showed that methylation of H3K4 by scCOMPASS and H3K79 by scDot1 is dependent upon the ubiquitination of H2BK123 by scRad6/Bre1 [69]. Additionally, this mechanism is conserved in mammals, including humans [70]. It was indicated that the binding of a protein to a particular modification could be disrupted by an adjacent modification. A great example is phospho-switch, where heterochromatin protein 1 (HP1) binds to H3K9me2/3, except during mitosis, where the binding is disrupted due to phosphorylation of H3S10 [71].

Finally, direct functional interplay between histone methylation and another epigenetic modification, DNA methylation, was demonstrated. In *Neurospora* and *Arabidopsis*, genetic evidence indicated that H3K9 methylation is necessary for DNA methylation to occur [72]. In addition, loss of specific histone methyltransferases, such as Suv39H1/2 in knockout mouse cells altered the DNA methylation patterns of heterochromatin [73], suggesting that DNA and histone methylation have a cyclical and mutually reinforcing relationship and both are required for stable and long-term epigenetic silencing. Even though not studied in the context of this project, the histones post-translational modifications and their connections with DNA methylation is of significant importance for further developing the perspectives of the study presented below.

1.1.4. DNA methylation

Despite the fact that covalent modifications of DNA bases were described since the late 1940s, it was only suggested in 1969 that these modifications might modulate gene expression [74,75]. The significant DNA modification is methylation of cytosine (C), followed by adenine (A) and guanine (G) methylation [74]. The functions of the cytosine modification remained uncharacterised until 1975, when two studies revealed essential roles of 5-methylcytosine (5mC) as an epigenetic modification that influences gene expression [76], bringing up the significance of this *fifth nucleotide* in eukaryotic biology [77]. Today, DNA methylation is widely recognised as a typical epigenetic mark because it satisfies the stringent criterion of an epigenetic system that is mitotically and meiotically heritable [78].

In prokaryotes, methylation at both A and C residues contributes to host restriction systems and protects the cell from foreign genetic materials such as bacterial and viral genomes [79]. However, DNA methylation in multicellular eukaryotes occurs predominantly but not exclusively at cytosine residues within CpG dinucleotides [7], where the CpG denotation refers to the occurrence of a cytosine linked to guanine by a phosphate bond. In general, vertebrate genomic CpGs are highly methylated, with 60-90% of genomic CpGs in a methylated state [80]. However, both CpG frequency and methylation patterns vary across a single genome.

Furthermore, specific genomic regions named CpG islands (CGIs), are CpG-enriched yet practically devoid of methylation. These sequences, which are on average 1000 bp long, show an elevated G+C base composition and little CpG depletion. They comprise around 1% of total genomic DNA [81] and are associated with 5' promoter regions of housekeeping genes, as well as a proportion of tissue-specific genes and developmental regulator genes [82–84]. Approximately 60%-70% of annotated vertebrate gene promoters are associated with CGIs, making this the most common promoter type [85]. Furthermore, an extensive genome-wide mapping of histone modifications by chromatin immunoprecipitation has established that H3K4me3 is a signature characteristic of most promoter CGIs, even when the associated gene is not expressed [66]. Recently, a large class of CGIs located remotely from the annotated transcription start sites was described [86]. These sites, named "orphan CGIs", show evidence for promoter function and strong correlation between CGIs and transcription initiation. It was demonstrated that they co-localise with peaks of H3K4me3 and evidence suggests that a large proportion recruit RNA polymerase II and give rise to novel transcripts, highlighting even more the CGIs importance in gene transcription [87].

DNA methylation is a significant form of epigenetic modification and is regulated during embryonic development to control tissue and cell differentiation [88]. Moreover, DNA methylation patterns are altered in cancers and embryos produced by somatic cell nuclear transfer [89]. These changes contribute significantly to the molecular pathology of numerous disease states [87].

1.1.4.1. The mediators of cytosine DNA methylation

The establishment and maintenance of DNA methylation is regulated by the family of methyltransferase enzymes (DNMTs) which convert cytosine to 5-methylcytosine (5mC), including those that establish methylation (DNMT3a and DNMT3b) and maintain methylation (DNMT1) (Table 1.3) [90]. Their catalytic domains appear highly conserved across species, and S-adenosyl methionine (SAM) appears to function as the only methyl donor (Figure 1.4.a) [91].

TABLE 1.3. OVERVIEW OF DNA METHYLTRANSFERASES AND THEIR FUNCTIONS.

DNA methyltransferases (DNMTs)		Functional role
De novo DNMTs	DNMT3a	embryonic development, methylation of CpG sites
	DNMT3b	embryonic development
	DNMT3L	maternal genomic imprinting, silencing of retrotransposons in spermatogonial stem cells
Maintenance DNMTs	DNMT1	cellular maintenance methylation, maintenance of imprinting, silencing of mobile elements during genomic demethylation, contribution to histone deacetylases
	DNMT2	still unclear

(adapted from [90])

DNMT3a and DNMT3b are *de novo* methyltransferases that target cytosines of previously unmethylated CpG dinucleotides (Figure 1.4.b) and have equal preference for hemimethylated and unmethylated DNA, which are essential for *de novo* methylation of the genome during development and newly integrated retroviral sequences [92]. Functional analysis indicated that DNMT3a and DNMT3b have regional specificity due to their respective N-terminal domains; and non-overlapping functions during development with different phenotypes and lethality stages [93]. It

was demonstrated that DNMT3a is necessary for maternal imprinting at differentially methylated regions, and DNMT3b is required for methylation of pericentromeric repeats and CGIs on inactive X-chromosomes [94].

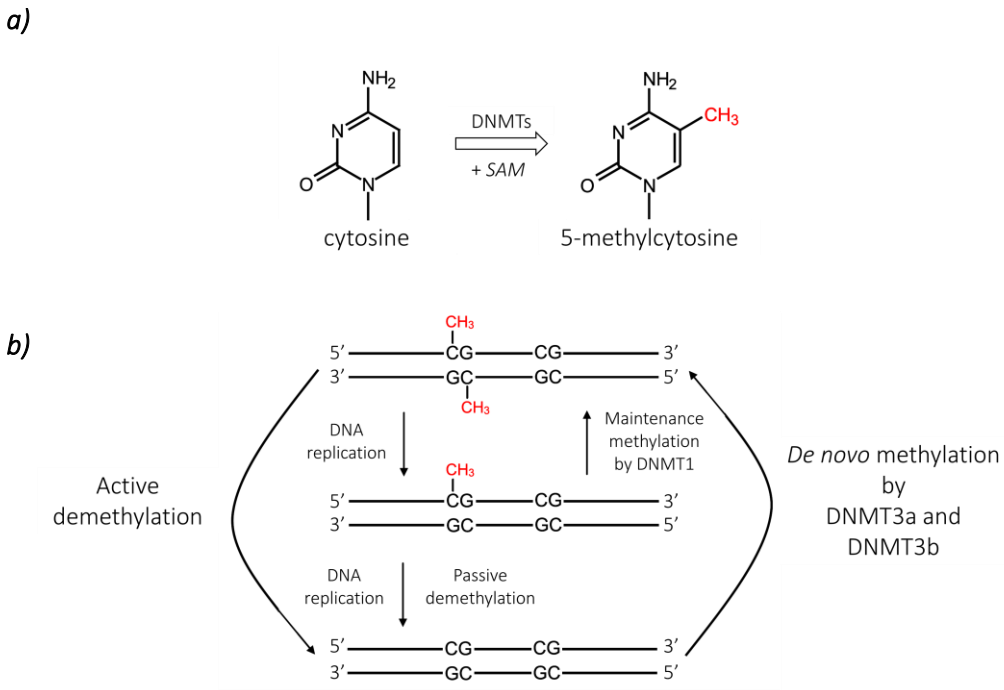

FIGURE 1.4. OVERVIEW OF MECHANISMS INVOLVED IN DNA METHYLATION AND DEMETHYLATION.
a) DNA methyltransferases (DNMTs) catalyse the transfer of a methyl group (in red) from S-adenyl methionine (SAM) to the fifth carbon of cytosine residue to form 5-methylcytosine (5mC). **b)** DNMT3a and DNMT3b are the *de novo* methyltransferases and transfer methyl groups (in red) onto non-methylated DNA. On the other hand, DNMT1 is involved in the *maintenance* of DNA methylation patterns during replication. When DNA undergoes semi-conservative replication, the parental DNA stand retains the original DNA methylation pattern. However, DNMT1 precisely replicates the original DNA methylation pattern by adding methyl groups onto the newly synthesised daughter strand (adapted from [95]).

However, it is unclear how DNMT3a and DNMT3b target specific DNA sequences. One of the proposed mechanisms is that transcription factors regulate *de novo* DNA methylation by binding to specific DNA sequence to either recruit enzymes for methylation or prevent DNA methylation [96]. To a large extent, CpG islands appear to be protected from methylation by transcription factor binding [97]. It was confirmed that when transcription factor binding sites are mutated, CpG islands are unable to retain their unmethylated state [98], resulting in now-exposed CpG sites to be targeted for DNA methylation [99]. Thus, DNMT3a and DNMT3b can either be recruited to promoters by specific transcription factors or may methylate all CpG sites unprotected by a bound transcription factor across the genome.

Additionally, methylation patterns are generally stable and are inherited by both daughter DNA molecules during mitosis. These patterns are sustained by DNMT1, which is known as a *maintenance* enzyme that guards existing methylated sites through its preference for hemimethylated DNA [100].

Furthermore, two other DNMTs were described. DNMT2 showed weak methyltransferase activity *in vitro*, and its depletion did not have any impact on global CpG methylation levels [100]. Moreover, while the DNMT3-like (DNMT3L) was shown to be catalytically inactive, it was highly expressed in germ cell lines and is an obligatory co-factor for *de novo* methyltransferases in embryonic stem cells (ESCs) [101]. It was indicated that DNMT3L stimulates the activity of DNMT3a or DNMT3b through physical interaction [102] and is a positive regulator of DNA methylation at gene bodies of housekeeping genes and a negative regulator of DNA methylation at promoters of bivalent genes in mouse ESCs [103].

1.1.4.2. DNA methylation and gene expression

For gene transcription to occur, the gene promoter should be readily accessible to transcription factors and other regulatory units. DNA methylation at CpG sites mostly suppresses transcription in several ways, either by directly preventing transcription factor binding or by leading to changes in chromatin structure that restrict access of transcription factors to the gene promoter [104].

Transcription factors (TFs) are key players in the activation of transcription, and their functions rely on binding to the DNA by recognising particular nucleotide motif through steric

interactions between the TF protein domains and the DNA molecule. Occurring chemical modifications to the DNA bases, such as methylation, can either increase or restrain these interactions [105]. Notably, the relationship between DNA methylation and TF binding is a complex process, often dependent on cell signalling and post-translational modifications. Interestingly, close to 70% of all mammalian gene promoters are associated with CpG islands that are mostly unmethylated [106]. The majority of mammalian transcription factors bind to GC-rich DNA motifs that contain CpGs; however, if their recognition sites are methylated or surrounded by methylated CpGs, the binding is hindered [107]. Hypermethylation of CGIs within promoter regions of genes leads to transcriptional repression of those genes (Figure 1.5) [108].

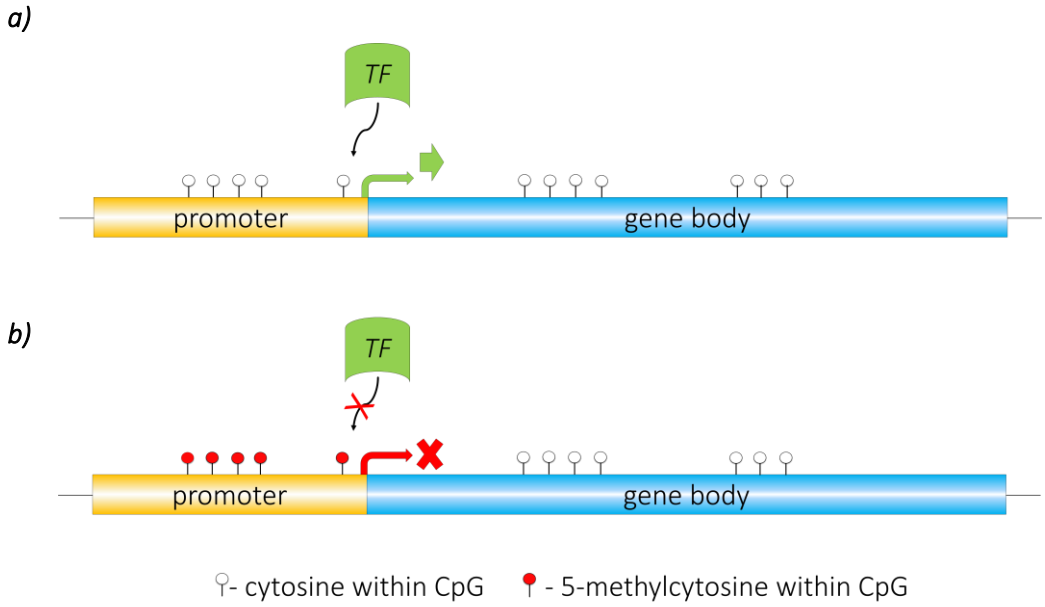

FIGURE 1.5. DNA METHYLATION AT CPG SITES SUPPRESSES TRANSCRIPTION BY DIRECTLY PREVENTING TRANSCRIPTION FACTOR BINDING.

a) When the CpG island located in the gene promoter is unmethylated (white circles) the binding of transcription factor (TF) is unhindered, resulting in the initiation of the transcription (green arrow). **b)** The methylated CpG island (red circles) located in the promoter region prevents TF binding and results in gene silencing (red arrow) (adapted from [108]).

Furthermore, it was revealed that DNA methylation in the close vicinity of TF binding sites not containing CpGs might also alter the strength of TF/DNA interaction. For example, TF AP-1 complex composed of cFOS and cJUN loses its ability to bind DNA when a CpG site adjacent to the core-binding motif is methylated [109].

Another well-described mechanism of gene regulation by DNA methylation is chromatin remodelling, which results in restricting the accessibility for protein complexes interacting with gene promoters or transcription start sites (TSS) (Figure 1.6) [110]. In this case, methylated CpGs are recognised by a class of proteins that induce a restrictive heterochromatin state [111]. Methyl-CpG-binding domain proteins (MBDs) bind methylated DNA and recruit chromatin remodelling corepressor complexes, such as the Nucleosome Remodelling and histone Deacetylation (NuRD) complex resulting in transcriptional repression [112].

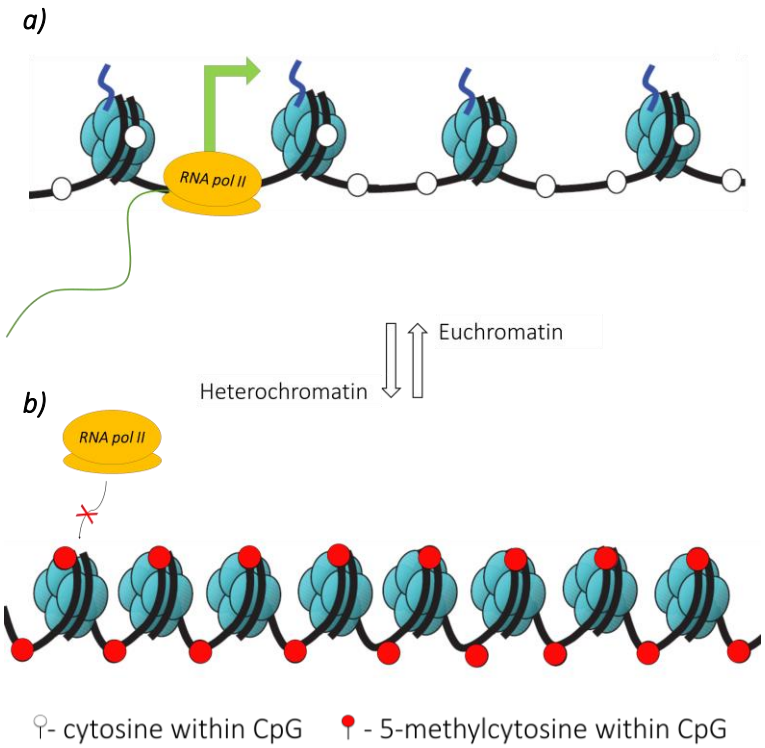

FIGURE 1.6. REVERSIBLE CHANGES IN CHROMATIN ORGANISATION THAT INFLUENCE GENE EXPRESSION.

a) Unmethylated cytosines (in white) and euchromatin allow the binding of transcription related complexes, such as RNA polymerase II (RNA pol II), guiding the transcription forward. **b)** Methylated cytosines (in red) influence the chromatin remodelling towards the heterochromatin that represses transcriptional activity by restricting the accessibility to gene promoters or transcription start sites (adapted from [113]).

1.1.4.3. Crosstalk between DNA methylation and other epigenetic mechanisms

DNA methylation machinery together with histone post-translational modifications and microRNAs establishes complex interplay that regulates gene transcription. Previously described chemical modifications on the N-terminal histone tails, which include methylation, acetylation, ubiquitination and phosphorylation, influence not only how DNA strands are packaged but also their transcriptional activity.

DNMTs directly interact with enzymes that regulate histone modifications typically involved in gene repression (Figure 1.7). It was established, that both DNMT1 and DNMT3a bind to the SUV39H1 histone methyltransferase that restricts gene expression by H3K9 methylation [114] and that DNMT1 and DNMT3b can both bind to histone deacetylases resulting in more tightly packed chromatin and restricted access for transcription [115]. In addition, histone modifications influence DNA methylation patterns. DNMT3L binds to unmethylated H3 histone tails and recruits DNMT3a and DNMT3b, however, the presence of the active histone modification H3K4me3 impairs the binding of DNMT3a, DNMT3b, and DNMT3L preventing *de novo* methylation [116].

As mentioned before, methyl-binding proteins (MBD) serve as the most definite link between DNA methylation and histone modification. The best-known member of the MBD family, methyl CpG binding protein 2 (MeCP2), recruits histone deacetylases to remove active histone modifications and repress gene transcription. Additionally, MeCP2 enhances the state of heterochromatin by recruiting histone methyltransferases that add repressive H3K9 methylation mark [114].

Furthermore, DNA methylation can regulate the expression of miRNA. The loss of DNMT1 and DNMT3b in colon cancer cells demonstrated that 10% of miRNAs were regulated by DNA methylation [117]. In this study, the relationship between DNA methylation and miRNA expression was studied using DNA methyltransferase knockout HCT116 cell line as a model. For 13/135 miRNAs screened, methylation status tightly controlled their expression, since a high level of CpG site demethylation was required for their re-expression. Treatment with DNA methyltransferase inhibitor resulting in partial demethylation, as seen in DNMT1 knockout cell line, was not sufficient to induce the re-expression of miRNAs.

Vice versa, miRNAs regulate histone modifications and DNMT expression resulting in the regulation of DNA methylation. For example, the depletion of miRNA-290 in mice, which

indirectly regulates DNMT3a and DNMT3b expression, lead to a loss of DNA methylation and an increase in repressive histone methylation at H3K9 [118].

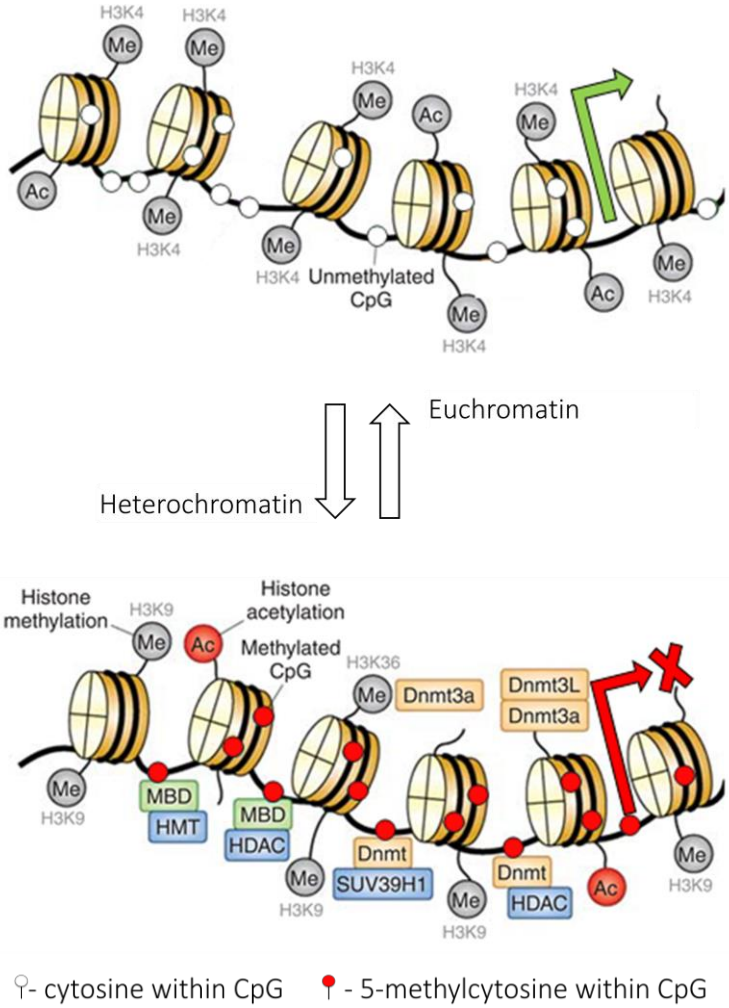

FIGURE 1.7. EPIGENETIC CROSSTALK BETWEEN DNA METHYLATION AND HISTONE POST-TRANSLATIONAL MODIFICATIONS.

Transcription is regulated by the complex interactions of multiple epigenetic mechanisms that cooperate to activate or silence gene expression. To suppress gene expression, DNA methyltransferases (DNMTs) target CpG sites and actively methylate DNA. For some DNMTs, association with histone tails and with DNMT3L enhances their catalytic activity. DNA methylation is recognised by methyl-binding proteins (MBDs) that together with DNMTs recruit enzymes that modify the histone tails. These enzymes include histone deacetylases (HDACs), which remove acetylation (Ac) and histone methyltransferases (HMTs), which methylate histones, and together with DNA methylation serve to repress gene expression (red arrow) further. In regions of DNA with active transcription, histone tails often contain H3K4me3 that inhibits DNMT binding to unmethylated CpG sites and maintains a permissive environment for transcription (adapted from [119]).

1.1.4.4. DNA demethylation pathways

DNA demethylation occurs by either passive or active mechanisms. Passive demethylation is a phenomenon characteristic for dividing cells. As DNMT1 actively maintains DNA methylation during cell replication, its inhibition or dysfunction allows newly incorporated cytosine to remain unmethylated and consequently reduces the overall methylation level following each cell division [100].

At the same time, active DNA demethylation is established in both dividing and non-dividing cells, however, this process requires enzymatic reactions that further modify 5mC, by deamination and oxidation reactions, to a product that is recognised by the base excision repair (BER) pathway to replace the modified base with unmodified cytosine (Cyt) [120]. Several mechanisms of active DNA demethylation were proposed (Figure 1.8). 5mC can be chemically modified at two sites, the amine (NH_2) group and the methyl (CH_3) group. Deamination of the amine to a carbonyl group by activation-induced cytidine deaminase (AID) and apolipoprotein B mRNA-editing enzyme complex (APOBEC) effectively converts 5mC into thymine, thus creating a G/T mismatch and inducing the BER pathway to correct the base [121].

Second DNA demethylation mechanism is mediated by the ten-eleven translocation (TET) family of enzymes that include TET1, TET2 and TET3. TET enzymes add a hydroxyl group onto the methyl group of 5mC to form 5-hydroxymethylcytosine (5hmC) [122]. Once 5mC is oxidised into 5hmC, two separate pathways convert 5hmC back into the cytosine. The first pathway includes sequential oxidation by TET enzymes that continues to oxidise 5hmC, first to 5-formylcytosine (5fC) and then to 5-carboxylcytosine (5caC) [123]. The second pathway is established when 5hmC is deaminated by AID/APOBEC to form 5-hydroxymethyl-uracil (5hmU) (Figure 1.8) [124].

Following the modifications of 5mC, the BER pathway utilises thymine DNA glycosylase (TDG) to cleave the modified residue (thymine, 5-hydroxymethyl-uracil, 5-formyl-cytosine, and 5-carboxylcytosine) and replaces it with a non-modified cytosine (Figure 1.8). TDG is essential for DNA demethylation and is required for normal development [125].

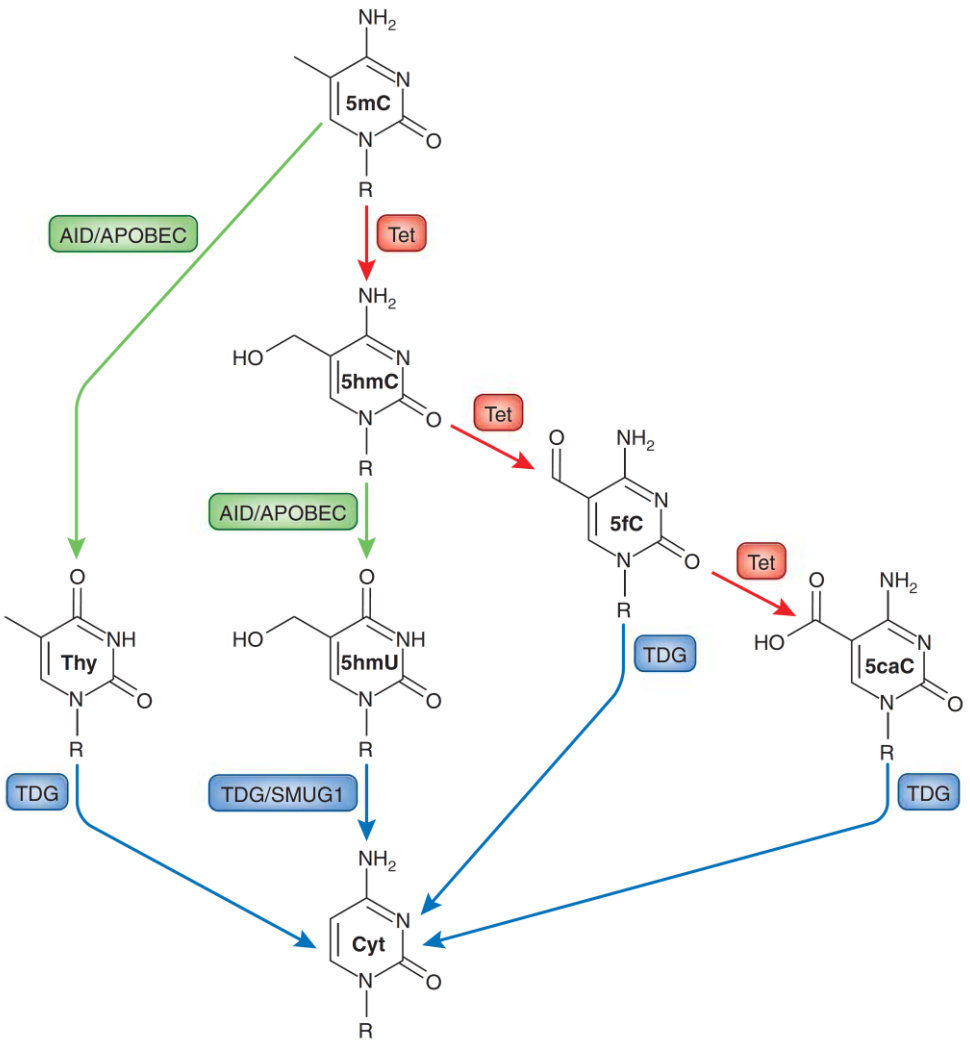

FIGURE 1.8. PATHWAYS OF ACTIVE DNA DEMETHYLATION.

5-methylcytosine (5mC) can be enzymatically modified at the amine group (NH_2) and the methyl group (CH_3). The amine group of 5mC can be deaminated (in green) by AID/APOBEC, converting 5mC into thymine (Thy). The methyl group of 5mC can be modified by the oxidation and addition of a hydroxyl group mediated by ten-eleven translocation (TET) enzymes to generate 5-hydroxymethylcytosine (5hmC). Newly synthesised 5hmC can be further enzymatically modified at the amine group and the hydroxymethyl group. AID/APOBEC deaminates (in green) 5hmC to produce 5-hydroxymethyluracil (5hmU) and TET enzymes further oxidize (in red) 5hmC to form 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Eventually, the products of each pathway (Thy, 5hmU, 5fC, and 5caC) are recognised and cleaved to replace with a cytosine (Cyt) by thymine DNA glycosylase (TDG) and single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1), both components of the base excision repair (BER) pathway (in blue) (adapted from [119]).

In addition, the second enzyme of BER pathway, single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1), from the same uracil DNA glycosylase family as TDG, is found to be involved in DNA demethylation pathway (Figure 1.8) [125].

The evidence for the role of 5hmC as a critical intermediate in the process of active demethylation is ever-expanding [126], and in addition, a growing number of studies are demonstrating, that like methylation, 5hmC may regulate gene expression. For example, the oxidation of 5mC to 5hmC impairs the binding of the repressive methyl-binding protein MeCP2 and resulting in chromatin changes [127], marking 5hmC as an essential player in regulating DNA demethylation and gene expression.

1.1.5. DNA hydroxymethylation and the elusive roles of 5-hydroxymethylcytosine

1.1.5.1. Proteins of TET family

TET proteins belong to TET/J-binding protein (JBP) family of α-ketoglutarate- and iron (II)-dependent dioxygenases, closely related to the JBP1 and JBP2 proteins found in kinetoplastids such as trypanosomas and leishmanias [122]. In mammals, the TET/JBP family is composed of the TET1, TET2 and TET3 that can, as previously described, oxidize 5mC into 5fC and 5caC. Interestingly, 5fC and 5caC seem to be of physiological importance due to their detection in ESCs and early embryos DNA [128].

The TET proteins share a conserved catalytic domain composed of a double-stranded β-helix (DSBH) region downstream of a cysteine-rich domain. Furthermore, TET1 and TET3 contain a CXXC DNA-binding domain in their N-terminus (Figure 1.9). Interestingly, even if it seems that TET2 lost CXCC domain during evolution, it exists as a separate IDAX gene (CXXC4), which encodes an inhibitor of Wnt signalling, suggesting a connection between the Wnt pathway and the TET proteins [122]. Due to its abundance in ESC, TET1 was the most studied member of TET family for which it was observed that it binds preferentially to methylated and unmethylated CpG-rich regions in promoters and within genes by the CXXC domain [129].

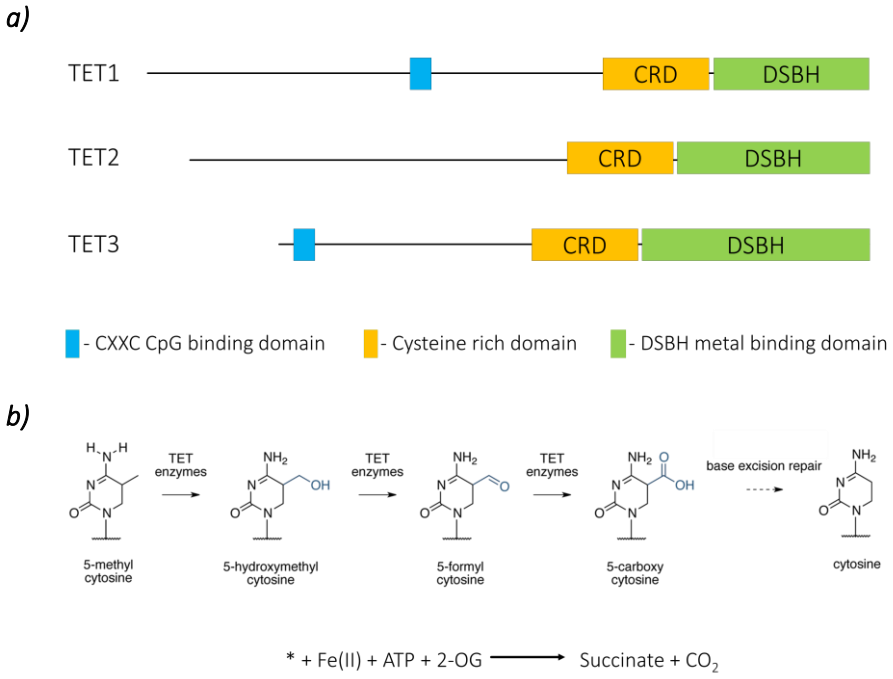

FIGURE 1.9. STRUCTURE AND FUNCTION OF THE MEMBERS OF THE TET PROTEIN FAMILY.

a) All members of the TET protein family have a highly conserved cysteine-rich region (in orange) and the double-stranded β-helix (DSBH) domain that exhibits 2-oxoglutarate (2-OG)- and iron (II)-dependent dioxygenase activity (in green). In addition, the N-termini of TET1 and TET3 contain a CXXC domain (in blue), which mediates their direct DNA-binding ability. **b)** All three enzymes of the TET family oxidise 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) in the presence of 2-OG and iron. 5mC and 5hmC can be further oxidised to 5-carboxylcytosine (5caC) by TET enzymes if the reaction system is supplemented with ATP (adapted from [130]).

Thus, by establishing CXXC-DNA bond, TET1 could contribute to maintaining CpG islands in a hypomethylated state by counteracting *de novo* CpG methylation [131]. The TET proteins have distinct expression patterns, suggesting possible non-overlapping functions. TET1 and TET2 are highly expressed in ESCs, and TET2 has a crucial involvement in haematopoiesis, with the discovery that TET2 mutations are frequent in human myeloid malignancies [132], as well as in B- and T-cell lymphomas [133]. Moreover, TET3 is highly expressed in oocytes and was implicated in epigenetic reprogramming in the zygote. It was shown that embryos derived from TET3-deficient oocytes have an increased incidence of developmental failures due to impossibility to properly erase 5mC from the paternal genome in the preimplantation embryos [134].

In recent years, the increase in evidence demonstrated that beside dynamic transcriptional control, the activity and recruitment of TET proteins are regulated at multiple levels that result in

the final effect on DNA methylation patterns. It was shown that the activity of TET enzymes could be stimulated or inhibited in the presence of distinct metabolites, co-factors, and post-translational modifications [135]. For example, aberrant accumulation of metabolites such as 2-hydroxyglutarate, succinate and fumarate, as a result in the mutations in the genes coding for the metabolic enzymes isocitrate dehydrogenases 1 and 2, succinate dehydrogenase and fumarate hydratase, inhibit TET protein enzymatic activity [136].

In addition, the increase in ascorbic acid (vitamin C) levels demonstrated a stimulating effect on TET protein enzymatic activity, that resulted in the higher levels of the cytosine oxidation products and in a reduction of global DNA methylation in cultured cells as well as in mouse tissue [137,138]. Vitamin C exists as ascorbate anion under physiological pH conditions, and in rodents, *de novo* synthesis of ascorbate occurs in the liver *via* glucose biosynthetic pathway. However, in humans, ascorbate cannot be synthesized due to a mutated and non-functional L-gulonolactone oxidase that catalyses the last step of ascorbate biosynthesis, and thus it needs to be supplemented through dietary sources [139]. The notion that vitamin C is another mediator in the interface between the epigenome and metabolic environment was further supported by the fact that vitamin C can globally modify the status of DNA methylation in mammals. For instance, human embryonic stem cells exhibit a widespread DNA demethylation of a large number of genes in response to ascorbate [140]. Ascorbate-dependent DNA demethylation was also shown to enhance the generation of induced pluripotent stem cells during somatic cell reprogramming, known to be accompanied by genome-wide DNA demethylation and the enrichment of 5hmC [141]. Although the precise molecular mechanisms underlying metabolic ascorbate-dependent TET activity remain to be entirely determined, it is likely that ascorbic acid interacts directly with the catalytic domain of TET proteins and provides a local, reducing environment that increases recycling efficiency of the Fe(II) co-factor [138].

1.1.5.2. Emerging roles of 5-hydroxymethylcytosine

As early as 1972, a novel cytosine modification, 5-hydroxymethylcytosine (5hmC), was observed, that accounted for approximately 15% of the total cytosine residues in rat and mouse brain DNA [142]. However, it was only in 2009 that the biological synthesis, role and importance of 5hmC was described [122,143]. Noticeably, contrasting 5mC levels that are relatively equal between cell types, 5hmC is variable between cell types and generally below the one observed in ESCs and the nervous system [144]. As depicted in the previous paragraph, genomic 5hmC acts as an intermediate in active DNA demethylation by replication-dependent or replication-independent pathways, and additionally, since 5hmC is a predominantly stable oxidation product of 5mC and relatively stable component of DNA, it is implicated in regulating gene expression [145]. This is exemplified in diverse tissues and cell types that display a positive association of gene expression with enrichment for 5hmC in the gene body that is actively or moderately transcribed, in comparison to those that are weakly transcribed [146]. Furthermore, 5hmC is recognised by proteins involved in cell metabolism regulation, including MeCP2, for example [147].

Profile analysis of 5hmC placements showed that it is localised at specific genomic regions, especially in enhancers, sites flanking promoters or CpG-islands and throughout gene bodies (Figure 1.10). Furthermore, an abundance of 5hmC at enhancers was signified to be positively correlated with enhancer activity [148]. At CGIs, suggested primary function of 5hmC is to maintain promoters in the unmethylated state, whereas in intragenic sequences, 5hmC could have an inhibitory action on antisense transcription initiation [131]. Interestingly, a more in-depth analysis of hydroxymethylomes from various mammalian samples indicated that 5hmC is enriched at 0.5–2 kilobases upstream and downstream of the transcription start site (TSS), and depleted closer to the TSS at moderately or highly transcribed genes [146]. Furthermore, for poorly transcribed or untranscribed genes, the peak of 5hmC was recorded at the TSS, indicating that in this region, 5hmC enrichment may have negative regulatory potential [149].

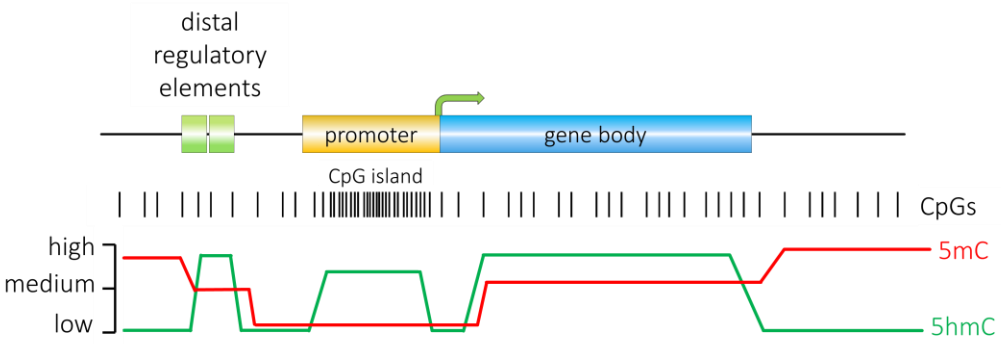

FIGURE 1.10. PROPOSED DISTRIBUTION OF CPGS, 5-METHYLCYTOSINE AND 5-HYDROXY--METHYLCYTOSINE LEVELS THROUGHOUT THE GENOME OF THE HEALTHY TISSUE.

CpGs are locally enriched at CpG islands that are found in numerous gene promoters. Most CpGs in the genome are hypermethylated except those in CpG islands that are generally unmethylated (red line). In contrast, the elevated levels of 5-hydroxymethylcytosine (5hmC) (green line) correspond to the areas of the promoter surrounding transcription start sites (green arrow), gene body as well as distal regulatory regions of enhancers (adapted from [150]).

Previously described association of 5hmC with the borders of promoters of actively transcribed genes, that are mostly unmethylated, could be explained by the need of these CpGs to be protected against DNA methylation spreading from adjacent regions that have much higher 5mC contents [151,152].

Altogether, this evidence provides a new emerging template of 5hmC as a significant player in the shaping of the epigenetic landscape of the cell through the regulation of dynamic DNA methylation patterns.

1.1.6. The interplay between epigenetic DNA modifications and cancer

Malignant transformation and tumour progression are complex processes intertwined with numerous disruptions of the regulatory mechanisms. During tumorigenesis, the cells undergo a disruptive change involving uncontrolled proliferation, a loss of checkpoint controls tolerating the accumulation of chromosomal aberrations and misregulated differentiation. This process is commonly thought to be triggered by point mutations, deletions or translocations, disrupting either oncogenes or tumour suppressor genes. In transformed cells, oncogenes are activated through dominant mutations or overexpression of a gene, while tumour suppressor genes become silenced or inactivated [153]. The deregulation of typical DNA modifications and gene expression helps transformed cells to evolve rapidly, generating intratumoral heterogeneity, thus contributing to increased metastatic potential and potentially drug resistance [154]. Thanks to decades of research, today's definition of cancer corresponds to both a genetic and epigenetic disease, as specific tumour types are frequently found to harbour standard sets of genetic mutations as well as recurrent changes to the epigenetic landscape [155].

1.1.6.1. DNA methylation abnormalities in cancer

Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality to be identified in cancer cells, when it was demonstrated that a substantial proportion of CpGs that were methylated in healthy tissues were unmethylated in cancer cells [156,157]. Later on, the site-specific hypermethylation in the promoters of tumour suppressor genes was established as another hallmark of cancerogenesis [158].

In transformed cells, hypomethylation usually occurs at repeated DNA sequences, such as long interspersed nuclear elements, whereas hypermethylation predominantly involves CGIs (Figure 1.11) [159]. CGI regions are preferentially located at the 5' end of genes that occupy close to 60% of human gene promoters. While most of the CpG sites located in large repetitive CpG sequences of the genome are methylated, the majority of CGIs are usually unmethylated in healthy tissues. Due to this pattern, if the corresponding transcription factors are available, histone

modifications are in a permissive state, and the CGIs remain unmethylated, the gene in question will be transcribed [160].

When hypomethylation of CGIs located in the promoter regions occurs in malignant cells, nearby genes become activated. This phenomenon contributes to genomic instability and, less frequently, to activation of silenced oncogenes, exemplified by the Ras oncogene, which is expressed generally in the testis and aberrantly in tumours [161]. Important to mention, DNA hypomethylation of individual genes in cancer cells is relatively uncommon. The majority of the promoters affected by the loss of DNA methylation belong to tissue-specific genes.

Furthermore, in cancer, despite global hypomethylation, specific genes undergo inactivation because of hypermethylation of CGIs in regulatory regions (Figure 1.11), which are unmethylated in non-malignant tissues (Figure 1.10). DNA hypermethylation was shown to result in abnormal silencing of several tumour suppressor genes in most types of cancer [162]. DNA hypermethylation-induced gene silencing was first indicated in the studies on the of the retinoblastoma tumour-suppressor gene (RB1) of retinoblastoma patients [163].

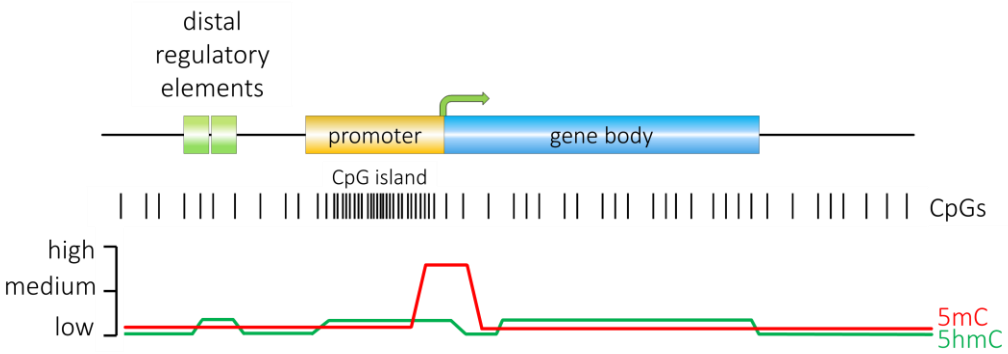

FIGURE 1.11. PROPOSED DISTRIBUTION OF CPGS, 5-METHYLCYTOSINE AND 5-HYDROXY--METHYLCYTOSINE LEVELS THROUGHOUT THE GENOME OF THE TUMOUR TISSUE.

DNA methylation (red line) and hydroxymethylation (green line) landscapes change drastically in cancer cells. Global hypomethylation is one of the hallmarks of carcinogenesis and occurs on the repetitive CpG sequences of the genome. On the other hand, hypermethylation of the CpG island located in the gene promoter regions is a typical pattern in tumour tissue. Analysis of 5-hydroxymethylcytosine patterns (in green) across genes revealed overall lower levels compared to healthy tissue (adapted from [150]).

In addition, methylated cytosines present highly unstable bases with the predisposition to gene mutation as the methylated cytosines are often deaminated and converted to thymine. Replacement of cytosine by thymine can as well lead to inactivation of tumour suppressor genes. For example, if the triplet CGA, which encodes an arginine residue, is changed to TGA, which specifies a stop codon, it may lead to a prematurely truncated protein [164].

1.1.6.2. Aberrant 5-hydroxymethylcytosine patterns in cancer

Due to the robust interplay between 5mC and 5hmC previously described, the changes in each modification in cancer are expected to be dynamically linked [150]. Initially, perturbation of the global level of 5hmC was observed conjointly with impaired TET activity in a wide range of malignancies, including breast cancer, colon cancer, prostate cancer and melanoma [165]. Moreover, it was shown that the loss of 5hmC affects various genomic regions, including promoters, exons, introns and intergenic regions, indicating that 5hmC depletion might occur across the whole genome [166]. Further studies of 5hmC gene patterns signified an apparent reduction around TSS and increased levels in gene bodies in both healthy and cancer cells. However, overall lower levels of 5hmC were observed in the cancer tissue (Figure 1.11) [167]. In addition, it was indicated that in cancer cells, similarly to 5mC, gene promoters can be affected by both aberrant gain and aberrant loss of 5hmC, which positively correlates with changes in gene expression [168]. Furthermore, as confirmed in leukaemia, intergenic regions and enhancers, in particular, are also widely affected by changes in 5hmC levels [169].

The reason behind the global 5hmC reduction could be the fact that the TET enzymes responsible for generating 5hmC, as well as their co-factors, are often mutated, transcriptionally downregulated or reduced at the protein level [150]. For example, the first mechanisms discovered to underlay altered 5hmC pattern included microdeletions and somatic mutations within the TET2 gene described in hematopoietic malignancies, where missense mutations affected the catalytic domain and thus TET2 activity [170].

Furthermore, another proposed mechanism for the general depletion of 5hmC in cancer is due to the inhibition of TET co-factors. As previously described, TET enzymes are 2-oxoglutarate (2-OG) dependant, which is produced through catalytic oxidative carboxylation of isocitrate by isocitrate dehydrogenases (IDHs) in the Krebs cycle [171]. It was shown that the mutations of

IDH1 and IDH2 identified in cancer cells produce both enantiomers of the oncometabolite 2-hydroxyglutarate (2-HG) [172], that are structurally similar to 2OG and can antagonise the 2OG-dependant reaction, thus inhibiting TET-mediated 5mC to 5hmC conversion [171].

1.1.7. Viral oncogenesis

Research done in the past three decades showed that several viruses play a significant role in the development of animal and human cancers, with 12% of human cancers associated with viral infections [173]. Contribution of the oncogenic viruses to different steps of the carcinogenesis and the association of a virus with a given cancer can vary from 15-100% [174]. Oncogenic viruses are also widespread among animals and can cause economic losses in animal husbandry and serve as valuable models to study viruses affecting humans (Annex A) [175].

ANNEX A: Viral oncogenesis: Lessons from homologous animal models

Srđan Pejaković, Benoît Muylkens

Namur Research Institute for Life Sciences, Integrated Veterinary Research Unit, Department of Veterinary Medicine, Faculty of Sciences, University of Namur, Namur, 5000, Belgium

Abstract

The interactions between viruses and the hosts entail complex machinery and numerous events that, in the end, drive viral infection, which sometimes leads to oncogenesis. The great number of information concerning viral pathogenesis and oncogenesis, as well as the host responses, have been obtained using animal models. These models are helpful for understanding virus-host interactions and better deciphering different steps of cancer development. Well-defined small animal models with the reliable kinetics of disease induction and progression, together with the fact that oncogenesis can be studied in a natural virus-host system, allows unique studies that are impossible to perform in other non-natural models of viral disease and oncogenesis. In particular, they are important for understanding viral replicative/pathogenic/oncogenic properties in humans. To this date, animal research models are necessary to describe *in vivo* situation and are essential for developing a detailed knowledge of virus-caused infectious diseases. This review aims to present the studies on well-established homologous animal models used for translational oncogenic research for three viral families: Retroviridae, Herpesviridae and Papillomaviridae, and the examples of lessons obtained that helped in deciphering the mechanisms of major steps of oncogenesis.

Keywords: Animal models; Viral oncogenesis; Herpesviridae; Papillomaviridae; Retroviridae

Seven different viruses were linked to oncogenesis of human cancers: DNA viruses such as Epstein-Barr virus (EBV), Kaposi Sarcoma–Associated Herpesvirus (KSHV), human papillomavirus (HPV) and Merkel cell polyomavirus (MCPyV) and RNA viruses hepatitis B virus (HBV), hepatitis C virus (HCV) and human T-cell lymphotropic virus (HTLV)[176].

In recent years, it became evident that viruses infecting animal and humans play central roles in shaping the epigenetic landscape of infected cells, and thus, deciphering the mechanisms of viral infections on the epigenetic control of their target cells will undoubtedly help to understand better the interplay between viruses and the hosts. Up to date, comprehensive knowledge of the methylation status of viruses, such as polyomaviruses, and especially herpesviruses, was obtained. DNA viruses use host transcription factors as well as epigenetic in order to affect epigenetic control of viral and hosts' gene expression [177].

One of the best-studied members of the herpesvirus family is EBV, and examples of viral epigenetic modifications induced in host's cells will be presented in the paragraph below.

1.1.7.1. Epigenetic modifications by oncogenic herpesviruses

EBV is a human gamma-herpesvirus that predominantly establishes latent infection in B-lymphocytes and epithelial cells. EBV is one of the most common viruses in humans, with 90% of the total population infected and is associated with mononucleosis and with several human cancers such as Burkitt's lymphoma, nasopharyngeal carcinoma, T- and NK-cell lymphoma and gastric carcinoma [178].

EBV, like other herpesviruses, establishes latent infections and reactivates to re-establish active replication. During latency, the circularised viral genome remains idle in an episomal state and can be replicated by the host cell replication machinery. Active viral replication is highlighted with the production of viral progeny and inevitably results in cell lysis [90]. The EBV latency is regulated by six EBV nuclear antigens EBNAs (1, 2, 3A, 3B, 3C, and LP), three latent membrane proteins LMPs (1, 2A, and 2B), BARF-1 protein, two small RNA molecules (EBER 1 and EBER2) and RNA transcripts [178]. Furthermore, the maintenance of viral latency and switching from the latent phase to the lytic phase, together with viral replication, are controlled by the viral immediate-early genes BZLF1 and BRLF [179].

In EBV-induced transformation as well as in EBV life cycle, epigenetic mechanisms such as DNA methylation/hydroxymethylation and histone modifications, which control the expression of latent viral oncogenes together with miRNAs, play an essential role (Table 1.4) [180].

TABLE 1.4. EPIGENETIC INTERACTIONS OF EBV VIRAL PROTEINS

Viral protein	Target	Mode of action	Reference
EBNA2	histone acetyltransferases	induce expression of LMP1	
EBNA3C	histone deacetylase 1	transcriptional repression of mRNA	[179]
LMP1	DNMT1, DNMT 3a, DNMT 3b	activation	
LMP2A	DNMT1, DNMT3B	upregulation	[179]
	TET2	downregulation	[181]
BARF	TET2	downregulation	

The EBV genome appears to be hypomethylated or unmethylated during lytic infection, highly methylated during latency and demethylated during reactivation [90]. Interestingly, the analysis of CpG methylation pattern in the EBV genome showed that only five promoters do not possess the DNA methylation marks during latency, EBER1, EBER2, BZLF1, LMP1 and LMP2B [182].

It was showed that LMP1 induces the expression and activity of DNA methyltransferase, and, in addition, LMP2A upregulates DNMT3B and DNMT1 by inducing the expression of phosphorylated signal transducer and activator of transcription 3 (pSTAT3), resulting in aberrant CpG hypermethylation [179]. Resulting hypermethylation of the EBV genome aids virus to escape from the host immune system, inhibiting expression of viral proteins that are recognised by cytotoxic T-cells [178]. Moreover, the hypermethylation of promoter regions in tumour suppressor genes and consequent gene silencing results in manipulation of gene expression that leads to a variety of oncogenic events [203].

EBV oncoproteins might also interact with components of histone modification machinery. The viral proteins EBNA2 and EBNA3C act through histone modifications and chromatin remodelling [183]. EBNA2 plays an essential role in inducing the expression of LMP1 by interaction with histone acetyltransferases, such as p300 and PCAF78 [184]. EBNA3C interacts with HDAC1 promoting deacetylation activity and causes the repression of transcription from the main promoter initiating the transcription of EBNA mRNA [185].

Furthermore, it was demonstrated that the 5-hydroxymethylation of EBV genome could affect lytic reactivation. Increased TET activity decreases methylation of lytic EBV promoters in EBV-infected cell lines affecting the ability of EBV immediate-early proteins, BZLF1 and BRLF1, to induce the lytic form of viral infection and initiate cancerogenesis [186]. It was attested that the EBV reactivation is methylation dependant and is initiated by the expression of the immediate-early BZLF1 gene, which encodes for Zta transcription activator. Interestingly, Zta recognises and binds to methylated CpGs on the viral promoters and activates the expression of the remaining lytic genes, thereby inducing a lytic infection [178]. This interaction suggests that the loss of TET function in EBV-infected epithelial cells, or the presence of other mutations that inhibit 5hmC conversion, may result in the promotion of EBV-induced cancerogenesis, by either enhancing methylation of cellular tumour suppressor genes or altering 5mC/5hmC equilibrium of lytic EBV promoters [186]. This was exemplified by the discovery that TET family enzymes, especially TET2, were repressed by EBV infection at both mRNA and protein levels. TET2 was found to be downregulated by BARF and LMP2A transcripts and also by EBV-upregulated miRNAs targeting TET2 [181].

Furthermore, one of the most detailed studied oncogenic animal herpesviruses is Gallid herpesvirus type 2 (GaHV-2). Early research has shown that GaHV-2 genome is methylated during latency and indicated that epigenetic modifications might be involved in regulating the switch from latency to the productive replication and play a role in the regulation of viral gene expression during GaHV-2 infection [187].

A more in-depth analysis of the region of the GaHV-2 genome (Figure 1.12) encoding for the productive phase protein pp38 showed significantly higher methylated patterns than a neighbouring region encoding for latency-associated transcripts (LATs) and major viral oncogene (Meq) (discussed in the paragraph 1.3.2.4). In addition, during latency, analysis of the pp38 promoter and the origin of replication (ori) have revealed restrictive epigenetic modification marks, including H3K27me3 and H3K9me3. In contrast, the promoters in the region involved establishing and maintaining latency (mRNA-M9-M4 cluster, meq and LATs) possessed permissive H3K4me3 and H3K9Ac marks [188].

Our team, together with the Transcription and Virus-Induced Lymphoma team (TLVI, Université François Rabelais de Tours, France), demonstrated the existence of differential DNA methylation patterns at promoters of GaHV-2. DNA methylation patterns for immediate early genes ICP4, ICP27 and late gene ICP22 showed that they are widely methylated during the viral latency, and returning to an unmethylated state during productive viral replication (Figure 1.12) [189–191].

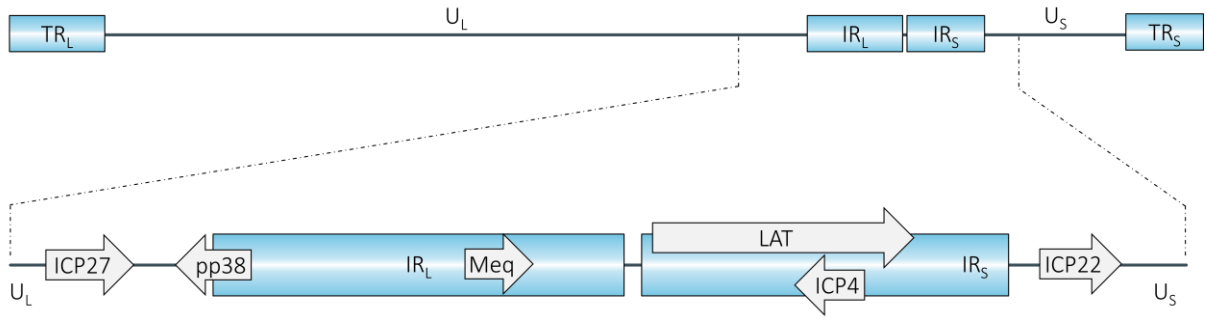

FIGURE 1.12. REPRESENTATION OF GALLID HERPESVIRUS 2 GENOME WITH THE POSITIONS AND ORIENTATIONS OF MAJOR VIRAL GENES INCLUDED IN THE DIFFERENTIAL DNA METHYLATION PATTERN STUDIES OCCURRING BETWEEN TWO STEPS OF THE VIRAL LIFE CYCLE.

The promoters of immediate early genes including ICP4, ICP27 and late gene ICP22, as well as promoters of productive phase protein pp38, major viral oncogene (Meq) and the region encoding for latency-associated transcripts (LAT), showed a distinctive difference in methylation patterns between latency and productive viral replication.

1.2. Telomeres and telomerase

1.2.1. General overview and roles in cancerogenesis

Evolution of mammalian cells resulted in complex mechanisms involved in the regulation of cellular life. The cells of healthy tissue demonstrate a strictly limited growth potential and senescence after a defined number of cell divisions, marking it one of the pillars of cellular ageing [192]. Furthermore, genomic stability and integrity must be maintained for an organism to function and propagate successfully.

Progressive shortening of the ends of the eukaryotic chromosomes, the telomeres, is an essential component of senescence and is involved in the control of the cell cycle, making telomeres one of several critical elements required for genomic stability. The telomeres protect enzymatic end-degradation and maintain chromosome stability as well as the solution for the DNA end-replication problem [193]. Early on, it was indicated that chromosomes with truncated telomeric ends fuse with other chromosome ends or become lost during cell division [194]. Further on, telomeres also play a role in the organization of the cellular nucleus by serving as attachment points to the nuclear matrix [195]. The telomeric DNA consists of non-coding tandemly repeated sequences, with the exact repeat sequence varying from one species to the other. In the vertebrates, the repeat unit is the hexanucleotide TTAGGG (5' - 3' direction) [192].

As mentioned above, all chromosomes lose a small portion of telomeric DNA during each cell division, due to a natural consequence of the cellular DNA replication machinery, known as DNA end-replication problem. However, the loss of genomic sequences at each replication cycle can be compensated by the addition of telomeric terminal sequences by telomerase [193].

Telomerase is a holoenzyme, composed of an RNA subunit (TR) and a reverse transcriptase enzymatic subunit (TERT), that together with accessory proteins such as dyskerin, NHP2, NOP10 and GAR, add telomeric repeat sequences to the 3' end of telomeres (Figure 1.13) [196]. Interestingly, human TR is expressed in most healthy cells, contrary to TERT, suggesting that TR may have functions other than the template for telomere extension [197].

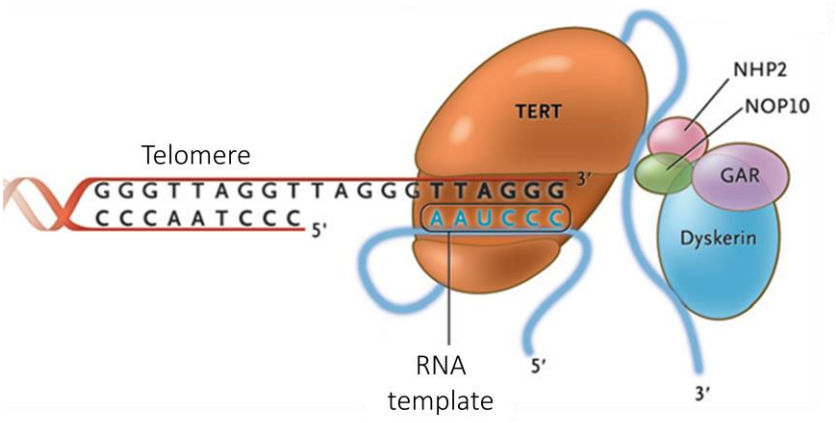

FIGURE 1.13. THE ACTION OF THE TELOMERASE COMPLEX AND ITS COMPONENTS.

The telomerase reverse transcriptase (TERT) and RNA subunit (TR), together with the protein dyskerin and other associated proteins (NHP2, NOP10, GAR), initiate the telomeric elongation. Telomerase catalytically adds TTAGGG hexameric nucleotide repeats to the 3' hydroxyl end of the leading telomeric strand, using a specific sequence presented by TR (blue ribbon) as the template (adapted from [198]).

Importantly, progressive telomere shortening occurs in all healthy dividing cells, eventually resulting in cellular growth arrest that is could be an initial proliferative barrier to tumour formation [196]. In contrast to healthy cell, tumour cells often exhibit an unlimited proliferation potential that leads to their immortalisation.

Telomerase is absent in most human somatic cells, with very low amounts of telomerase activity in healthy human tissues found in hematopoietic progenitor cells upon their proliferation and differentiation and T- and B-lymphocytes [199], germ cells, ovaries and testicles [200], and in physiologically regenerating epithelial cells [201]. However, during tumour development, telomerase activity is often essential in order to maintain the telomeres and is mainly dependant on TERT over-expression, suggesting that TERT is the limiting component of the telomerase activity [197]. Numerous mechanisms involved in either engaging telomerase activity or activating a telomere maintenance mechanism have been proposed, including mutations/deletions in the TERT promoter [202], engagement of TERT alternative splicing [203], epigenetic changes [204] and alternative lengthening of telomeres (ALT) [205], respectively.

Furthermore, research done in past years suggested a strong requirement for oncogenic viruses to regulate telomerase activity and telomere length. Several human oncogenic viruses such as HTLV, HPV, HCV as well as EBV and KSHV and animal oncogenic alphaherpesvirus GaHV-

2 have an effect on the telomerase activity during the infection. The increase of telomerase activity by viruses may be part of a complex transformation mechanism. Many oncogenic virus proteins act as transcription factors or act by other means to increase telomerase expression [206].

1.2.2. Telomerase activity during oncogenic herpesvirus infection

In patients with EBV-induced nasopharyngeal carcinoma, 94.9% showed an increase in telomerase activity [207], which was also observed in EBV-immortalized B-lymphoblastoid cell lines [208], demonstrating an important role for telomerase reactivation in EBV-infected tissues. It was shown that LMP1 is partially responsible for the increase in telomerase activity in nasopharyngeal epithelial cells infected with EBV [209]. Additionally, targeting LMP-1 with small-interfering RNAs (siRNAs) substantially reduced hTERT protein levels and telomerase activity in B-cell lymphomas and overexpression of LMP1 in an EBV-negative nasopharyngeal carcinoma cell line increased hTERT protein expression [210]. Furthermore, studies revealed that LMP2A prevents infected cells from entering the lytic replication cycle, is involved in the transformation of epithelial cells [183] and act as a negative regulator of the hTERT promoter [211]. In addition, analysis of telomere lengths in EBV-positive Burkitt lymphoma cell lines showed increases in telomere length compared with those in EBV-negative cells [212], making EBV, to date, the only human oncogenic virus for which infection was linked to an actual increase in telomere length.

For the other well-studied human oncogenic herpesvirus, KSHV, is was indicated that virus-transformed endothelial cells had elevated telomerase activity compared with uninfected cells [213]. Here, the latency-associated nuclear antigen (LANA) protein plays a vital role in latent episomal persistence of the viral genome in infected cells and B-cell lymphoma development [214,215]. It was indicated that LANA increases expression at the hTERT promoter through interaction with Sp1 transcription factor in fibroblasts and the B-cell lines [216]. Furthermore, avian GaHV-2 encodes for a viral telomerase RNA subunit that interacts with the chicken TERT enhancing telomerase activity and contributing to the efficient and rapid onset of lymphoma [217–219], and will be discussed in detail in following sections.

In a normal cell, TERT expression is regulated primarily at the transcription level [220]. The hTERT promoter contains several E-boxes and GC-rich elements, which can bind c-Myc and Sp1, respectively [221]. It was demonstrated that hTERT gene expression is increased following c-

Myc binding to E-box elements within TERT promoter [222]. However, in most cells, c-Myc and Sp1 act together for regulating hTERT expression [221]. During viral infection, the mechanisms of viral activation of telomerase could be divided in the *trans*-activation of the hTERT promoter by viral oncoproteins, epigenetic control of hTERT in virus-infected cells, post-transcriptional regulation of telomerase activity and *cis*-activation of the hTERT promoter through viral integration.

The knowledge on viral trans-activation of hTERT gene expression has been obtained studying the papillomavirus system, in which the HPV E6 oncoprotein has been shown to directly trans-activate the hTERT promoter [223]. Mutations in the GCs or the E-box elements of the hTERT promoter partially inhibited telomerase expression, demonstrating that independent binding of c-Myc or Sp1 to the hTERT promoter is not sufficient for E6-induced telomerase activation. However, telomerase expression is strongly induced by E6 when c-Myc and Sp1 are cooperatively bound to the hTERT promoter [223]. However, less is known about the mechanisms used by other viral oncoproteins to *trans*-activate hTERT gene expression. For the Tax protein of HTLV-1 was shown to stimulate hTERT promoter through activation of the NF-kB pathway [224]. The LMP1 protein of EBV has been found to induce c-Myc-mediated *trans*-activation of the hTERT promoter, where C-terminal end of LMP1 was found to stimulate hTERT gene expression through NF-kB activation [209]. Lastly, the LANA protein of KSHV has been shown to *trans*-activate the hTERT promoter in various cell lines [225], by activating hTERT gene expression through interactions with Sp1 [216].

Furthermore, since activation of hTERT transcription may involve histone acetylation, its repression may involve histone deacetylation. For example, HPV E6 expression promotes acetylation of histone H3, providing epigenetic control of the hTERT gene [226]. Acetylation of histone H3 at the hTERT promoter was increased in late passage E6- and E7-immortalized keratinocytes, whereas p300 expression was decreased. E6 has been shown to target the p300 acetyltransferase, and cells that expressed E6 and p300 antisense RNA showed increased acetylation of histone H3 and activation of the hTERT gene. Therefore, p300 may act as a repressor of telomerase activation in the context of E6 expression [226].

Telomerase activity can also be regulated at the post-transcriptional level. For example, protein kinase C (PKC)-mediated phosphorylation of telomerase results in the interaction of telomerase and Hsp90 protein necessary to maintain the integrity of the telomerase holoenzyme. Inhibitors of PKC significantly reduced telomerase activity present in human nasopharyngeal and head and neck cancer cells [227]. Moreover, PKC has been shown to modulate telomerase activity in human cervical cancer cells [228]. Thus, EBV and HPV may activate PKC to increase telomerase

enzymatic activity. Furthermore, the RelA/p65 subunit of NF-kB has been shown to bind directly to hTERT and to facilitate its translocation to the nucleus [229]. In nasopharyngeal carcinoma cells, EBV LMP1 can increase telomerase activity post-transcriptionally by promoting NF-kB RelA/p65-mediated binding to hTERT [230].

Furthermore, in addition to encoding telomerase regulators that act in *trans* on the hTERT promoter, the HPV and HBV genomes have been found to integrate within proximity to the hTERT gene [221,231]. In a subset of cervical and hepatocellular carcinoma tumours, the integration resulted in the placement of viral enhancers near the hTERT promoter without perturbing the hTERT coding region, resulting in the increased telomerase expression. The analysis showed that HBV integration acted as an enhancer for *cis*-activation of the hTERT gene [232]. A recent study demonstrated that the expression of the gene encoding for RNA component of telomerase (TR) was progressively amplified with the development of HPV-associated cervical intraepithelial neoplasias to advanced invasive carcinomas The overexpression of the hTR gene was predominantly associated with the integration of oncogenic HPV. Therefore, it appears that the expression of the genes for hTERT and/or hTR may be specifically activated by virus integration in a population of infected cells [233].

1.3. Gallid herpesvirus type 2: A well-established model for studying virus-induced oncogenesis in a natural virus-host system

As discussed throughout previous sections, oncogenic herpesviruses use a broad array of mechanisms to establish and maintain infection, including epigenetic modulation and regulation of telomerase activity, which at the end for some lead to tumour development. Thus, in order to understand all the processes involved in virus-induced oncogenesis well-defined, small-animal models with the reliable kinetics of disease induction and progression, are essential. The fact that oncogenesis can be studied in a natural virus-host system makes these models unique and allows studies that are impossible to perform in other non-natural models of viral disease and oncogenesis.

Similar to EBV, GaHV-2 is one of the few oncogenic herpesviruses that induces tumours in its natural host [234] and serves as a versatile small-animal model for studying different aspects of herpesvirus pathogenesis and induced oncogenesis in a natural virus-host system with remarkable reliability [235].

1.3.1. The Herpesviridae family

1.3.1.1. General overview

The virions of *Herpesvirales* order have complex and characteristic structures that morphologically distinct them from all other viruses. The spherical virion is composed of the core, capsid, tegument and envelope. The core consists of linear, double-stranded DNA genome of 125 to 290 kbp (kilobase pairs) that is contained within a T = 16 icosahedral capsid, which is surrounded by a tegument protein matrix. Finally, the viral particle is covered by an envelope that is pirated from the host nuclear membrane and comprised of the viral glycoproteins (Figure 1.14) [231].

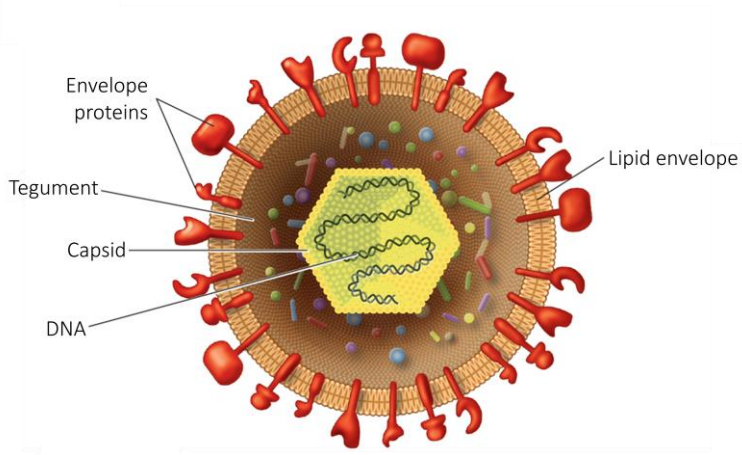

FIGURE 1.14. SCHEMATIC REPRESENTATION OF A HERPESVIRALES VIRION.

Herpesviruses are large, enveloped viruses with a linear double-stranded DNA ranging from 125 to 290 kilobase pairs (kbp). DNA is associated with an icosahedral capsid composed of 162 capsomers surrounded by tegument proteins. The virion is enveloped by pirated host nuclear membrane that is comprised of the viral glycoproteins (adapted from [236]).

The new taxonomy resulting from a considerable genetic distance among the members of *Herpesvirales* order has provided three families, *Herpesviridae* that infect mammals, birds and reptiles, the family *Alloherpesviridae* incorporates the fish and frog viruses, and the family *Malacoherpesviridae* that contains the viruses infecting bivalves (Figure 1.15) [237].

Furthermore, the *Herpesviridae* family is best described with a wide variety of hosts including animals and humans, and based on the biologic and genetic properties can be further divided into three subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. Up to date, *Herpesviridae* family contains more than 130 species sharing common features (Figure 1.15) [238].

Viruses belonging to the *Alphaherpesvirinae* are characterized by a wide range of hosts, a short replication cycle and a rapid proliferation in cell culture. Members of this subfamily mostly infect epithelial or neuronal cells before establishing latency, mainly in non-dividing sensory neurons [239]. Four genera of *Alphaherpesvirinae* subfamily include *Mardivirus*, *Iltovirus*, *Simplexvirus* and *Varicellovirus*. *Mardivirus* and *Iltovirus* infect avian hosts while *Simplexvirus* and *Varicellovirus* infect mammals (Figure 1.15) [240]. Typical members of *Simplexvirus* genus are human Herpes simplex virus 1 (HHV-1) and Herpes simplex virus 2 (HHV-2), while for *Varicellovirus* genus is Human herpesvirus 3 (HHV-3) also known as Varicella-Zoster virus (VZV) [238]. *Mardivirus* member Gallus herpes virus type 2 (GaHV-2) is an oncogenic herpesvirus causing Marek's disease (MD).

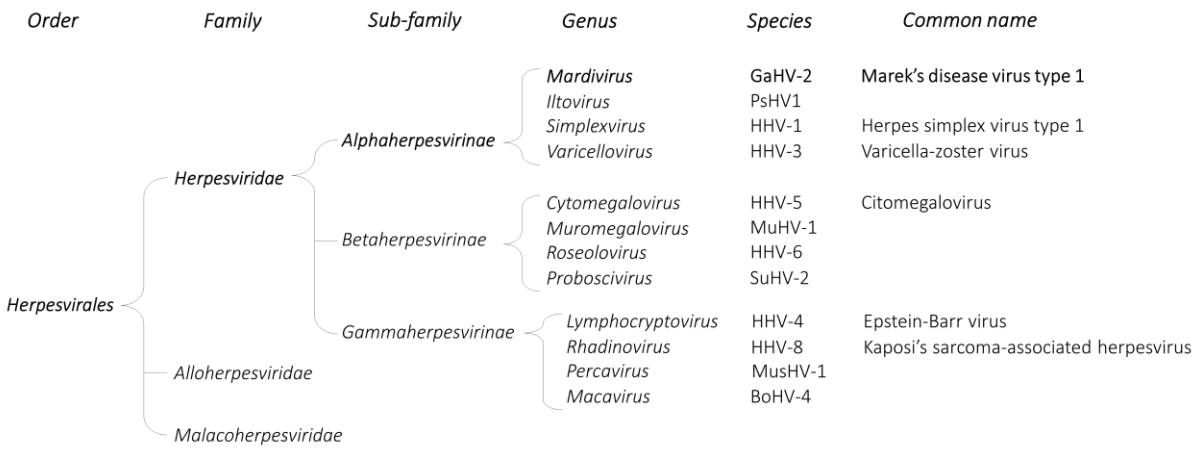

FIGURE 1.15. TAXONOMICAL CLASSIFICATION OF *HERPESVIRALES* ORDER.

Examples of viral species for each genus are given. GaHV-2 - Gallid herpes virus type 2; PsHV-1 - Psittacid herpesvirus 1; HHV - human herpesvirus; MuHV-1 - Murid herpesvirus 1; SuHV-1 - Suid herpesvirus 2; MusHV-1 - Mustelid herpesvirus 1; BoHV-4 - Bovine herpesvirus 4 (adapted from [240]). Gallid herpesvirus type 2 is followed throughout the taxonomy tree and represented in bold.

Unlike most of the members of this subfamily, GaHV-2 latently infects T-CD4$^+$ lymphocytes and can trigger tumorigenesis in avian species, making it closely related to the members of the *Gammaherpesvirinae*. One of the main attributes of *Alphaherpesvirinae* is that the replicative infection does not lead to cell lysis and that viral intra-organism infection is exclusively mediated through cell-to-cell spreading [235].

Contrary to *Alphaherpesvirinae, Betaherpesvirinae* is characterized by a narrow host range with a long replication cycle within the host. *Betaherpesvirinae* subfamily is divided into four genera designated *Cytomegalovirus*, *Muromegalovirus*, *Roseolovirus* and *Proboscivirus* (Figure 1.15). They can establish latency in lymphocytes, secretory glands and kidney cells in mammalian hosts [241].

Gammaherpesvirinae subfamily includes four genera, *Lymphocryptovirus*, *Rhadinovirus*, *Percavirus* (Perissodactyla and carnivore) and *Macavirus* (Malignant catarrhal fever). Their cellular targets in mammalian hosts for latent infection are monocytes, dendritic cells and usually B- or T-lymphocytes (Figure 1.15). Under specific circumstances, they are also able to transform latently infected cells and induce lymphoproliferative diseases and other non-lymphoid cancers in infected hosts [237].

Additionally, *Herpesviruses* are classified according to their genomic arrangement into six groups from A to F (Figure 1.16) [242].

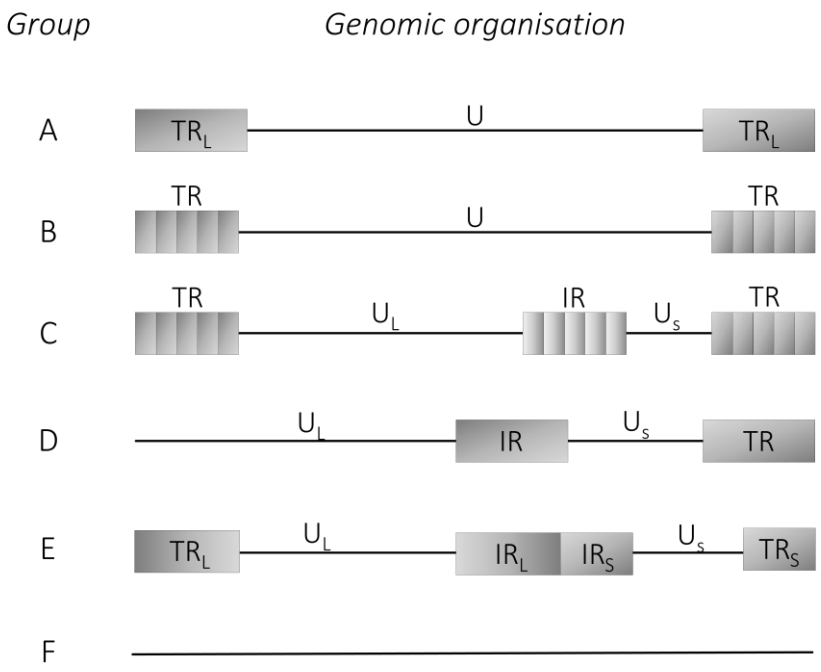

FIGURE 1.16. SIX CLASSES OF HERPESVIRAL GENOMIC ORGANISATION.

The large genomes of *Herpesviridae* family are divided into six different classes of organisation. Class **A** consists of a unique region sequence (U) that is flanked by relatively long terminal repeats (TR_L) and is represented in *Betaherpesvirinae* (HHV-6) [243]. For class **B**, represented in *Gammaherpesvirinae*, the terminal repeats consist of multiple tandem repeated sequences (TRs), with up to 30% of the genome belonging to the repeated regions [244]. Class **C** presents a modified class B arrangement, bearing internal sets of the direct repeats (IRs) unrelated to the terminal repeats and is common to Epstein-Barr virus genome [245]. Class **D** genomes include unique long (U_L) and unique short region (U_S) that are flanked by inverted internal (IR), and terminal (TR) repeats, characteristic for *Alphaherpesvirinae* [246]. The **E** group, represented by Gallid herpesvirus type 2, is structured of two unique regions unique long (U_L) and unique short (U_S) surrounded by inverted repeats. U_L and U_S regions are surrounded by the long terminal (TR_L) and the long internal (IR_L) repeat and short internal (IR_s) and short terminal (TR_s) short repeat, respectively [247]. Finally, for class **F**, no repeats were described and is the most common genomic organisation for *Betaherpesvirinae* [248] (adapted from [242]).

As mention throughout the previous section, a major characteristic of herpesviruses following the productive infection is the establishment of the latency, a phase where the virus remains dormant within the host cells, and the viral replication is suppressed. All along latency, herpesviruses are able to undergo reactivation that ultimately results in a new production of viral particles [249].

1.3.1.2. Herpesviral replication cycle

Despite limited nucleotide or amino acid homology and deduced proteins between the three *Herpesvirales* families, their characteristic virion morphology suggests that the underlying mechanisms of virion formation are comparable [250].

One of the best-studied human herpesviruses is the Herpes simplex virus type 1 (HHV-1) and represents the reference model for studying the herpesvirus life cycle. Herpesvirus infection of a host cell starts with the interaction of viral envelope glycoproteins (gC and gB) with the receptors present at the cellular plasma membrane (Figure 1.17) [251]. The entry then occurs by fusion of the virion envelope with the plasma membrane. The fusion is enhanced with the interaction of glycoprotein gD, which interacts with cellular receptors (CRs), such as tumour necrosis factor superfamily and cell adhesion molecules of the immunoglobulin superfamily [252]. gD-CRs interaction induces gD conformational changes, resulting in interplay with other glycoproteins that facilitate virus entry by mediating fusion of the envelope to the cellular membrane and the delivery of the viral capsid into the cytoplasm (Figure 1.17.a) [253].

Once inside, the nucleocapsid travels along microtubules to the nuclear pore where viral DNA is transported into the nucleus and circularised [254,255]. DNA-to-nucleus transport is facilitated with viral genes, such as in case of HHV-1 the inner tegument protein VP1-2, that displays nuclear localization signal and is responsible for the delivery of the viral capsid to the nucleus [256]. In addition, at the entry, a part of the tegument dissociates from the nucleocapsid, with the indication that tegument proteins may modulate host cell to create a beneficial environment for viral replication (Figure 1.17.b) [257]. For example, the UL41 tegument protein of *Alphaherpesviruses* was implicated in virally induced host cell shut-off by the degradation of host mRNAs [258].

Circularised DNA triggers the initiation of viral genes transcription that occurs in three-phasic cascade-like fashion, where the viral mRNA is synthesized by the host cell using the cellular RNA polymerase II (Figure 1.17.c) [259]. The first, immediate-early (IE) phase of transcription is mediated by the regulatory VP16 tegument protein and allows the transcription of IE-genes, such as ICP4 and ICP27, which are essential for inhibition the cellular defence against the virus [259,260]. It was demonstrated that VP16 is able to regulate methylation and demethylation of histone H3 that binds with viral DNA promoters during infection (Figure 1.17.d).

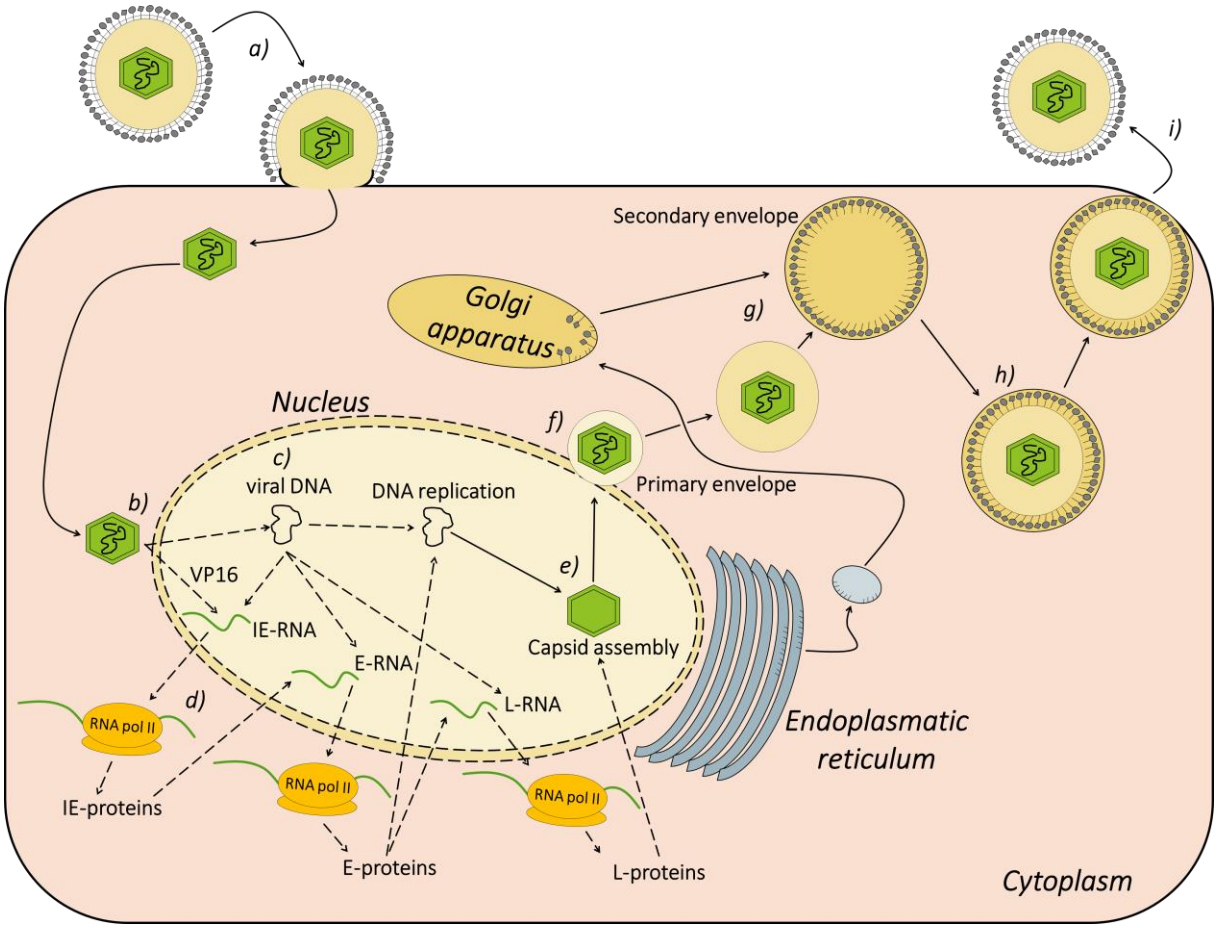

FIGURE 1.17. SCHEMATIC REPRESENTATION OF THE HERPESVIRAL REPLICATION CYCLE.

a) The viral replication cycle starts with the viral attachment to the specific receptors on the cell surface. After the fusion, the viral capsid is released into the cytoplasm of the host cell. **b)** The capsids are transported to the nucleus *via* interaction with microtubules, where the viral genome is delivered into the nucleus through the nuclear pore, where it becomes circularized. **c)** In the nucleus, transcription of viral genes and genome replication occurs. The transcription of viral genes occurs in three sequential phases driven by the cellular RNA polymerase II. **d)** Initiated with the aid of tegument VP16 protein, the first phase marks the transcription of immediate-early (IE) genes. IE proteins act as transactivators of transcription by stimulating the transcription of the early (E) genes that are essential for DNA replication, and late (L) genes necessary for the synthesis of structural proteins, such as the envelope glycoproteins and the capsid proteins. **e)** Concatemeric replicated viral genomes are cleaved during encapsidation into assembled capsids, which leave the nucleus by budding at the internal nuclear membrane **(f)**. **g)** Following the tegumentation of newly formed capsid and synthesis of the secondary envelope containing viral glycoproteins by trans-Golgi network, final maturation results in an enveloped virion within a cellular vesicle **(h)**. **i)** Finally, following transport to the cell surface, vesicle and plasma membranes fuse, releasing a mature, enveloped virion from the cell (adapted from [254]).

VP16 triggers a cascade of viral gene expression by directly or indirectly activating the viral IE-gene promoters and removing bound histone H3, that is most likely the result of the cellular response to foreign DNA in order to inactivate it [261].

The primary function of the IE-gene-encoded proteins is the activation of early (E) gene expression. Proteins and enzymes encoded by the E-genes are involved in viral genome replication, regulation of nucleotide metabolism *via* thymidine kinase, suppression of IE-genes and activation of late (L) genes [259]. L-genes encode mainly structural components of the virion, including capsid proteins, which are translated in the cytoplasm and then imported into the nucleus where capsid assembly occurs. After replication, concatemeric viral DNA is cleaved to unit length during encapsidation with the preformed capsids (Figure 1.17.e) [262]. The maturation of herpesvirions occurs in the cytosol after nucleocapsid was translocated through the nuclear membrane. Both nuclear egress and final maturation involve budding processes, which occur in different subcellular compartments and involve different viral proteins (Figure 1.17.f) [263].

First, the mature capsid buds into the inner nuclear membrane, producing the primary envelope. Non-enveloped virions, localized between the inner and the outer nuclear membrane, acquire primary envelope during budding through the inner nuclear membrane. The primary membrane is lost by its fusion with the outer nuclear membrane, resulting in the release of the naked capsid into the cytoplasm (Figure 1.17.g) [254]. Furthermore, in the cytoplasm, the naked capsid undergoes the tegumentation process that follows an intricate network of protein-protein interaction [263]. The final virion maturation includes re-enveloping with the secondary envelope that buds from Trans-Golgi-network (Figure 1.17.h). The mature enveloped virion is eventually secreted by exocytosis from the infected cell (Figure 1.17.i) [254].

1.3.1.3. Cellular responses to viral infection

An active viral replication will result in various host reply mechanisms, involving the immune system or internal signalling in the cell, leading to the death of the infected cells [264]. Viral infection induces a wide variety of defence mechanisms in the host, including innate and adaptive immune responses. The innate immune defences are initiated *via* pathogen recognition receptors of the Toll-like receptor (TLR) family or a family of DExD/H box RNA helicase [265]. These cellular responses promote the expression of type I interferons (IFN-α/β) and a variety of IFN-stimulated genes and inflammatory cytokines [266]. Different TLR molecules recognize specific viral products, including single- (TLR3) and double-stranded RNA (TLR7/8) or double-stranded DNA (TLR9) [267]. IFNs-α/β act by binding to the IFN receptor that results in the transcription of numerous IFN-stimulated genes. Additionally, INFs activate natural killer (NK) cells and induce other cytokines, such as interleukin 2 (IL-12), that promote NK responses. Several cytokines and chemokines induced by virus infection also play a role in defence, including cytokines TNF-α, IFN-γ, IL-6 and chemokines such as MIP-1α. Inflammatory chemokines may also play an important role in innate antiviral defence by orchestrating macrophage, neutrophil and NK responses at the site of infection [268]. Innate immunity generally serves to slow down, rather than stop the viral infection, allowing the adaptive immune response to begin. The two major pathways of adaptive immunity, B-lymphocytes and T-lymphocytes mediated responses are directed at different targets. Adaptive immunity activation is closely dependent upon early innate mechanisms that activate antigen-presenting cells (APC). APC and lymphocytes are accumulated in the lymphoid tissues by chemokine and cytokine signals [269]. B-cell activation occurs following antigen encounter in the B-cell follicles in the spleen or lymph nodes [270]. Antibody binding to epitopes expressed by native proteins at the surface of free virions usually blocks viral attachment or penetration of target cells. The antibody involved response to viral infection in humans is predominantly secretory immunoglobulin A (IgA). Like B-cell responses, T-cell responses to viral infections also begin within the lymphoid tissues. T-cell immunity against a particular virus commonly involves both $CD4^+$ and $CD8^+$ T-cell subsets, which recognize peptides derived from viral antigens bound to surface major histocompatibility complex (MHC). $CD4^+$ T-cells participate in antiviral immunity in several ways. First, the $CD4^+$ subset acts as helper cells for the induction of both antiviral antibodies and $CD8^+$ T-cell responses to most virus antigens [271]. $CD4^+$ T-cells also function as antiviral effector cells and generate stable memory cell populations similar to those of $CD8^+$ T-cells [272]. However, effector $CD4^+$ T-cells act by synthesizing and releasing numerous cytokines following their reaction with the antigen. The T-cell type most often involved in antiviral

defence are designated T-helper 1 (Th1) cells. Th1 cells primarily produce IFN-γ, LTα, TNF-α, and IL-2 to help the inflammatory responses and antiviral defence [273]. Thus, in order to avoid host cell immune responses herpesviruses enter latent phase.

In addition, for promyelocytic leukaemia (PML) nuclear bodies (NBs) it was shown to possess antiviral activity. It was based on the observation that PML protein is known to contribute to an IFN-induced antiviral state in HSV-1 and influenza infections [274]. However, the precise molecular mechanisms by which PML protein stimulates IFN pathways are still not well understood. PML protein is thought to associate with transcription factors, such as STAT1, which are involved in regulating IFN and IFN-stimulated gene expression [275]. Furthermore, PML NB-associated transcriptional repressors, such as Sp100 and DAXX, have been shown to restrict viral gene expression in HSV-1, HPV and EBV infections [276–278]. To overcome the antiviral environment induced by PML NBs, viruses have evolved effector proteins to serve as a defence against PML NBs [279]. These viral proteins are products of immediate early genes, due to their critical role in the establishment of infection. The best-studied interaction of a viral protein with PML NBs is HSV-1 immediate-early protein ICP0. ICP0 localizes to PML NBs at early time points of viral infection and was shown to disrupt and reorganize PML NBs, mainly by mediating the degradation of specific PML isoforms [280]. ICP0 is also thought to be involved in the degradation of Sp100, as knockdown of Sp100 results in increased expression of ICP0-null mutant HSV-1 [281].

1.3.1.4. Herpesviral latency

The hallmark of latency is the maintenance of the virus in the host cells, highlighted with the absence of progeny virus production and expression of only a small subset of viral genes. During latency, viral DNA is copied by cellular DNA polymerases, together with the chromosomes during mitosis. In contrasts, lytic replication relies on the viral DNA polymerase, reflecting a viral takeover of the cell. During latency, the virus depends on the host's epigenetic mechanisms for the silencing of viral genes, such as histone modifications and DNA methylation [282]. In most cases, viral latency is established in specialized host cell types and microenvironments that provide essential conditions. For example, the predominant latent forms of EBV are found in $CD19^+$ memory B-cells and HPV in basal epithelial cells [283].

Latency is the principal characteristic of the herpesvirus life cycle, and they evolved numerous mechanisms to regulate latent switch and keep the integrity of their genome. For example, in order to establish latency, the viral genome is circularized in the form of an episomal DNA that is fully chromatinized like the cellular DNA. Consequently, the transcriptional activity of the viral latent genome form can be regulated by histone modifications using cellular mechanisms [284].

For example, viruses like EBV and KSHV recruit the host cell replication machinery to the episome and maintain a stable episome copy number by segregating newly replicated genomes equally to daughter cells after each cell division [283]. Moreover, the EBV latent proteins allow the successful persistence in the infected cells. EBNA-LP and EBNA-2 are responsible for the transcriptional regulation of other latency proteins. EBNA-1 plays an important role in binding the viral genome to cellular chromosomes and participating in viral genome segregation and latent replication, as well as acting like a transcriptional activator [285]. Additionally, LMP-1 belongs to the tumour necrosis factor superfamily of proteins and mimics the $CD4^+$ T-cell signals and promotes B-cell survival by constitutively upregulating NF-$\varkappa$B signalling [286]. In the case of KSHV, LANA origin binding protein is responsible for latent replication of the viral genome as well as tethering the genome to cellular chromosomes [284]. Additionally, LANA also inhibits the action of p53 tumour suppressor, promoting the survival of infected cells and inhibits the function of Rb [287,288].

Furthermore, several herpesviruses, including GaHV-2 and HHV-6, establish latent infections in $CD4^+$ T-cells with viral genomes integrated into host chromosomes [289]. The integration repeatedly occurs in telomeric repeats by homologous recombination [290]. In addition, some herpesviruses belonging to *Alphaherpesvirinae* (GaHV-2) and *Gammaherpesvirinae* (EBV and KSHV) subfamilies are associated with tumorigenesis during latency in their natural hosts [291]. Finally, herpesviruses from all the three subfamilies can periodically reactivate, causing symptomatic recurrent infection or are asymptomatically shed to new hosts. Viral reactivation and second productive infection can be triggered by a broad range of physiological and environmental factors [284]. Several external factors and stimuli that trigger viral reactivation in humans are known. For example, in *Alphaherpesviruses*, latent HSV-1 in neurons of various ganglia can be reactivated by local injury to tissues innervated with latently infected neurons, systemic physical/emotional stress, fever and microbial co-infection and hormonal imbalance [292]. Reactivation occurring in *Betaherpesviruses*, for example, CMV, is observed commonly in the setting of immunosuppression, mainly where pro-inflammatory cytokines are present and stimulate cellular differentiation to macrophages or dendritic cells [283,293,294]. Factors that induce

reactivation of *Gammaherpesviruses*, for example, EBV, are the differentiation of B-cells into plasma cells through antigen stimulation of the B-cell receptor. Additionally, host cell stress, induced for example, by chemotherapy or body irradiation, can reactivate latent EBV. In cell culture, EBV reactivation can also be triggered by sodium butyrate [295].

However, in GaHV-2 context, despite the ability to mimic some aspects of reactivation using different chemical stimuli, the *in vivo* mechanisms mediating changes in virus gene expression and reactivation during GaHV-2 infection are still unknown [296].

1.3.2. Gallid herpesvirus type 2 (GaHV-2)

1.3.2.1. GaHV-2 genome structure

Due to the biological properties, Gallid herpesvirus 2 (GaHV-2) also known as Marek's disease virus (MDV), was long thought to be closely related to EBV, mainly because of its ability to induce T-cell lymphoma and slow growth in cell cultures [235]. However, based on genomic organization analysis, GaHV-2 was reclassified as an Alphaherpesvirus, genetically more closely related to HSV-1 and VZV (Figure 1.18). GaHV-2 belongs to the genus Mardivirus, into which two other closely related but distinct species were grouped, represented by Gallid herpesvirus type 3 (GaHV-3) and Meleagrid herpesvirus type 1 (MeHV-1). Only GaHV-2 causes clinical disease in chickens with the other two species non-pathogenic [270].

The GaHV-2 genome belongs to a class E genome with a size of 175 to 180 kbp. GaHV-2 genome consists of a unique long (U_L) and a unique short (U_S) segments, bracketed by inverted repeats known as terminal and internal repeats long (TR_L and IR_L) and terminal and internal repeats short (TR_S and IR_S) (Figure 1.18.a) [235]. GaHV-2 genes, similar to those of other herpesviruses, also belong to three kinetic classes of immediate-early, early and late genes based on the requirements for viral protein synthesis and DNA replication [297].

In addition, GaHV-2 harbours telomeric repeats (TMR) that are located within both ends of the linear genome as well as in IR_L-IR_S junction. Each junction contains two telomeric repeat sequences, multiple telomeric repeats (mTMR), with a variable number of repeats, and short telomeric repeats (sTMR), with a fixed number of six repeats (Figure 1.18.b). Both mTMR and sTMR regions are located adjacent to the conserved packaging signals (Pac-1 and Pac-2) and the genome cleavage site (direct repeat 1, DR-1), both of which are essential for virus replication. It was demonstrated that both mTMR and sTMR play an important role in GaHV-2 integration and that this process is crucial for pathogenesis and tumour formation [298].

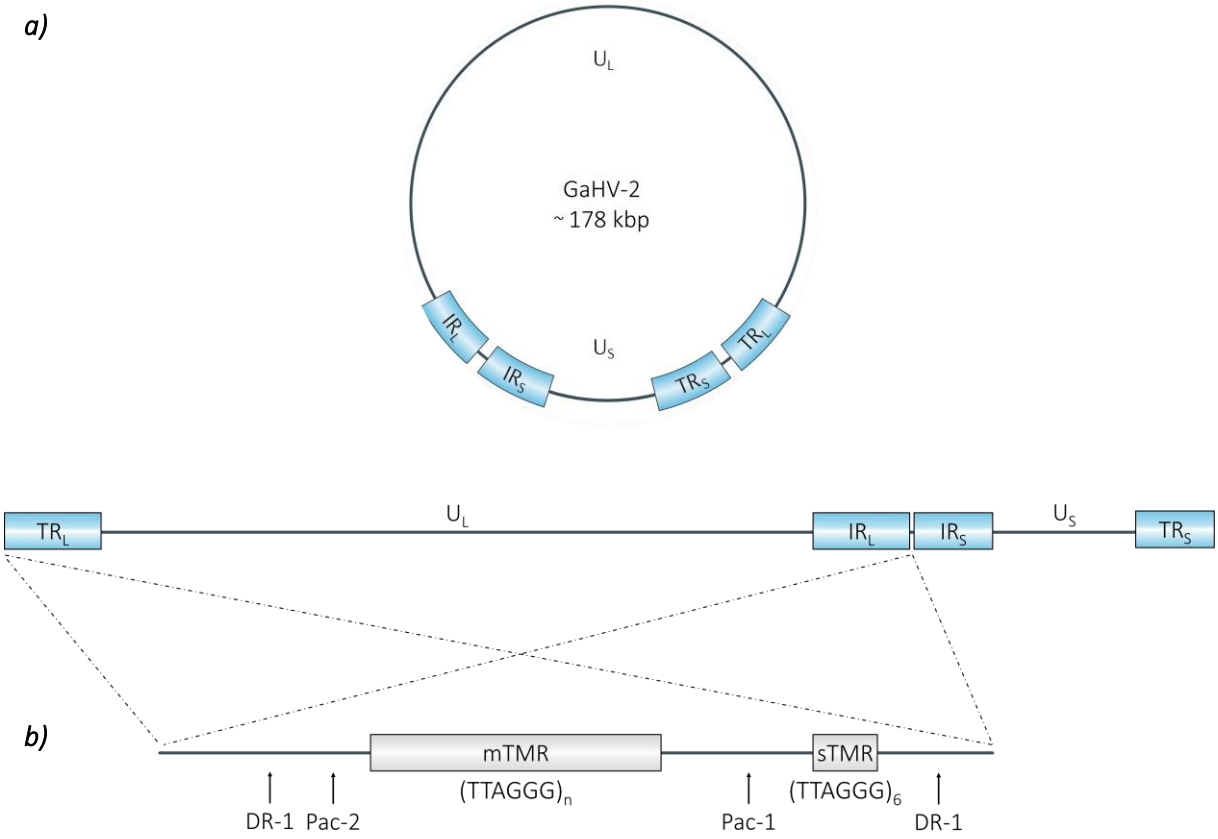

FIGURE 1.18. SCHEMATIC REPRESENTATION OF GAHV-2 GENOME ORGANISATION.

a) The circular form represented during viral replication and linear form of GaHV-2 genome consists of unique long U_L and unique short U_S regions flanked by the long terminal (TR_L) and internal (IR_L) repeats, and terminal (TR_S) and internal short repeats (IR_S), respectively. **b)** Telomeric repeat sequences (TMRs) which are located at the genome termini and the junction between IR_L and IR**s** are essential for viral integration in the host's telomeres (modified from [235,298]).

1.3.2.2. Marek's disease induced by GaHV-2

Gallid herpesvirus 2 (GaHV-2), the etiologic agent for Marek's disease (MD) that targets avian species (*Gallus gallus domesticus*) where it establishes chronic infection. MD is a multifaceted disease, characterized with immunosuppression, neurological disorders and neoplastic transformation of $CD4^+$ T-lymphocytes, localised around peripheral nerves and visceral organs of the host [299]. GaHV-2 is extremely infectious and routinely causes more than 90% morbidity and mortality in susceptible, unvaccinated animal populations.

MD is a highly contagious lymphoproliferative disease of domestic chickens, first described in 1907 by József Marek as fowl paralysis and generalized polyneuritis [300]. However, it was until the 1960s when it was discovered that the virus is a causative agent of the MD [301]. Today, MD has the highest economic impact in modern poultry production worldwide, partially due to lower feed conversion, weight loss and decreases in egg production [302,303]. Furthermore, a secondary indirect economic impact is a result of increasing the need for farming hygiene, vaccinations and inducing immunosuppression, making chicken more susceptible to secondary infections [304]. The introduction of vaccines to control MD in the early 1970s significantly reduced mortality and was essential for the sustainability of the modern poultry industry. Although MD vaccines were very successful at protecting poultry against tumours and mortality, they do not provide sterilizing immunity and vaccinate chickens still support viral replication and shedding.

Since the first implementation of the vaccines, their wide use has contributed to the evolution of GaHV-2 field viruses towards greater virulence (Figure 1.19) [305]. Up to date, GaHV-2 group contains all the oncogenic viruses and includes four pathotypes: moderate (m), virulent (v), very virulent (vv), very virulent + (vv+) (Table 1.5) [235].

TABLE 1.5. ONCOGENICITY OF DIFFERENT PATHOTYPES IN *MARDIVIRUS* GENUS.

Genotype	Pathotype	Oncogenicity	Strains
GaHV-2	very virulent + (vv+)	+++	RK-1, 584A, 648A
	very virulent (vv)	++	RB-1B, Md-5, Md-11
	virulent (v)	+	GA, HPRS-16, JM
	moderate (m)	-	Rispens, CU-2, HPRS-17
GaHV-3	moderate (m)	-	SB-1, HPRS-24, HN-1
MeHV-1	moderate (m)	-	FC-126, WTHV, HPRS-26

In the late 1960s, introduction of the MeHV-1 (strain FC-126) vaccine led to a sharp decrease in condemnation of chicken carcasses, with noticeable reduction and delayed tumour development [306]. For a few years, MD was controlled in the field, but since it did not induce sterile immunity, by the late 1970s, new GaHV-2 very virulent (vv) strains began to break through the vaccine protection (Figure 1.19).

The emergence of vv strains in the 1980s was followed by the introduction of bivalent vaccines that consisted of the GaHV-3 (strain SB-1) and MeHV-1 (strain FC-126) that was able to protect against vv viruses [307]. However, by the early 1990s, hypervirulent (vv+) GaHV-2 strains began to emerge and overcome protection provided by bivalent vaccines. In response to a new increase in virulence, trivalent vaccines featuring an attenuated GaHV-2 strain (CVI988 Rispens), used in Europe since the early 1970s for layer chickens, together with GaHV-3 (strain SB-1) and MeHV-1 (strain FC-126) were implemented in animal-dense areas and high-risk farms [308].

In the last decade, MD was controlled by vaccination of approximately 4.2 billion layers, 0.5 million breeders and 17 billion broilers worldwide annually [309]. For example, the high efficacy of MD vaccines was demonstrated by a decrease of losses from the condemnation of young broiler chickens in the United States from 1.5% in 1970 to 0.003% in 2006. Control of MD by vaccination was a crucial step for the viability of the modern poultry industry. However, it has a dark side, with the evolution of more virulent strains that will break the current vaccination protocols and with no improved or more efficacious vaccine available [305]. Moreover, increasing virulence of GaHV-2 appears not to be due to an increase in the ability of the virus to cause tumours but is the result of the virus evolving to replicate faster and more efficiently in the chicken [310].

Since there are no vaccines superior in protection than today's gold standard CVI988, the urgent priority is to develop other strategies to improve the control of MD [305]. For example, the development and the use of DNA vaccines, in the form of infectious BAC clones, would allow the injection of a well-characterized genome, which would result in the *in vivo* reconstitution of the modified virus with the desired protection factor. BAC clones derived from cell cultures of attenuated strains of a GaHV-2 vv+ (584A) and CVI988 were tested as potential MD vaccines, with a partial level of protection compared to CVI988 [311,312]. These studies have demonstrated a potential role of DNA vaccines in future MD control strategies and a more manageable model of production, storage and administration of vaccines.

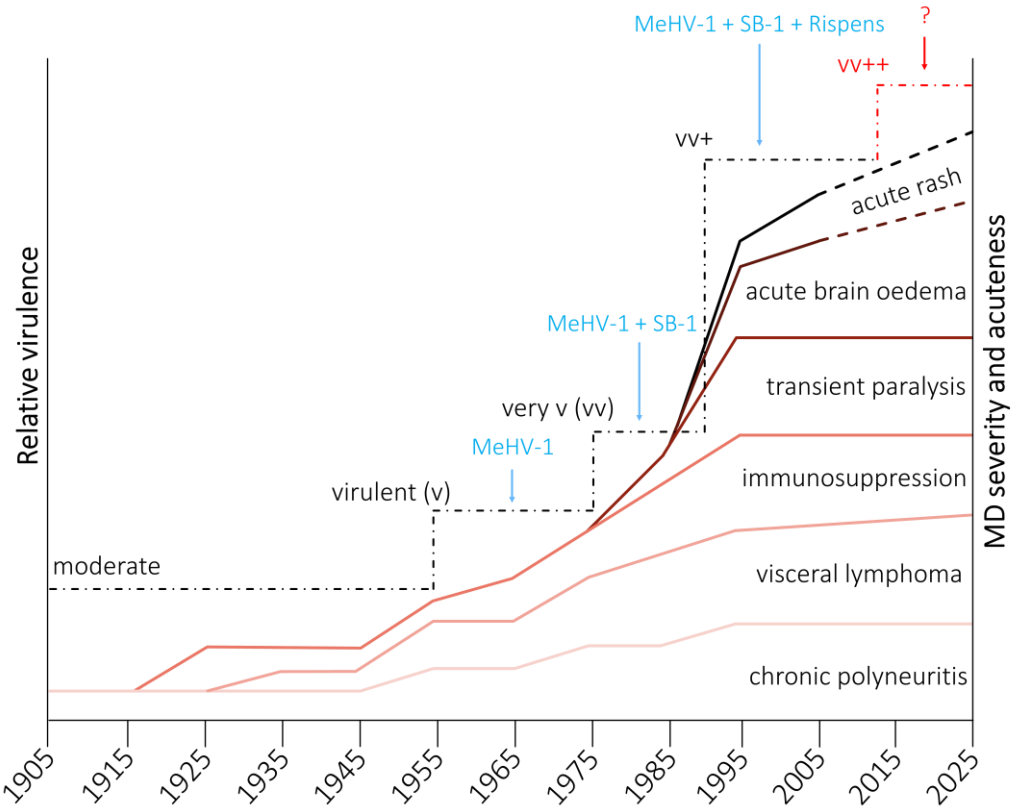

FIGURE 1.19. THE FAST-PACED EVOLUTION OF GALLID HERPESVIRUS TYPE 2 UNDER VACCINATION PRESSURE.

The clinical picture of Marek's disease (MD) has changed dramatically since its first recognition in the early 20th century, showing increasing virulence of Gallid herpes virus type 2 (GaHV-2) over time. The chronic polyneuritis was prevalent until 1925, followed by visceral lymphoma from 1925 to 1950. More aggressive and faster-developing tumours were observed from 1950 onwards. During the past three decades, GaHV-2 virulence has continued to increase, and the clinical picture of the disease has changed, now including severe brain oedema and acute deaths, even in fully vaccinated animals. GaHV-2 strains range from moderately virulent (m) to virulent (v), very virulent (vv) and very virulent plus (vv+). Most v and vv strains induce transient paralysis in most chicken lines, and vv+ strains, usually isolated from vaccine breaks, cause massive brain lesions. It was suggested that there is a close relationship between the evolution of strains towards increased virulence and their potential to overcome vaccine protection. After each introduction of a new vaccine (in blue), an increase in virulence was observed (black dotted line). Introduction of Meleagrid herpesvirus type 1 (MeHV-1) vaccine in the 1970s, resulted in the emergence of v strains, the use of bivalent vaccines containing MeHV-1 and the non-oncogenic GaHV-3 SB-1 strain in the 1980s and 1990s, resulted in vv+ breakout. Today, the use of trivalent MeHV-1, SB-1 and attenuated GaHV-2 Rispens vaccine will ultimately lead to the evolution of new very virulent ++ (vv++) strains (red dotted line) with new clinical pictures (red question mark) (adapted from [235,308]).

1.3.2.3. GaHV-2 pathogenesis

The proposed model of GaHV-2 life cycle (Figure 1.20) describes infection starting with the inhalation of dust particles containing the virus. Initial virus replication occurs in macrophages and B-cells in the lung of infected animals between 2 to 7 days post-infection (dpi) [313]. Macrophages act as the transporters of the virus to lymphatic tissues, the bursa of Fabricius and the spleen, spreading the infection to other immune cells [314]. Additionally, GaHV-2 secretes a viral chemokine vIL-8 (vCXCL13) that recruits B-lymphocytes and a subset of $CD4^+$ T-lymphocytes, for which was shown to be crucial for the establishment of infection [315]. It was shown that B-lymphocytes represent the majority of infected cells and appear to be the most susceptible cells for productive replication. However, recently was demonstrated that B-lymphocytes are entirely dispensable for GaHV-2 pathogenesis and tumour formation [316].

Starting between 7 and 10 dpi, GaHV-2 establishes latency in target cells, mainly $CD4^+$ T-lymphocytes that result in systemic viral dissemination [317]. In latently infected cells, GaHV-2 genome is integrated into the telomeres of the host chromosomes, ensuring the maintenance of the virus genome. Telomeric repeat sequences at the ends of the viral genome facilitate telomere integration, which likely occurs by a homologous recombination pathway. Viral integration appears to enhance cellular transformation and tumour formation [290]. However, why and how $CD4^+$ T-lymphocytes are targeted and chosen for latency establishment and what are specific cell tropisms involved is still not known, and furthers studies are needed in order to answer these questions.

In the late productive phase, GaHV-2 reactivates from the subpopulation of latently infected T-cells, with viral replication occurring within epithelial cells in feather follicles epithelium (FFE). The mechanisms by which GaHV-2 infects skin and FFE are poorly understood. Since B- and T-lymphocytes are the major targets of GaHV-2, infected early on [313], these cells are probably primarily involved in FFE infection. However, this hypothesis has not been yet demonstrated; therefore, the involvement of macrophages and/or dendritic cells cannot be excluded. In addition, for most pathogenic strains, replication in FFE starts at 7 dpi, well before tumour development [318], indicating that it is probably not transformed cells that migrated into the skin, since there are no or very few transformed cells [319]. Numerous questions have to be answered in order to obtain the full picture of mechanisms involved in FFE infection, such as: Why is the virus mainly present in the FFE and not in the epidermis of the skin? How does the virus infect the upper epidermis layers: directly, or indirectly by infecting the basal layer first and

then replicating when basal layer differentiate? How does the virus cross the basal membrane? Up to this date, only speculative hypotheses were proposed. For example, infected cells lymphocytes infiltrate the skin epithelium to transmit the virus to the upper epithelial cells of the epidermis, resulting in the viral propagation infecting neighbouring cells. Furthermore, lymphocytes could infiltrate the dermis or the dermal papilla, infecting neighbouring cells such as fibroblasts or melanocyte precursors, which in turn transmit the virus to the basal epithelial cells. Alternatively, lymphocytes could directly infect the follicle stem cells located in the bulge of the feather follicle, spreading the infection to transient amplifying cells that are involved in the repair of the follicle wall and the feather during feather regeneration [319].

Shedding occurs from FFE that release infectious particles encased in keratin or released by exocytosis. Released particles remain infectious for 16 to 28 weeks, ensuring the horizontal spread of the virus [320]. Finally, the transformation phase, occurring around 28 dpi within latently infected cells, leads to viral spread in peripheral nerves and visceral organs, causing T-cell lymphoma and paralysis. The rapid formation of T-cell lymphomas is the dominant characteristic of GaHV-2 infections, where GaHV-2-induced tumours consist mostly of transformed and clonally expanded $CD4^+$ T-lymphocytes [321].

During all the steps of the viral replication cycle, transcriptional modification and epigenetic changes, together with post-transcriptional and posttranslational modifications, regulate expression of cellular and viral genes, which allow GaHV-2 to switch between the productive and latent phases and to induce transformation of infected cells, resulting in visceral lymphomas.

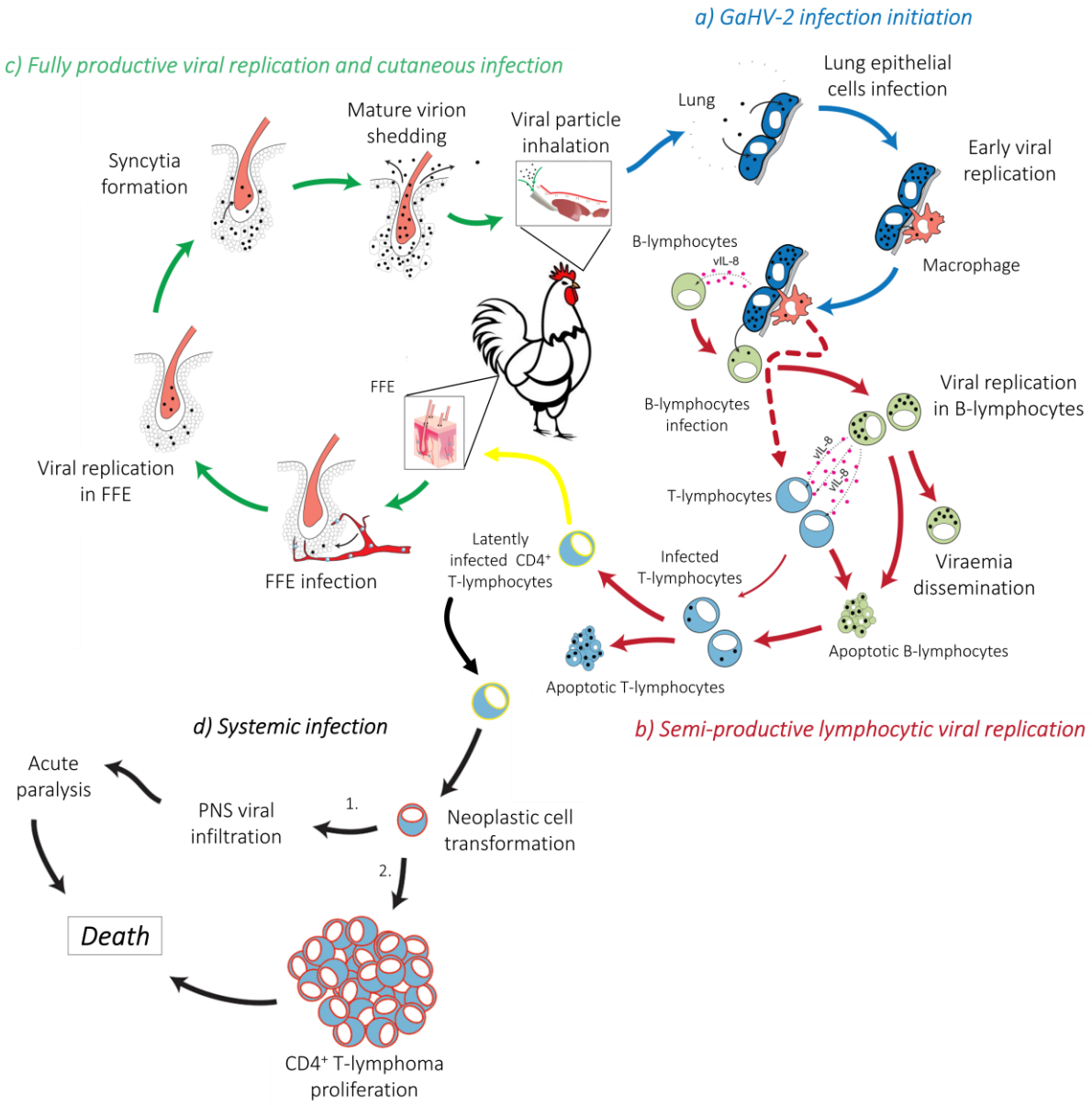

FIGURE 1.20. PROPOSED MODEL OF GALLID HERPESVIRUS TYPE 2 INFECTIOUS LIFE CYCLE.

a) Primary infection (in blue) starts with virus particle breaking mucosal tolerance in the epithelial lung cells, resulting in inflammatory responses by macrophages. Local viral replication establishes infection in B-lymphocytes supported by viral interleukin-8 (vIL-8). **b)** In B-lymphocytes, semi-productive viral infection (in red) and disease progression are initiated. Infected B-lymphocytes secret vIL-8 that acts as a chemotactic factor and infect T-lymphocytes, resulting in disseminated viraemia and immunosuppression. It was demonstrated that B-lymphocytes are dispensable for pathogenesis and tumour formation, and the virus can readily replicate in T-lymphocytes (red dotted line). GaHV-2 genome integration in $CD4^+$ T-lymphocytes enables escape from host immune response and initiates latent viral infection. Early latently infected and activated $CD4^+$ T-lymphocytes migrate (yellow arrow) to cutaneous sites of replication, feather follicle epitheliums (FFE). **c)** Infection of FFEs enables fully productive viral replication (in green). Infection of FFEs results in the secretion of mature virions in skin dander and dust that act as the primary source of infectious materials. **d)** Latently-transformed $CD4^+$ T-lymphocytes proliferate and undergo neoplastic transformation due to transcriptional and trans-repression activity of viral oncogenic protein Meq. Latent neoplastically transformed

$CD4^+$ T-lymphocytes infiltrate and establish a viral reservoir in peripheral nerve system (PNS) (1), leading to neuropathy presented as transient or acute paralysis of legs, wings and weight loss. Reactivation from latency enables the second phase of replication where viral oncogenic protein Meq acts on T-lymphocytes signalling pathways causing uncontrolled cellular proliferation leading to disseminated lymphoma formation in visceral organs, peripheral and central nervous system, musculoskeletal systems, skin and eyes (2), eventually causing death (adapted from [299]).

1.3.2.4. GaHV-2 induced lymphomagenesis

The most critical and obvious outcome of GaHV-2 infection is the transformation of latently infected $CD4^+$ lymphocytes, followed by multifocal lymphoma formation [235]. Rapid onset of lymphomas in most of the susceptible animals strongly indicates a direct effect of GaHV-2-encoded genes. Despite the differences in the number of latent viral genes expressed among transformed cell lines, it is apparent that GaHV-2 gene expression in latent-transformed cells is restricted to the IR_L-TR_L regions of the viral genome [322]. The crucial genes highly expressed in these cells are the Meq (Marek's EcoRI-Q-encoded protein), GaHV-2-encoded CXC chemokine viral interleukin 8 (vIL-8), the latency-associated transcript (LAT), the viral telomerase RNA (vTR) subunit and the GaHV-2-encoded miRNAs [323].

Of the genes, only Meq was shown to have direct transformational abilities and fulfils several of the criteria consistent with being an oncogene, making it putative GaHV-2 oncoprotein. It was shown that Meq is consistently expressed in tumours and cell lines derived from tumours, and inhibition of its expression decreases the proliferation of these cell lines [324,325]. Moreover, Meq expression in cell lines induces proliferation and apoptosis resistance [325]. Finally, Meq deletion and mutations of particular binding motifs were shown to be sufficient to block tumorigenesis [326]. Meq is a 339-amino-acid protein that is expressed during both the productive and the latent/tumour phase of infection, and is a basic leucine zipper (bZIP) protein with characteristics similar to oncoproteins such as v-Jun [324]. bZIP structure located at the N-terminal domain, similar to that of the cellular transcription factors c-Jun/c-Fos [327], allows Meq to form homodimers or to dimerise with other proteins possessing a bZIP domain, such as c-Jun, for which it has a strong affinity (Figure 1.21.a) [328]. In addition to homo- and heterodimerization with proto-oncoproteins, Meq can bind to several factors that are involved in cell cycle control, including retinoblastoma protein (pRb), p53 and cyclin-dependent kinase 2 (CDK2), which can also explain the role of Meq in oncogenic transformation of T-lymphocytes [329]. The heterodimer Meq/Jun and the homodimer Meq/Meq bind to specific nucleotide sequences termed the Meq responsive elements I (MERE I) and II (MERE II), respectively (Figure 1.21.b).

MERE I sites are located within the viral promoters, such as ICP4, a transactivator of the replicative phase and glycoprotein B (gB) implicated in cell-to-cell spread of GaHV-2 in cultured cells. In addition, MERE I sites are found and the cellular promoters as well, including interleukin-2 and B-cell lymphoma 2 (Bcl-2) which is anti-apoptotic and facilitates cell survival (Figure 1.21.b) [328,330,331].

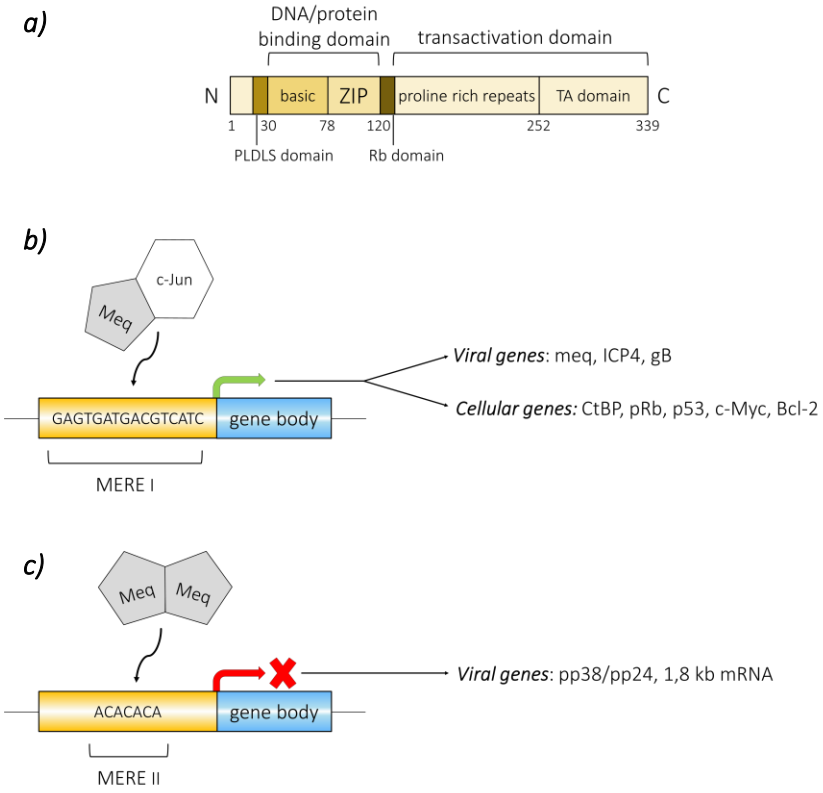

FIGURE 1.21. DOMAINS AND DIMERIZATION PARTNERS OF MEQ ONCOPROTEIN DURING GALLID HERPESVIRUS TYPE 2 INFECTION.

a) Meq basic leucine zipper domain (bZIP) has homology with the bZIP of the cellular transcription factors c-Jun and c-Fos. The transactivation (TA) proline-rich domain triggers either activation or repression of the promoter's activity. In addition, PLDLS and Rb binding domains allow Meq interaction with cellular genes such as C-terminal binding protein (CtBP) and retinoblastoma protein) (pRb), which can also contribute to oncogenic transformation of T-lymphocytes. **b)** Meq can form heterodimers (Meq/c-Jun) which bind to Meq response elements I (MERE I) located within viral (ICP4-infected cell protein 4, gB-glycoprotein B) and cellular (Bcl-2-B-cell lymphoma 2) promoters, inducing gene transcription. **c)** Meq can homodimerise (Meq/Meq) and bind to MERE II sites located within viral promoters, which results in repression of gene expression. (pp-phosphoprotein) (adapted from [289,327]).

Contrasting the effect of the Meq/c-Jun heterodimer binding to the MERE I site, the binding of the Meq/Meq homodimer to MERE II represses gene expression (Figure 1.21.c). A MERE II site was localized within bidirectional viral promoter controlling pp38/pp24 genes and 1,8 kb mRNA expression. It was demonstrated that pp38 is involved in a productive infection of B-lymphocytes in the spleen and maintained the viability of infected T-lymphocytes by limiting apoptosis [271] and 14 kDa protein, encoded by the 1.8 kb mRNA promotes the transition from

G1 to S phases of the cell cycle and enhances viral DNA replication [272]. By targeting their promoters, Meq prevents viral DNA replication due to their involvement in productive infection [328,330].

Furthermore, Meq-non-bZIP interactions are regulated *via* Pro-Leu-Asp-Leu-Ser (PLDLS) motif that is known to bind a cellular transcriptional co-repressor, the C-terminal binding protein (CtBP) that has an important role in the regulation of development and oncogenesis [326]. Interestingly, Meq shares CtBP-binding motif with EBV nuclear antigens EBNA-3A and EBNA-3C [332], both crucial for efficient EBV-transformation of human B-lymphocytes [333].

Although Meq has a clear role in oncogenesis, its transforming properties are significantly weaker comparing to retroviral oncoproteins such as v-Src [235]. Moreover, Meq is not sufficient for GaHV-2 transformation since it is also encoded and expressed by attenuated non-oncogenic viral strains [334]. Thus, other GaHV-2 gene products, that contribute to lymphoma development and progression or play a supportive role to Meq, must be involved in oncogenesis.

For example, lncRNAs localized in the IR_S and TR_S of the GaHV-2 genome, termed latency-associated transcripts (LATs) are mainly expressed during viral latency and lymphomagenesis, suggesting that they have a role in the maintenance of latency and/or cell transformation [335]. A cluster of microRNAs (cluster miR-M8-M10), identified in the first intron of the LATs gene [336] is overexpressed in GaHV-2 transformed and latently infected cells. The miR cluster overexpression is induced by binding of a tumour suppressor p53 to the LATs promoter, which consists of a series of 60 bp repeats, each containing a conserved functional p53 binding site [337]. Interestingly, it was shown that the number of 60 bp repeats may vary according to viral strain and its virulence [338].

Furthermore, GaHV-2 encodes for a viral homologue of telomerase RNA (TR), named vTR, which has extensive secondary structures that are similar to those of the EBV EBERs that are produced during latent EBV infection [217].

1.3.2.5. Virus-encoded telomerase RNA subunit

As previously discussed in section 1.2., oncogenesis does require not only a transformation event but also avoidance of cellular senescence and apoptosis. The enzyme involved in controlling cellular survival is telomerase that consists of two core components, a protein subunit TERT and its template RNA structure TR [308]. Furthermore, several oncogenic viruses, including the human papillomavirus HPV-16, hepatitis C virus, HHV-8 and EBV, are known to affect cellular telomerase activity [217]. In the context of virus-induced lymphomagenesis, as demonstrated in GaHV-2 homologous model, viral infection has two significant impacts on the telomerase complex. Firstly, it modifies TERT splicing, that partially regulates telomerase activation, thus playing a pivotal role in the upregulation of telomerase activity *in vivo* [339], and secondly, by an over-expressing viral homologue of chTR efficiently establishes tumour formation [340]. The detailed mechanism of how GaHV-2 modifies TERT splicing is not well known; however, a recent study indicated the possible involvement of nonsense-mediated mRNA decay (NMD) pathway. Amor et al., [339] demonstrated that the low basal telomerase activity in normal T-cell is controlled by in-frame non-functional isoforms (>99% isoforms detected) and was associated with low levels of constitutively spliced active chTERT transcripts (0.5%). This observation was consistent with a previous study demonstrating that telomerase-positive cell lines contain only a few molecules of functional hTERT mRNA [341]. Additionally, it was observed that telomerase activation during GaHV-2 infection was associated with an increase in constitutively spliced transcript levels accompanied by a switch in the profile of alternative transcripts from in-frame transcripts encoding non-functional isoforms to NMD-sensitive transcripts [339]. Furthermore, in the human context, it was demonstrated that the overexpression of the c-Myc oncogene inhibits NMD pathway and that this inhibition both stabilises and up-regulates multiple c-Myc targets, suggesting that the inhibition of NMD may play an important role in the dynamic regulation of genes by c-Myc [342]. However, further studies are needed in order to investigate this phenomenon in the context of GaHV-2 infection.

Vertebrate TR subunits consist of four main structural domains. First is the CR1 pseudoknot domain, which consists of the template sequence and the CR2 and the CR3 conserved regions. Second and third are the CR4/CR5 domain, essential for functional telomerase activity and the H/ACA box, respectively. Final domain is the CR7 domain, required for the 3-end processing, stability and nucleolar localization within cells [343–345].

Fascinatingly, two copies of viral non-coding RNA having structure and function homologous to a cellular TR were found to be encoded in TR_L and IR_L of GaHV-2 genome (Figure 1.22.a). Moreover, it was shown that the viral TR (vTR) is encoded by the genome of all GaHV-2 strains, regardless of their pathotype [346]. Further analysis demonstrated that the CR4/CR5 domains, conserved between vTR and chicken (chTR), are essential for telomerase activity. Furthermore, their secondary structures, consisting of the P6.1 stem and the L6.1 loop, are required for interaction with the TERT and essential for efficient telomerase activity. Additionally, the CR7 domain responsible for the 3-end processing, the maintenance and the TR stability within cells were conserved between vTR and other vertebrates (Figure 1.22.b) [217].

It was established that vTR shares 88% sequence homology with chTR, and it was likely acquired from the chicken genome during virus-host co-evolution. A recent study demonstrated that tumorigenesis of a virus lacking vTR could be restored by the insertion of chTR into the virus genome, highlighting that overexpression of a chTR can drive tumorigenesis and that the virus acquired the gene from its host [347].

Exact mechanisms of how GaHV-2 acquired host gene homologues, such as chTR or IL-8, is not known. One proposed hypothesis suggests involvement and co-infection of the host cells with retroviruses. Cell co-infection with GaHV-2 and avian retroviruses, such as reticuloendotheliosis virus (REV) or avian leukosis viruses (ALVs), could establish a base for retrotransposition events that result in the "shuffling" of the genetic material between the genomes of GaHV-2 and its host [348]. Retroviruses can easily integrate into GaHV-2 or any double-stranded DNA (dsDNA), cellular or viral. It was shown that inserts of avian retroviral sequences were mainly the long terminal repeats (LTRs), and were located at the junctions between the unique (long or short) GaHV-2 sequences and the terminal or internal repeated regions (TR_L and TR_S and IR_L and IR_S) [349]. Co-cultivation of GaHV-2 and REV in the same tissue culture resulted in the recombinant virus with altered *in vitro* replication and *in vivo* biological properties [350,351]. Moreover, two other studies reported that integration of retroviral LTR into GaHV-2 genome occurs not just because of co-cultivation of both viruses, but also as a result of culture maintenance and the presence of endogenous avian viruses in the host cells [352,353]. Further studies are needed in order to understand how GaHV-2 acquired homologues of host genes.

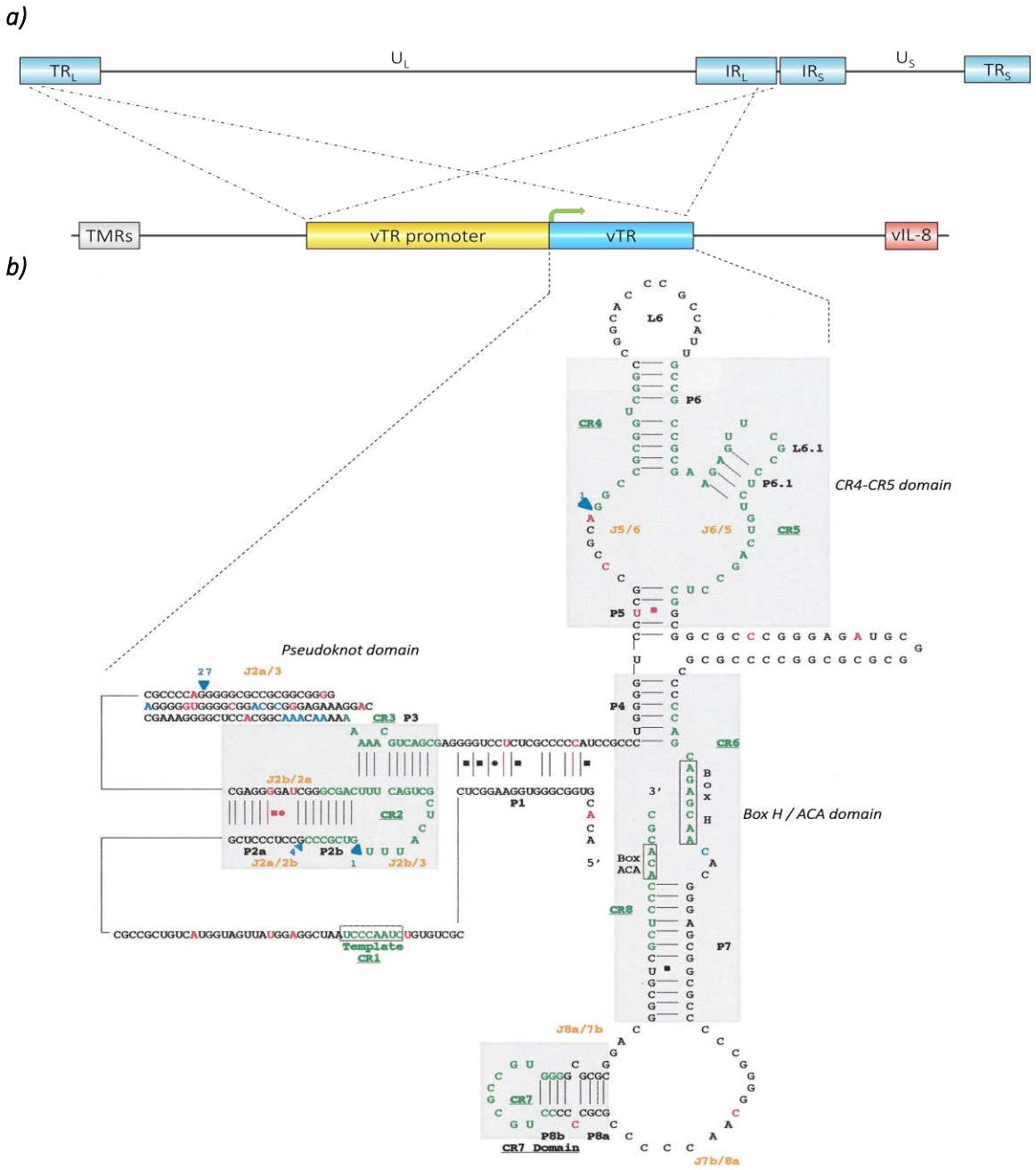

FIGURE 1.22. GENOMIC LOCATION AND PROPOSED SECONDARY STRUCTURE OF THE GALLID HERPESVIRUS TYPE 2 TELOMERASE SUBUNIT.

a) Two copies of vTR are located within in the TR_L and IR_L regions of the Gallid herpesvirus type 2 (GaHV-2) genome between the telomeric repeat sequences (TMRs) and the viral interleukin 8 (vIL-8) gene. **b)** Proposed secondary structure of GaHV-2 with four universal structural domains conserved (grey rectangles) in all vertebrate TRs. Paired regions (P) are numbered, and junction regions (J) between them are shown in orange. The H/ACA motifs are boxed, and conserved nucleotides are shown in green. Mutations in the vTR sequence and additional base pairing are shown in red. Additional nucleotides in the vTR sequence are shown in blue and deleted regions are indicated by blue arrows together with the nucleotide length of the deletion (adapted from [217]).

Furthermore, analyses have shown that vTR can reconstitute telomerase activity by interacting with chicken TERT (chTERT) more efficiently than chTR [354]. Previous studies done on healthy chicken examined the mRNA expression of both chTR and chTERT, as well a telomerase activity during growth and development of the chicken in order to better understand mechanisms which regulate telomerase activity [355,356]. Organ-specific profiles were established for brain, heart, liver, intestine, spleen and gonads from the embryos and adults, that showed transcript levels either similar or down-regulated relative to the early differentiation embryo stages (Table 1.6). Organs which were known to lose telomerase activity between the embryo and adult stages (brain, heart, liver) revealed down-regulation of chTR and either no change or an increase in chTERT transcripts. Whereas, organs which maintain high telomerase activity even in adults (intestine, spleen, gonad), generally exhibited up-regulation of transcripts for both components [356]. (Table 1.6). These results indicated that chTERT and chTR transcript levels correlate with telomerase activity profiles and suggest that chTR is the rate-limiting component in telomerase-negative tissues, however, to establish same profiles for other organs, tissues or cells (such as FFE or $CD4^+$) further studies are needed.

TABLE 1.6. TRANSCRIPT PROFILES OF TELOMERASE COMPONENTS AND TELOMERASE ACTIVITY DURING CHICKEN ONTOGENY

	Telomerase activity [355]		**Transcript profile** [356]	
Organ	Embryo	Adult	chTERT	chTR
Brain	+	-	↔	↓
Heart	+	-	↔	↓
Liver	+	-	↑	↓
Intestine	+	+	↑	↑
Gonad	+	+	↑	↓
Spleen	+	+	↑	↑

+ = increase in telomerase activity; - = reduction in telomerase activity

↔ = no change; ↑ = increase; ↓= decrease in transcript level between embryo and adult

Since all functional domains are conserved between chTR and vTR, the differences in telomerase activity could be explained by variances in their pseudoknot domains (PKD), such as four unpaired-nucleotide deletion and a mutation in the J2a-2b and J2b-2a junction regions of vTR PKD, which could stabilize the P2 stem-loop, enhancing telomerase activity (Figure 1.22.b) [217].

In addition, apparent differences in vTR and chTR promoters could explain the increase in telomerase activity due to higher levels of vTR expression [357]. Functional comparative analysis of vTR and chTR promoters indicated several *cis*-elements specifically involved in vTR transcriptional regulation, resulting in higher transcriptional efficiency of the viral promoter (Figure 1.23) [357]. It was established that the vTR promoter has additional AP-1 site, c-Myc transcription factor binding sites, namely E-box 1, E-box 2 and E-box 3, and EBS transcription factor binding site, which are absent in the chTR promoter, indicating that the vTR promoter is the homolog of the avian cellular TR promoter with certain responsive elements related to oncogenesis.

The vTR expression is explicitly regulated by an E-box 3, located two nucleotides downstream from the TSS (Figure 1.23.a) [357]. E-boxes (5'-CACGTG-3') serve as binding sites for proteins of the basic helix-loop-helix-Zip (bHLH-Zip) transcription factor superfamily, including the Myc/Mad/Max [358], that act as positive regulators of cell proliferation and death [359]. Additionally, for the E6 oncoprotein encoded by HPV-16 was shown to interact directly with c-Myc, increasing telomerase activity in infected tumour cells [360], demonstrating that the c-Myc oncoprotein is a common element of the pathways leading to higher levels of telomerase activity in GaHV-2- and HPV-16-transformed tumour cells.

Furthermore, it was shown that E-box 2 and the Ets binding site (EBS) are exceptionally functional in GaHV-2-transformed lymphoblastoid cell line (MSB-1), providing evidence that vTR is specifically regulated in transformed T-lymphocytes. However, it was demonstrated that E-box 1 was not active [357]. In addition, it was shown that vTR promoter contains AP-1 (MERE) binding sites that can be recognised by Meq oncoprotein, which heterodimerize with c-Jun and binds on AP-1 sites [328], however, it was revealed that Meq had no direct regulatory effect on the transcriptional activity of the vTR promoter *in vitro* [357]. These results demonstrate that cooperative action of factors that bind on EBS together with c-Myc oncoprotein are main elements inducing high levels of vTR expression and are required for the full expression of the oncogenic properties of vTR, especially its specific overexpression in lymphoblastoid cells during the latent period and the transcriptional regulation [357].

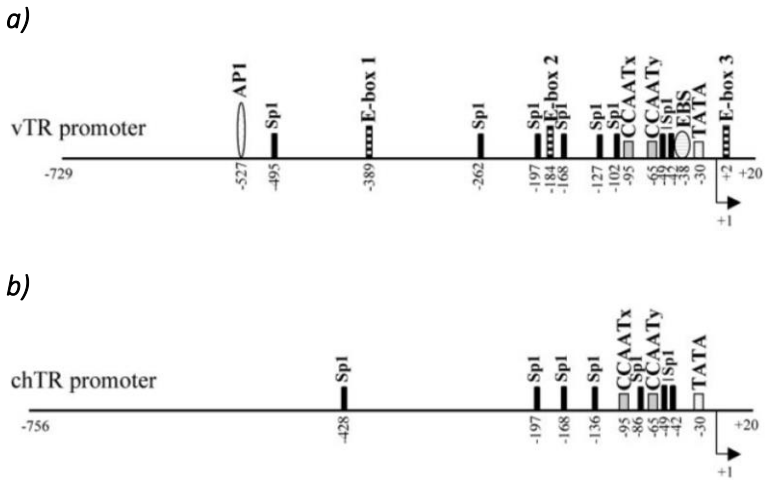

FIGURE 1.23. COMPARISON OF THE PROMOTER REGIONS OF THE VIRAL AND THE CHICKEN TR GENES.

Predicted transcription factor binding sites together with the transcriptional start sites (+1, arrow) are indicated for viral (vTR) and chicken (chTR) telomerase RNA subunit promoter sequences. **a)** Sites present in vTR promoter sequences and absent from chTR promoter **(b)** include AP-1, E-boxes and the EBS (adapted from [357]).

1.3.2.6. The role of vTR in tumorigenesis

As indicated in previous sections, vTR has a vital role in GaHV-2-induced T-cell lymphomagenesis by out-competing the chTR for chTERT [217,354]. This was further characterised, by generating and analysing mutant viruses that lacked either one or both copies of the vTR gene. vTR deletions from the genome the highly oncogenic GaHV-2 strain RB-1B did not abolish oncogenicity GaHV-2-susceptible birds. GaHV-2 mutants lacking both vTR copies were significantly impaired in T-cell lymphomas induction, with unaffected productive replication *in vivo*. Additionally, tumorigenesis was reduced by >60% in chickens infected with vTR^- viruses compared with animals inoculated with virus bearing one intact vTR copy. Moreover, gross tumours in animals infected with the vTR^- virus were also significantly smaller and less disseminated [218].

This "auxiliary" vTR function during oncogenesis was further studied by comparative *in vitro* analysis of the viral and chicken TR promoters [340]. The study showed that the vTR promoter was up to 3-fold more efficient than the chTR promoter in avian cells and that the more robust transcriptional activity of vTR resulted mainly from c-Myc response element (E-box 3) located two

nucleotides downstream of the TSS of the vTR gene. Furthermore, the vTR promoter importance in vTR expression and efficient tumour formation was tested by a recombinant virus harbouring chTR promoter as a replacement for vTR promoter. Viral replication rates *in vivo* showed no difference compared to parental GaHV-2 virus. However, tumour induction was reduced by >50% with smaller and less disseminated lymphomas. This highlighted that vTR promoter is essential for efficient vTR transcription and thus subsequently critical for efficient GaHV-2 lymphoma development [340]. Furthermore, the over-expression of vTR induced cellular proliferation increased expression of cell-surface adhesion molecules such as integrin alpha V, suggesting that vTR possesses functions beyond its telomerase activity [218].

To investigate if the tumour-promoting properties of vTR are dependent on the formation of a functional telomerase complex, mutations and disruption of the P6.1 stem-loop of vTR were introduced [219]. The P6.1 stem-loop of TR is known to mediate TR-TERT complex formation, and base pairing of the P6.1 stem-loop is conserved in all vertebrates [343]. The disruption of the base paring was shown to interfere with proper TR-TERT interaction and resulted in the absence of telomerase activity *in vitro* and *in vivo* [361]. In the GaHV-2 context, the introduced mutations in the P6.1 stem-loop interfered with proper vTR-TERT interaction and abrogated telomerase activity. Contrasting the recombinant viruses lacking vTR, all animals infected with the P6.1 mutant viruses developed lymphomas, with significantly delayed tumour formation and similar viral replication as a parental virus. Furthermore, P6.1 mutant viruses enhanced metastasis, highlighting the functionality of non-complexed vTR in tumour dissemination [219].

Furthermore, it was shown that vTR, similarly to EBV EBER-1 [362] and human TR [363], interacts with the cellular ribosomal protein L22 (RpL22) [219], which plays an important role in T-cell development and lymphoma formation [364,365], the main targets of GaHV-2 transformation. The interaction of EBER-1 with RpL22 results in re-localization of RpL22 from the nucleolus to the nucleoplasm and is associated with enhanced potential for cellular proliferation The mechanisms of RpL22 binding and re-localisation during EBV infection, they influence on viral replication and viral latency and moreover, the cellular role of RpL22 remain to be elucidated [366]. It is speculated that the binding and re-localisation of RpL22 may serve in direct functional role during EBV latency, or its sequestration may modulate cellular function, which may have negative consequences on viral replication or maintenance within the cell. To support this speculation, results demonstrating that RpL22 is re-localised to viral replication compartments during HSV type 1 infection suggest that RpL22 plays an active role during viral infection [367].

Furthermore, RpL22 may serve in a regulatory capacity and its re-localisation during viral infection. This is supported by results suggesting that RpL22 does serve a regulatory and

nonessential role within cells. It was demonstrated that RpL22 is not required for basal levels of translation and the RpL22 knockout is not lethal in mice [368,369]. Described vTR/RpL22 interaction indicates to an alternative GaHV-2 transformation mechanism that may be similar to that demonstrated for EBER-1 [219], that needs to be further studied.

In conclusion, vTR possesses two distinct functions during virus-induced tumorigenesis. Firstly, during the stages of early infection, vTR is part of the telomerase complex and contributes to the survival of rapidly dividing transformed cells. Secondly, the vTR functions independently of the telomerase complex and presents an essential part in the establishment of lymphomagenesis and metastasis. vTR involvement in lymphomagenesis and metastasis is likely a consequence of vTR-mediated gene regulation, partially controlled by interaction with RpL22 that is involved in T-cell development and virus-induced transformation [219].

Importantly, in order to unravel transformational mechanisms of vTR independent of its function within an active telomerase complex and regulatory processes controlling the vTR expression, further studies involving *in vitro* approach, as well as GaHV-2 natural small model for virus-induced tumorigenesis, are essential.

1.4. Project outline and objectives

Gallid herpesvirus type 2 (GaHV-2) is an avian alphaherpesvirus that causes the most frequent clinically diagnosed cancer in the animal kingdom, resulting in highly malignant T-cell lymphomas [235,321,370]. In susceptible chicken, the transformed $CD4^+$ T-lymphocytes, the ultimate consequence of interactions of GaHV-2 with the host cell [327], proliferate to form visceral tumours approximately four weeks after infection, leading to the death of the animal [371].

GaHV-2 is one of the few oncogenic herpesviruses that induces tumours in its natural host [234] and thus serves as a versatile small-animal model for studying different aspects of herpesvirus pathogenesis and induced oncogenesis in a natural homologous virus-host system [235].

During the viral life cycle, epigenetic changes, together with post-transcriptional and post-translational modifications, regulate expression of cellular and viral genes. Altogether, they allow GaHV-2 to switch between the productive and latent phases, and to induce infected cell transformation [191]. During latency, GaHV-2, like other herpesviruses, expresses a minimal subset of transcripts, including miRNAs, protein-encoding genes and other ncRNAs, all of which have a direct or indirect role in the maintenance of the latent state. For latency to be achieved, there must be a controlled shutdown of the lytic genes in concert with the expression of transcripts needed to establish and maintain the latent state [315]. The opposite situation (shutdown of the latent genes and expression of the transcripts needed to complete the lytic infection) is expected to induce the reactivation.

In this context, epigenetic modifications of the viral genome both at the DNA level and at the chromatin level are likely involved in the gene expression switch observed in the alternative stages of infection.

Furthermore, GaHV-2 encodes two copies of viral telomerase RNA subunit (vTR), which is expressed both in productive infection and in virus-transformed T-cell lines. vTR is driven by a robust viral promoter and is highly overexpressed compared to cellular TR in virus-infected cells. Its expression is regulated by promoter E-boxes (E-box 2 and E-box 3) that bind the oncoprotein c-Myc. vTR interacts with the chicken telomerase reverse transcriptase subunit (TERT) enhancing telomerase activity and contributing to the efficient and rapid onset of lymphoma [217–219]. Telomerase activity, absent in most somatic cells, is commonly up-regulated in transformed cells and is significantly elevated in over 85% of human cancers and over 70% of immortalized human

cell lines [206,221,372]. Moreover, vTR functions independent of the telomerase complex are responsible for tumour progression and dissemination.

An understanding of the mechanisms and regulatory processes controlling the genome-wide repression and transcription of productive genes is essential in studies of oncogenic herpesviruses.

Thus, the focus of this project was on the mechanisms involved in this switch between the productive and latent phase of GaHV-2 life cycle. The primary goal was to determine whether and how epigenetic modifications are involved in this transition and to establish patterns of DNA methylation in the promoter of the vTR gene involved in the regulation of GaHV-2 life cycle.

In order to do so, the first part of the project was designed to:

- Measure the impact of methylation on the telomerase activity,
- Establish a detailed DNA methylation and hydroxymethylation patterns of vTR promoter at different stages of the infection *in vitro* and *in vivo,*
- Investigate the impact of DNA methylation on the vTR expression levels.

Following the conclusions and outcomes from the first part that indicated the involvement of c-Myc binding sits in regulating vTR promoter activity, a second study was designed in order to investigate the importance of these binding sites in virus-induced tumorigenesis. To assess functional aspects of c-Myc binding sites, a recombinant virus bearing mutations in E-box 2 and E-box 3, as well as revertant, were produced by *en passant* mutagenesis using bacterial artificial chromosome of a highly oncogenic RB-1B strain. To assess if the mutation of E-boxes influenced virus replication, replication properties of recombinant viruses were investigated *in vitro* and *in vivo* by infecting a susceptible chicken line.

The objectives of the second study were to decipher the functionality of specific response elements in a viral promoter that shows discriminative DNA methylation signatures at crucial steps of GaHV-2 infection and will allow further assessment of the impact and importance of E-boxes on GaHV-2 pathogenesis and tumour formation.

The results of these studies, presented below, were accepted for publication in the Journal of Virology.

CHAPTER 2. RESULTS

2.1. Epigenetic modulation of viral telomerase RNA subunit promoter over-expressed during Gallid herpesvirus type 2 induced lymphomagenesis

2.1.1. Introduction

As previously discussed, Gallid herpesvirus type 2 (GaHV-2) is an avian alphaherpesvirus that causes the most frequent clinically diagnosed cancer in the animal kingdom, resulting in highly malignant T-cell lymphoma [235,370]. In susceptible chicken, the transformed $CD4^+$ T-cells, the ultimate consequence of interactions of GaHV-2 with the host cell [327], proliferate to form visceral lymphomas approximately four weeks after infection, leading to the death of the animal [371]. During GaHV-2 infection, numerous viral factors, proteins and transcripts contribute to lymphomagenesis and include the major oncoprotein Meq [324], the viral interleukin-8 [373] and GaHV-2-encoded miRNAs [374–376].

In addition, GaHV-2 encodes two copies of viral telomerase RNA subunit (vTR), which is expressed both in productive infection and in virus-transformed T-cell lines. vTR interacts with the chicken telomerase reverse transcriptase subunit (TERT) enhancing telomerase activity and contributing to the efficient and rapid onset of lymphoma [217–219]. Telomerase activity, absent in most somatic cells, is commonly up-regulated in transformed cells and is significantly elevated in over 85% of human cancers and over 70% of immortalized human cell lines [206,221,372]. In the absence of telomerase activity, progressive telomere shortening results in cellular senescence and cell cycle arrest [377]. Previous studies have demonstrated that telomere length and the mtDNA copy number are associated with numerous specific types of cancer [378–381], and positive correlation between two was observed [382], suggesting an early and essential effect on carcinogenesis. Moreover, vTR functions independent of the telomerase complex are responsible for tumour progression and dissemination. Furthermore, vTR re-localizes ribosomal protein L22 that plays an essential role in T-cell development and transformation [290].

It is the most abundant viral transcript detected in GaHV-2-induced tumour cells with higher expression than chTR in infected cells, consequence likely due to differences in their promoters. The vTR promoter has additional AP-1 sites, c-Myc transcription factor binding sites (namely E-box 1, E-box 2 and E-box 3) and EBS transcription factor binding sites, which are

absent in the chTR promoter. However, it was indicated that E-box 1 was not functional [24]. It was shown that the c-Myc oncoprotein is involved in the regulation of vTR during GaHV-2-induced lymphomagenesis [357] and that increased expression of vTR is essential for the oncogenic function of the virus [340].

During the viral life cycle, transcriptional modifications and epigenetic changes, together with post-transcriptional and post-translational modifications, regulate expression of cellular and viral genes. Altogether, they allow GaHV-2 to switch between the productive and latent phases, and to induce infected cell transformation [191].

An understanding of the mechanisms and regulatory processes controlling the genome-wide repression and transcription of productive genes is essential in studies of oncogenic herpesviruses. The focus of this study was on the epigenetic mechanisms involved in the switch between the productive and latent phase of GaHV-2 life cycle. We established methylation (5mC) and hydroxymethylation (5hmC) patterns and measured the impact of methylation on the telomerase activity and c-Myc response elements (c-Myc REs) of the vTR promoter. In addition, mtDNA copy number during latency and after viral reactivation was determined.

2.1.2. Materials and Methods

2.1.2.1. Cell lines

The chicken embryo fibroblasts (CEFs) infected with highly virulent GaHV-2 RB-1B strain were used for the productive phase of the viral life cycle. The CEFs were obtained from eleven days old chicken embryos treated by trypsinization (Lonza). The primary CEFs were cultured in Dulbecco's modified eagle medium (DMEM) (Lonza) supplemented with 2.5% foetal bovine serum (FBS), 1.25% chicken serum (CS), 1% penicillin (50 units/mL) and streptomycin (50 µg/mL), 1% fungizone (GIBCO), 1.475 g/L tryptose phosphate (Sigma). Four days after primary CEFs culture, cells were passaged to produce secondary CEFs. Secondary CEFs were transfected with an infectious clone of the RB-1B bacmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols.

The latently infected and transformed MSB-1 cell line, derived from GaHV-2 induced lymphoma [383], was cultured in RPMI 1640 medium (Gibco). Reactivation of the virus in the MSB-1 cells was induced with three mM sodium butyrate (Sigma), an inhibitor of class I histone deacetylase (HDAC) resulting in histone hyper-acetylation, as previously described [291] or by treatment with five μM 5-azacytidine (Merck), an inhibitor of the DNA methyltransferase, both described as reactivation stimuli for GaHV-2 [296]. The viral reactivation was monitored and confirmed by RT-qPCR measuring relative expression of major viral capsid protein VP5, 48h after the treatment with specified inhibitor. Relative expression levels were normalized against chicken small nuclear RNA U6 gene, using primers presented in Table 1b.

The avian fibroblast cell line DF-1 derived from primary chicken embryonic fibroblasts was cultured in DMEM medium (Lonza).

The LMH cell line established from chicken liver carcinoma epithelial cells [384] was cultured with 0.2% gelatine to maximize cell adhesion in DMEM medium (Lonza).

The human epithelial HeLa cell line, derived from a cervical carcinoma and transformed by human papillomavirus type 18 [385] was maintained in EMEM medium (Lonza).

All media were supplemented with 10% FBS, 5% CS (except EMEM), 1% of non-essential amino acids, 1% penicillin (50 units/mL) and streptomycin (50 μg/mL). The MSB-1, DF-1 and LMH cell cultures were maintained at 41°C, and HeLa cells at 37°C, under 5% CO2.

2.1.2.2. Plasmids

The pGEM®-T Easy (Promega) was used as a vector for the direct cloning of the various polymerase chain reaction (PCR) products. This plasmid is linearized by the EcoRV endonuclease leaving a 5' thymine overhang. Thus, the pGEM®-T Easy vector is adapted for ligation of PCR products obtained with Taq polymerase that provides a 3' adenine overhang at the end of PCR amplicons. This 3015 bp plasmid carries the beta-galactosidase alpha-subunit gene (LacZ cassette), located in the multiple cloning site, allowing the white-blue colony screen test. Moreover, this plasmid codes for a beta-lactamase, the ampicillin resistance gene, which was used to select transformed bacteria by selective medium containing ampicillin (Figure 2.1).

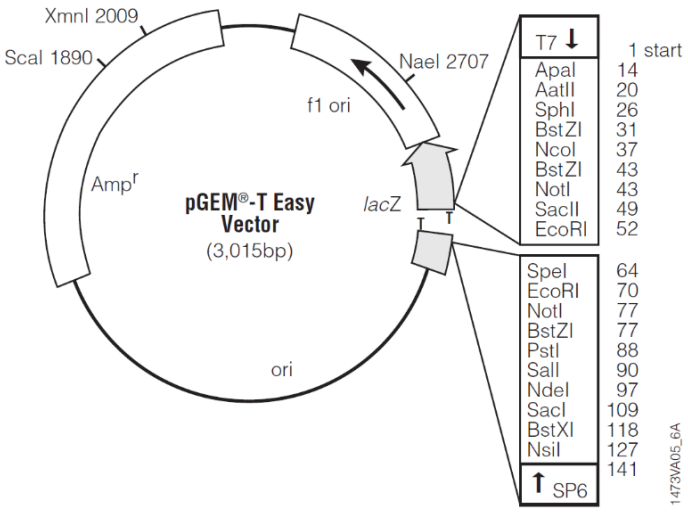

FIGURE 2.1. PGEM®-T EASY CLONING VECTOR MAP.

The plasmid contains a resistance gene to ampicillin, allowing the isolation of the transformed bacteria using a selective medium. In addition, the blue-white colony screen is possible due to the LacZ gene, which expression is abolished by the integration of an inserted amplicon into the vector.

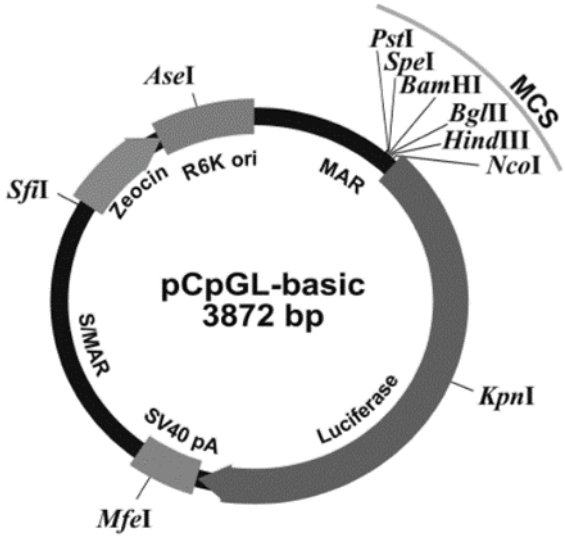

FIGURE 2.2. PCPGL-CPG-FREE REPORTER VECTOR MAP.

The vector, including its multiple cloning site (MCS), is entirely free of CpG dinucleotides. It contains a Zeocin resistance gene and a reporter gene coding for firefly luciferase

The pCpGL-Basic reporter vector, devoid of the CpG dinucleotides and thus not sensitive to methylation was used to study the activity of the constructed vTR promoters. vTR promoters were cloned at the multiple cloning site located upstream of the reporter gene coding for firefly luciferase. In this plasmid, a short CpG-free linker containing several restriction sites replaced the enhancer/promoter region of the CpG-free plasmid pCpG-mcs (Invivogen). In addition, the CpG-free luciferase coding region was released from pMOD-LucShS (Invivogen) by enzymatic restriction with MfeI and NcoI, and sub-cloned into linker ligated CpG-free backbone [386] (Figure 2.2).

The pCpGL-EF1/CMV plasmid, insensitive to methylation was used as a control. This plasmid contains the gene encoding firefly luciferase under the control of the hybrid promoter EF1/CMV. For constructing a completely CpG-free control reporter vector, the EF1A-promoter/CMV-enhancer cassette was released from pCpG-mcs (Invivogen) using PstI/HindIII restriction enzymes and inserted into corresponding sites of the pCpGL-basic vector [386].

The pCpGL-basic and pCpGL-EF1/CMV plasmids were generously provided by Dr. Michael Rehli (Department of Haematology and Oncology, University of Regensburg, Germany).

The pRL-TK vector (Promega) containing the gene encoding for Renilla luciferase was used to standardize the activity of Firefly luciferase (Figure 2.3). This vector contains the cDNA encoding Renilla luciferase (Rluc) cloned from the anthozoan coelenterate *Renilla reniformis* (sea pansy). The gene coding for the Rluc is expressed under the control of the thymidine kinase (TK) promoter of the Human Herpes Virus type I (HHV-1). The expression of this reporter gene in eukaryotic cells helps standardize the activity of firefly luciferase. Like the vector pGEM®-T Easy, the pRL-TK encodes the gene for resistance to ampicillin, which allows the selection of transformed bacteria using a selective medium.

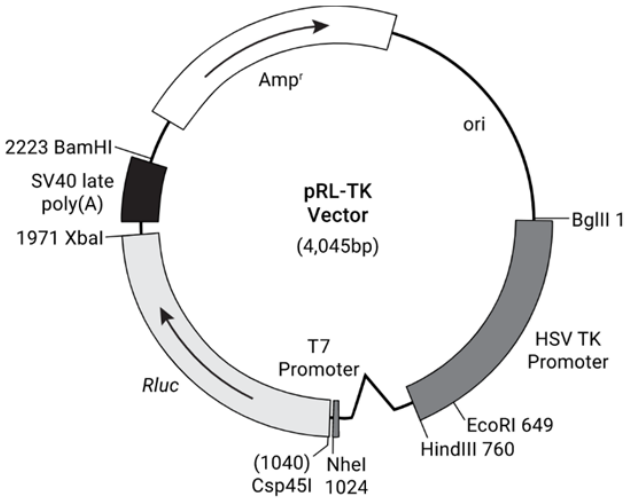

FIGURE 2.3. PRL-TK CONTROL REPORTER VECTOR MAP.

The pRL-TK vector provides a constitutive expression of Renilla luciferase, which can be used in combination with a firefly luciferase vector for cell co-transfection. Expression of Renilla luciferase provides an internal control value to which expression of the experimental firefly luciferase reporter gene may be normalized.

2.1.2.3. Telomeric repeat amplification protocol assay

Telomerase activity of 1 μg of protein extracted from the MSB-1 cell line was quantified using the semiquantitative fluorescence-based telomeric repeat amplification protocol (TRAP) assay, as previously described [217]. Full protein extracts were collected every 12h. Briefly, 10^6 cells were centrifuged at 1,400 × g for 20 seconds. Cell pellets were lysed on ice for 30 minutes with 200 μl ice-cold 1X CHAPS lysis buffer (Millipore), a protease inhibitor (0,1 mM AEBS Sigma) and RNAsin (0,5 unit/μl Promega). Cell lysis was followed by centrifugation for 30 minutes at 10,000 × g and 4 °C. Protein concentrations were determined with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and adjusted to 500 ng/μl.

The polymerase chain reaction (PCR) was carried out using tetramethylrhodamine (TAMRA)-labelled forward TS primer and CX-ext as a reverse primer as initially described (Figure 2.4) (Table 2.1.a) [387]. An internal amplification standard (ITAS) was included to verify PCR amplification efficiency.

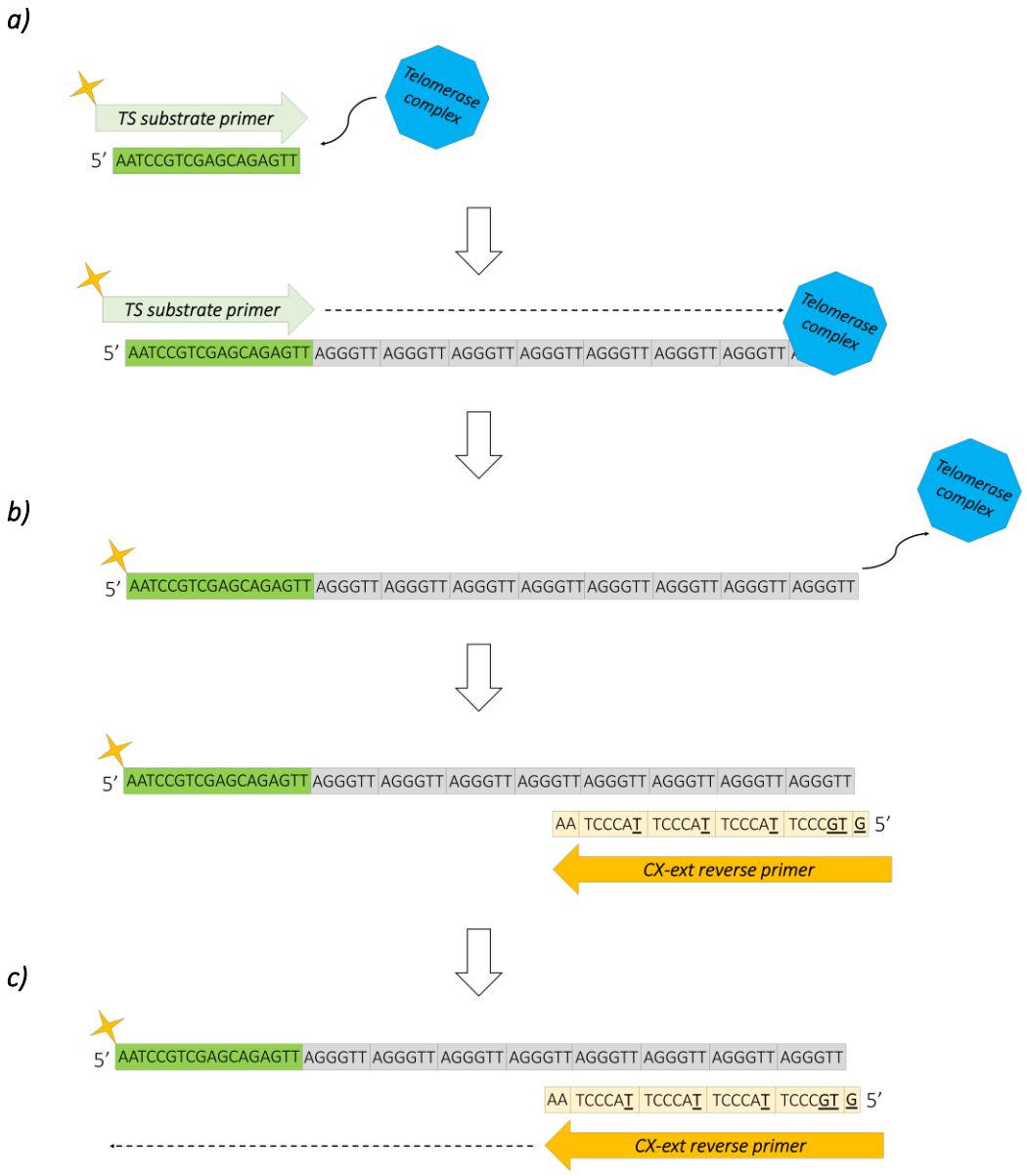

FIGURE 2.4. SCHEMATIC REPRESENTATION OF PCR STEPS DURING THE TELOMERIC REPEAT AMPLIFICATION PROTOCOL FOR MEASURING TELOMERASE ACTIVITY.

a) Telomerase complex (blue octagon), from total cell protein extract, first extends TS substrate primer (green rectangle) of non-telomeric sequence with telomeric repeats (grey rectangles). TS substrate primer is labelled with TAMRA fluorophore (yellow star). **b)** After telomerase inactivation, in the first PCR step, reverse primer (CX-ext) anneals to the primary telomerase products. Bold underlined nucleotides indicate designed mismatches in CX-ext primer that reduce the formation of primer–dimers. **(c)** PCR with CX-ex and TS as a forward primer finalize targeted amplification, and the product is further analyzed using capillary electrophoresis in order to measure relative telomerase activity (adapted from [388]).

ITAS was prepared by PCR as described before [389] using primers presented in Table 2.1.a. Shortly, a 150 bp internal standard, sufficiently long so it would not interfere with the visualization of the telomerase ladder was used. ITAS was prepared by synthesizing TS and CX-ext oligonucleotides that contained an additional 15 bases at their 3' ends that overlapped with sequences encoding 97-132 aa of rat myogenin. Amplification of the myogenin cDNA with these primers generated a 150 bp product, which could be reamplified using the same TS, and CX-ext primers used to amplify the telomerase ladder in the standard TRAP assay.

The telomerase amplification PCR was performed as described before [217]. 25 μL of PCR reaction were prepared, including GoTaq® G2 Colorless Master Mix (Promega), 20 pmol TS primer, 10 pmol CX-ext, 1 μg of protein extract and ITAS concentration of 0,0001 atmol. The reaction mix were incubated for telomerase extension at 30 °C for 30 minutes, followed by 38 cycles at 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 1 minute. Following amplification, 2 μL of PCR product were denatured for 5 minutes at 94 °C in a mix composed of 22,5 μL of formamide and 0,5 μL of Genescan-500 ROX size marker, resulting in simple strand DNA fragments from 35 to 500 bp, labelled with ROX.

Finally, treated PCR products were analyzed by capillary electrophoresis (Applied Biosystems 3130xl Genetic Analyzers). The relative telomerase activity level of each protein extract was calculated by adding values of fluorescence intensity of all telomerase products containing 5 and up to 15 telomeric repeats and normalized to the fluorescence intensity value of the ITAS, as previously described [390].

2.1.2.4. Mitochondrial DNA copy number evaluation

Cellular DNA, from MSB-1 cells mock-treated or treated with sodium butyrate or 5-azacytidine, was extracted using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's recommendations. Quantitative PCR (qPCR) for measuring mitochondrial DNA (mtDNA) amplification was performed on the gene encoding for NADH Dehydrogenase 2 (ND2) using the primers specified in Table 2.1.b. The normalization with nuclear DNA was done using the gene encoding for Beclin amplified by primers described in Table 2.1.b. Real-time qPCR was performed with SYBR Select Master Mix (ThermoFisher) following the manufacturer's

recommendations. mtDNA copy number was calculated as previously described according to the following formula [391]:

$$2 \times 2^{-\Delta Ct} \ (\Delta Ct = Ct\ mean_{\ ND2} - Ct\ mean_{\ Beclin})$$

2.1.2.5. Animal experiment, cell isolation, magnetic cell sorting and DNA extraction

White Leghorn specific pathogen-free $B^{13}B^{13}$ chickens highly susceptible to GaHV-2 were used for animal experiment. The animals were housed in isolated biosecurity level 3 facilities at Veterinary and Agrochemical Research Centre CODA-CERVA (Brussels, Belgium). Animals were injected intramuscularly at the age of 3.5 weeks with 1,000 PFU of the highly oncogenic GaHV-2 RB-1B strain, using infected peripheral blood leukocytes (PBL) suspension. Blood samples and feather follicle epithelium (FFE) were collected once a week, and tumours were collected from euthanized chickens at 28 days post-infection (dpi). PBLs from anticoagulated blood and tumour tissue were isolated using Histopaque-1077 density gradient (Sigma-Aldrich) according to the manufacturer's recommendations at 14 dpi (representing the start of the latency) and 28 dpi (representing viral reactivation). Magnetic cell sorting with Dynabeads anti-mouse IgG kit (Invitrogen) was used to isolate $CD4^+$ and $CD30^+$ lymphocytes from tumour tissue according to the manufacturer protocol. Mouse anti-chicken CD4 monoclonal antibody (AbD Serotec, Bio-Rad) was used as the secondary antibody for $CD4^+$ magnetic isolation, and monoclonal antibody AV37 (generously provided from Prof. Venugopal Nair, The Pirbright Institute, UK) was used to isolate $CD30^+$ lymphocytes. Genomic DNA from each sample was isolated using the DNeasy®blood and tissue kit (Qiagen) as described by the manufacturer.

2.1.2.6. Ethics statement

The animal study was conducted following Belgian law for animal protection and the European Directive, 2010/63/EU. The Ethics committee of CODA-CERVA (file n° LA1230174) approved all animal experiments.

2.1.2.7. Bisulfite genomic sequencing assay, 5-hydroxymethylation mapping, polymerase chain reactions and vector cloning

Bisulfite genomic sequencing assay (BGSA) was used for 5-methylcytosine mapping, as described before [392]. Bisulfite treatment was performed with the EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's recommendations. This technique involves treating methylated DNA with bisulfite, which converts unmethylated cytosines into uracils (Figure 2.5). Methylated cytosines remain unchanged during the treatment. Briefly, 500 ng of genomic DNA of interest, were submitted to bisulfite treatment. In the first step, CT Conversion Reagent was added to the DNA sample followed by incubation first at 98 °C for 10 minutes and then 64 °C for 2.5 hours.

Following the sulphonation step, M-Binding Buffer was added to a Zymo-Spin™ IC Column, and the treated sample was loaded. Following the centrifugation, M-Wash Buffer was used for washing the column, and M-Desulphonation Buffer was added to the sample to the column and incubated at room temperature for 20 minutes. Afterwards, the second washing step was performed, and the bisulfite converted DNA sample was eluted with dH_2O and stored at -20 °C.

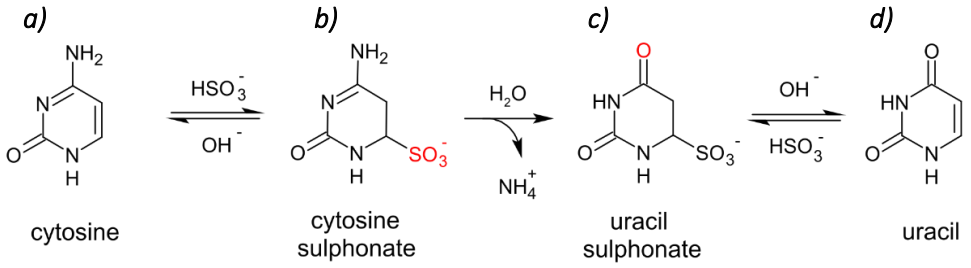

FIGURE 2.5. OUTLINE OF THE CHEMICAL REACTIONS THAT DRIVE THE BISULFITE-CATALYSED CONVERSION OF CYTOSINE TO URACIL.

a) A fragmented genomic DNA sample is treated with sodium bisulfite. **b)** Sodium bisulfite deaminates cytosine residues to 5,6-dihydrocytosine-6-sulphonate, which are converted to **(c)** 5,6-dihydrouracil-6-sulphonate. **d)** Incubation at high pH removes sulphite group resulting in uracil. In contrast, 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) are not susceptible to bisulfite treatment and remain intact, which allows downstream differentiation of methylated and non-methylated cytosines in the DNA sequence.

For 5-hydroxymethylation mapping, the 5hmC-TAB-Seq Kit (WiseGene) was used according to the manufacturer's recommendations. Briefly, in order to differentiate 5-hydroxymethylcytosine (5hmC) from 5-methylcytosine (5mC) and non-modified cytosine (C), two enzymatic steps were introduced before bisulfite conversion. Firstly, β-glucosyltransferase (βGT) is utilized to convert 5hmC present in genomic DNA to β-glucosyl-5-hydroxymethylcytosine (5gmC) thereby protecting it from further oxidation to 5-formylcytosine (5fC) or 5-carboxylcytosine (5caC) by the TET enzymes. Next, the glucosylated genomic DNA is treated with an excess of highly active TET1 and TET2 enzymes to oxidize most 5mC to 5caC, which can then together with cytosine be deaminated using standard bisulfite treatment (Figure 2.6) [393]. After bisulfite treatment and PCR amplification, 5hmC is sequenced as cytosine, whereas both non-modified cytosine and 5mC are sequenced as thymine, therefore distinguishing 5hmC sites from C and 5mC sites. Rates of 5mC conversion and 5hmC protection were monitored with the specific spike-in controls (primers presented in Table 2.1.c).

Finally, nested PCR, comprised of two successive PCRs, was performed on the genomic locus of interest. The vTR promoter was amplified using the specific primers modified for bisulfite converted DNA and are presented in Table 2.1.c. Shortly, PCR mix was prepared using the Epimark HotStart Taq DNA polymerase (New England BioLabs, NEB), 0.2 mM of deoxyribonucleotide mix and 10 mM of primer pairs specific for first or second PCR. PCR was performed as follows: 94 °C for 5 minutes, followed by 35 cycles of denaturation (94 °C, 1 minute), annealing (target dependant) and extension (72 °C, 1 minute). Following the PCR, gel electrophoresis, amplicon excision and agarose gel purification were performed. For agarose gel purification, NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) was used according to the manufacturer's protocol.

Produced amplicons were cloned into the pGEM-T Easy vector system (Promega) as suggested by the manufacturer. Briefly, previously prepared PCR amplicons of interest, bearing site-directed mutations, were ligated into pGEM-T Easy vector using the T4® DNA ligase (Promega). The ligation mix contained Ligase 10x Buffer, 1 µl of the vector, 3 µl of the PCR amplicon and 1000 units of T4® DNA ligase. Negative control was performed by replacing the insert with 3 µl of water. The ligation reaction was incubated overnight (O/N) at 12 °C.

The ligation was followed by transformation of the competent bacteria *E. coli* TG1 strain and plated on Luria-Bertani (LB) agar plates containing X-gal, IPTG and ampicillin as a resistance selection marker (100 µg/ml).

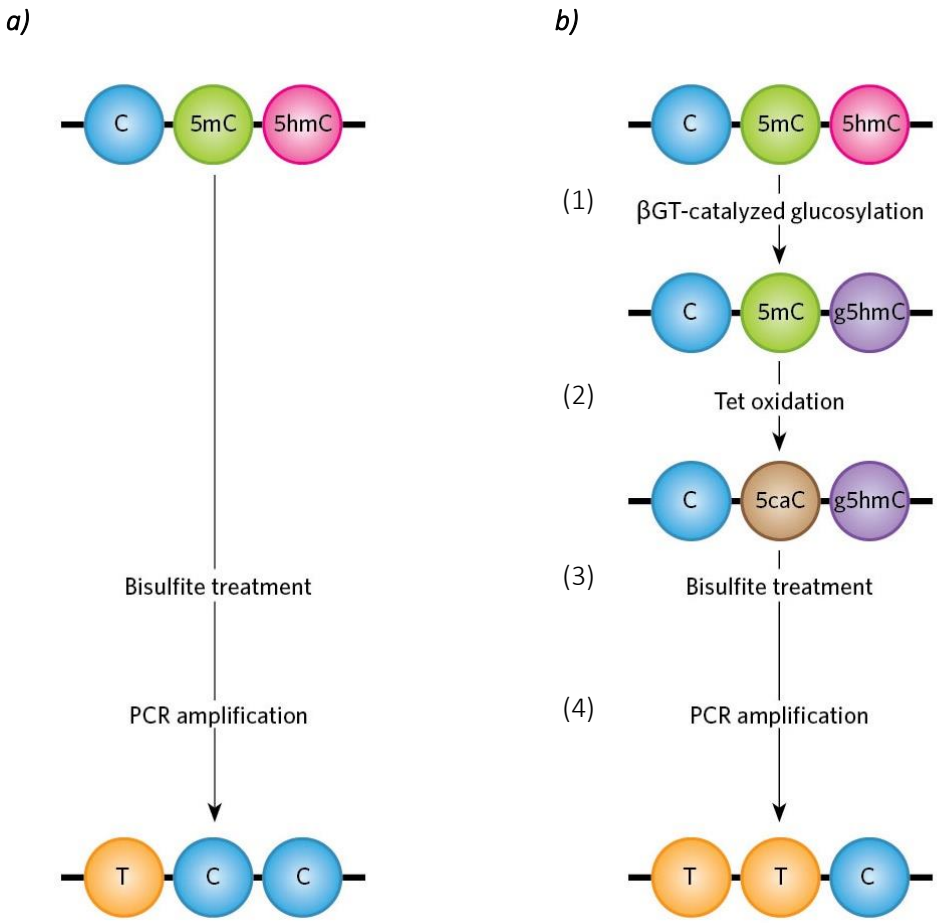

FIGURE 2.6. OVERVIEW OF TET-ASSISTED BISULFITE SEQUENCING (TAB-SEQ).

a) Bisulfite sequencing alone is not able to determine whether a cytosine base is modified as 5-methylcytosine (5mC) or 5-hydroxymethylcytosine (5hmC). **b)** With TAB-Seq 5hmCs are protected from oxidation by glucosylation (1), while 5mCs are oxidized using TET enzymes, which converts 5mC to 5-carboxylcytosine (5caC) (2). Bisulfite treatment then converts the 5caCs and unmodified cytosines that read as thymines following PCR (3). The 5hmCs continue to read as cytosines, therefore distinguishing them from C and 5mC sites that are read as thymines (4) (adapted from [393]).

Positive bacterial colonies were screened using the blue-white colony screen PCR. Screen PCR was performed using the GoTaq® DNA Polymerase (Promega) with 10 mM of universal primer pairs (Table 2.1.e) and performed as follows: 94 °C for 5 minutes, followed by 35 cycles of denaturation (94 °C, 1 minute), annealing (30 °C, 1 minute) and extension (72 °C, 1 minute).

Clones positive for amplicon of interest were sent for Sanger sequencing, and 5mC or 5hmC positions on the vTR promoter were analysed by Geneious software.

2.1.2.8. Genome-wide mapping of methylation patterns during latency and after viral reactivation

Cell line

The latently infected and transformed MSB-1 cell line, derived from GaHV-2 induced lymphoma [383], was cultured in RPMI 1640 medium (Gibco), supplemented with 10% foetal bovine serum, 5% chicken serum, 1% of non-essential amino acids, 1% penicillin (50 units/mL) and streptomycin (50 μg/mL) The cell cultures were maintained at 41°C under 5% CO2.

Viral reactivation

Reactivation of the quiescent virus genome in MSB-1 cells was induced after 48h treatment with 5 μM 5-azacytidine (Merck), an inhibitor of the DNA methyltransferase, described as reactivation stimuli for GaHV-2 [296]. The viral reactivation was monitored and confirmed by q-PCR measuring relative expression of major viral capsid protein VP5 after the treatment with specified inhibitor.

DNA extraction

Genomic DNA was extracted from mock-treated MSB-1 cells as well as cells treated with 5-azacytidine in order to induce virus reactivation. DNA from each sample was isolated using the DNeasy®blood and tissue kit (Qiagen) as described by the manufacturer. DNA purity was assessed via a Thermo Scientific™ NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). DNA concentrations were measured using a Qubit DNA Assay Kit with a Qubit 2.0 Fluorometer (Life Technologies).

DNA quantification and qualification

Genomic DNA degradation and contamination were monitored on agarose gels. DNA concentration was measured using the Qubit® DNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies).

Library preparation and quantification

A total amount of 5.2 μg genomic DNA spiked with 26 ng lambda DNA was fragmented by sonication to 200-400 bp fragments with Covaris S220, followed by end repair and adenylation. Cytosine-methylated barcodes were ligated to sonicated DNA as per manufacturer's instructions. Then these DNA fragments were treated twice with bisulfite using EZ DNA Methylation-Gold TM Kit (Zymo Research). In addition, the resulting single-strand DNA fragments were PCR amplified using KAPA HiFi HotStart Uracil + Ready-Mix (2X). Library concentration was quantified by Qubit® 2.0 Fluorometer (Life Technologies, CA, USA) and quantitative PCR, and the insert size was checked on Agilent 2100 system.

Clustering and sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform, and paired-end reads were generated. Image analysis and base calling were performed with the standard Illumina pipeline, and finally, paired-end reads were generated.

Data Analysis

Reads mapping to the reference genome

Bismark software [394] was used to perform alignments of bisulfite-treated reads to a reference genome with the default parameters. The reference genome was firstly transformed into

bisulfite-converted version (C-to-T and G-to-A conversion) and then indexed using bowtie2 [395]. Sequence reads were also transformed into fully bisulfite-converted versions (C-to-T and G-to-A conversion) before they are directionally aligned to similarly converted versions of the genome. Sequence reads that produce a unique best alignment from the two alignment processes (original top and bottom strand) were then compared to the genomic reference sequence, and the methylation state of all cytosine positions in the read was inferred. The same reads that aligned to the same regions of genome were regarded as duplicated ones. The sequencing depth and coverage were summarised using reduplicated reads. The results of methylation extractor were transformed into big Wig format for visualisation using IGV browser. The sodium bisulfite non-conversion rate was calculated as the percentage of cytosines sequenced at cytosine reference positions in the lambda genome.

Estimating methylation level

A binomial distribution tests the methylated and unmethylated counts at each site. Assuming the number of methylated cytosines is x in a specific site, where the read coverage is n, and BS conversion rate is p, so the reliability of x methylated cytosine needs to be tested in above conditions. In order to find accurate methylated sites, a set of thresholds are set in analysis process: (1) the sequencing depth is equal to or greater than 5; (2) q-value less than or equal 0.05. We employed a sliding-window approach, which is conceptually similar to approaches that have been used for bulk BS-Seq. With window size w = 3,000 bp and step size 600 bp [396], the sum of methylated and unmethylated read counts in each window were calculated. Methylation level for each cytosine (C) site shows the fraction of methylated Cs.

2.1.2.9. PCR site-directed mutagenesis

Site-directed mutagenesis was used to accurately induce specific mutations in the sequence of the vTR promoter. Mutations of the vTR promoter at the c-Myc transcription factor binding sites were performed by overlapping PCR using primer pairs described in Table 2.1.d. The backbone DNA used to generate the E2 and E3 mutations was the recombinant pGEM-T Easy

vector (Promega) containing the wild-type vTR promoter. E-box 2 (E2) and E-box 3 (E3) mutations were introduced in two stages using primer pairs with overlapping fragments containing the mutation (Figure 2.7). Two overlapping fragments containing the mutation were initially constructed by PCR using two pairs of specific primers (Table 2.1.d). Firstly, for the starting stage of site-directed mutagenesis, we used a primer pair composed of a sense oligonucleotide bordering the targeted insert and containing PstI restriction site extension and reverse oligonucleotide bearing the internal mutation (Figure 2.7.a). Secondly, we used a sense oligonucleotide bearing the internal mutation and a reverse oligonucleotide bordering the targeted insert and containing the HindIII restriction site extension (Figure 2.7.b). Finally, after hybridization of two fragments containing the mutation, the complete mutant sequence was amplified using the bordering oligonucleotides presented in Table 2.1.d (Figure 2.7.c).

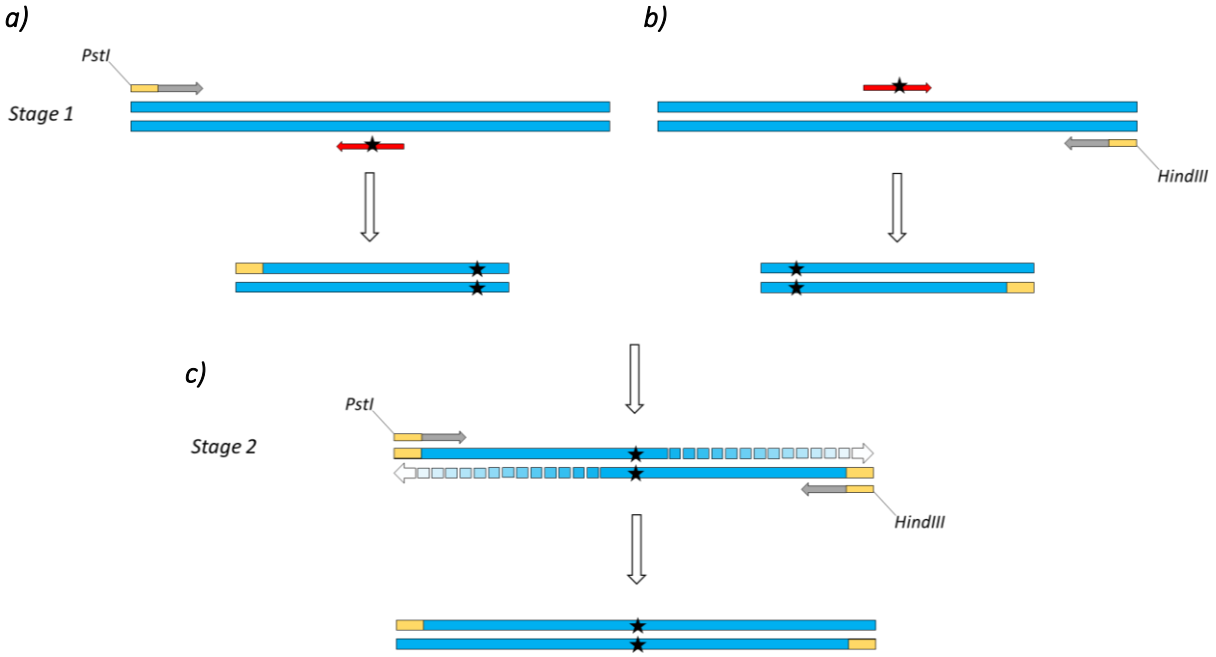

FIGURE 2.7. PRINCIPLES OF TWO-STAGE PCR SITE-DIRECTED MUTAGENESIS.

Two overlapping fragments containing the mutation were initially constructed by PCR using two pairs of specific primers. **a)** A targeted DNA sequence is first amplified using a forward primer (grey arrow) containing restriction site extension (gold rectangle) and reverse primer (red arrow) bearing the desired internal mutation (black star). **b)** The second overlapping fragment was constructed using forward primer (red arrow) with the internal mutation (black star) and a reverse primer bordering the targeted insert and containing restriction site extension (gold rectangle). **c)** The final mutated construct was obtained after hybridization of two overlapping fragments containing the mutation (black star) by PCR amplification using the bordering primers (grey arrows) with specific restriction site extensions (gold rectangles).

Using the same principle, the double E-box 2 and E-box 3 mutation (E2E3) was generated by introducing the E3 mutation on the vector bearing E2 mutation that served as a PCR backbone, followed by confirmation by Sanger sequencing.

2.1.2.10. Construction of the reporter vectors

The pCpGL-basic reporter vector, free from the CpG dinucleotides and thus not sensitive to methylation, was used to study the activity of the vTR promoter. Wild-type and mutated vTR promoter constructs were cloned at the multiple cloning site upstream of the reporter gene coding for firefly luciferase. Plasmid pCpGL-EF1/CMV was used as the control vector. The plasmid pRL-TK (Promega) containing the gene encoding for Renilla luciferase was used to standardize the activity of Firefly luciferase. Construction of the reporter vectors was done as follows:

DNA digestion catalysed by restriction enzymes

Plasmid enzymatic digestion was carried out using HindIII (NEB) and PstI (NEB) restriction endonucleases following manufacturer's instructions. Briefly, 10 µg of plasmid DNA were added to 50 µl reaction mixture containing 10 units of a specific enzyme, 5 µl of NEBuffer 3.1 (10×) and incubated at 37 °C for one hour.

Dephosphorylation of linearized plasmids

Next, the linearized vector was dephosphorylated to avoid recircularization by removal of 5' terminal groups. The linearized plasmids were incubated in a dephosphorylation mix composed of NEB cutsmart buffer 1x, NEB Calf Intestinal Phosphatase (CIP, 50U) in a final volume of 100 µl. The reaction mix was incubated for 20 minutes at 37 °C. Immediately after, the reaction mixture was cooled down on ice for 5 minutes. A second cycle was carried out after further addition of 4 µl of CIP followed by incubation for an additional 20 minutes at 37 °C. Finally, the reaction was stopped by incubation at 65 °C for 20 minutes. The dephosphorylated vectors were purified using phenol-chloroform extraction.

Plasmid purification by phenol/chloroform

A phenol/chloroform purification was performed on dephosphorylated vectors. Firstly, an equal volume of phenol/chloroform was added to the treated plasmids samples. The phenol/chloroform triggers a lipophilic and hydrophilic phase separation, after which the samples were centrifuged at 16,000 × g for 15 minutes. Following the centrifugation, the upper aqueous phase containing the dephosphorylated plasmid was collected. Next, an equal volume of chloroform and isoamyl alcohol (3-methylbutane-1-ol) (49:1) was added to the aqueous phase, in order to remove the proteins from the aqueous phase. The separation of the phases induced by the addition of isoamyl alcohol was followed by the centrifugation at 16,000 × g for 15 minutes. The aqueous phase was collected, and the two volumes of 100% ethanol and 1/10 volume of sodium acetate were added and mixed to precipitate DNA. Next, the mix was incubated for 20 minutes at -80 °C. Following thawing, the mix was centrifuged at 15,000 × g for 10 minutes, and the supernatant was then discarded. Next, the pellet was washed with 1 ml of 70% ethanol and centrifuged at 7,500 × g for 10 minutes at room temperature. Finally, after drying, the pellet was resuspended in 100 µl of dH_2O.

Ligation of PCR products into the plasmid vectors

Ligation of the previously prepared vTR promoter PCR products bearing site-directed mutations into a linearized and dephosphorylated vector was performed using the T4® DNA ligase (Promega). The ligation mix contained Ligase 10× Buffer, 1 µl of the digested and dephosphorylated vectors, 3 µl of the insert and 1000 units of T4® DNA ligase. Negative control was performed by replacing the insert with 3 µl of dH_2O. The ligation mix was incubated O/N at 12 °C.

Electroporation of ligated products in the electro-competent 100D strain of E.coli

The strain 100D of *E.coli* was used for the transformation of the pCpGL-basic vectors with the ligated products. This strain allows the replication of plasmids containing an R6K origin of replication, dependent on the PIR protein.

Briefly, one microliter of the ligation product was added to 50 µl of competent bacteria. The electroporation was induced with 2,500 V for five milliseconds in 2 mm electroporation cuvette. Immediately after electroporation, 150 µl of LB culture medium were added to the electroporated bacteria. After 30 minutes incubation at 37 °C, bacteria were spread on LB agar plates containing zeocin selective medium (25 µg/L) and incubated O/N at 37 °C.

Finally, in order to confirm proper integration, a screen PCR on bacterial colonies was performed using the GoTaq® DNA Polymerase (Promega) with 10 mM of specific primer pairs (Table 2.1.e). The PCR was performed as follows: 94 °C for 5 minutes, followed by 35 cycles of denaturation (94 °C, 1 minute), annealing (30 °C, 1 minute) and extension (72 °C, 1 minute).

Plasmid DNA midi-preparation

To amplify and isolate plasmid DNA, NucleoBond® Xtra Midi plasmid DNA purification kit (Macherey-Nagel™) was used following the manufacturer's protocol. Briefly, bacteria were grown in 200 ml of LB medium with zeocin antibiotic selection (25 µg/ml) and incubated O/N at 37 °C under shaking at 200 rpm. O/N cultures were centrifuged at 4,200 × g for 15 minutes and 4 °C. After centrifugation, the supernatant was discarded, and 8 ml of Resuspension Buffer were added to resuspend the bacterial pellet. Following resuspension, the equivalent volume of Lysis Buffer was added to the bacterial cells and incubated for 5 minutes at room temperatures. After that, the equivalent volume of Neutralization Buffer was added to stop the cell lysis and to precipitate the proteins and the DNA. The precipitate was pelleted by centrifugation for 20 minutes at 4,200 × g, and the supernatant was loaded on the equilibrated NucleoBond® Xtra Column. Next, the column was washed twice, first with Equilibration Buffer and then with the Wash Buffer respectively. Following washing steps, the plasmid DNA was eluted using the Elution Buffer. Next, the isopropanol was used to precipitate the eluted plasmid DNA, followed by the final wash with 1 ml of 70% of ethanol. Finally, plasmid DNA was resuspended in dH_2O and stored at -20 °C.

Confirmation of the correct insertion and plasmid construction was done using Sanger sequencing.

2.1.2.11. Hypermethylation of plasmid DNA

The hypermethylation of the CpG dinucleotides of the newly constructed plasmids containing wild-type or mutated vTR promoters was carried out using an M.SssI methyltransferase (NEB) and S-adenosyl-L-methionine (SAM) (NEB) as described by the manufacturer. Briefly, the CpG methyltransferase methylates all cytosine residues (C5) within the double-stranded dinucleotide recognition sequence 5'...CG...3'. One μg of plasmid DNA was added to a mixture containing 2 μl of NEBuffer 2 (10×), 2 μl of SAM (1,600 μM), 1 unit of the M.SssI methyltransferase in a reaction volume of 20 μl. The reaction mix was incubated for one hour at 37 °C, followed by the incubation for 20 minutes at 65 °C to inhibit the enzyme activity. In order to objectively compare the effect of methylation, the unmethylated vectors were mock-treated under the same conditions in the absence M.SssI methyltransferase. After the reaction, the plasmids (methylated and unmethylated) were purified by phenol-chloroform-isoamyl alcohol (50-49-1).

2.1.2.12. Cell transfection and dual-luciferase reporter assay

Twenty-four hours before transfection, 1.5×10^4 DF-1 and 3×10^4 LMH and HeLa cells were seeded per well in 96-well plates. These cell lines were co-transfected with 150 ng of luciferase reporter constructs containing vTR wild-type or mutant E-box target sites and 30 ng of luciferase control vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols.

A suspension of 1×10^6 MSB-1 cells in Nucleofector solution T (Amaxa Biosystems) was co-transfected with 1 μg of luciferase reporter constructs containing vTR wild-type or mutant E-box target sites and 40 ng of luciferase control vector. Co-transfection with electroporation was done using Nucleofector program X-001 (Nucleofector II, Amaxa) following the manufacturer's instructions.

Luciferase activity was quantified in the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. Firefly and Renilla luciferase activities were measured 24 hours after transfection. The firefly luciferase activity obtained for each sample was normalized by the corresponding Renilla luciferase activity. For the standardization of luciferase activity, the

control vector pCpGL-EF1/CMV from which the firefly luciferase gene is expressed under the control of the CMV promoter was used.

Three independent experiments were carried out in triplicates. The significant difference between the analysed promoter constructs was determined using the student t-test and comparisons for which $p \leq 0.05$ was considered to be statistically significant.

2.1.2.13. TABLE 2.1. PRIMERS USED IN THE STUDY OF TELOMERASE ACTIVITY AND DNA METHYLATION PATTERNS.

a) Primers used for TRAP assay

Target/construct	Orientation	Sequence (5'-3') (the fluorescent label is in bold)	Reference
ITAS	For	AATCCGTCGAGCAGAGTTGTGAATGAGGCCTTC	[389]
	Rev	CCCTTACCCTTACCCTTACCCTAATAGGCGCTCAATGTA	
Telomeric amplification	For (TS)	**(TAMRA)**-AATCCGTCGAGCAGAGTT	[387]
	Rev (CX-ext)	GTGCCCTTACCCTTACCCTTACCCTAA	

b) Primers used for RT-qPCR confirming viral reactivation

Target/construct	Orientation	Sequence (5'-3')
GaHV-2-VP5	For	CAAGGGGATCCCGCATATCCATTTCG
	Rev	CAGGGGTCCTCGGTCAATTCGGTGG
U6	For	CTCGCTTCGGCAGCACATATAC
	Rev	TTTGCGTGTCATCCTTGCGC

c) Primers used for mtDNA copy number assay

Target/construct	Orientation	Sequence (5'-3')	Reference
ND2	For	TGTTGGTTATACCCTTCCCGTACTA	[391]
	Rev	CTGCAAAGATGGTAGAGTAGATGA	
Beclin	For	CCCTCATCACAGGGCTCTCTCCA	
	Rev	GGGACTGTAGGCTGGGAACTATGC	

d) Primers used for nested PCR for BGSA and 5hmC TAB-Seq Kit

Target/construct	Orientation	Bisulfite modified sequence (5'-3')	Original sequence (5'-3')
vTR promoter	For-1	TTAATATTTTYGATTAGGGTTAG	TCAATACCTCCGATTAGGGTTA
	Rev-1	AACAAACAATTATACACCTACCT	GACAGACAGTTGTACACCTGCCT
	For-2	GATTAGGGTTAGATATAGYGGAG	GATTAGGGTTAGACACAGCGGAG
	Rev-2	CACCTACCTACACTACTACATCC	CACCTGCCTGCACTACTACATCC
5mC-spike-in control	For	TTTGGGTTATGTAAGTTGATTTTATG	
	Rev	CACCCTACTTACTAAAATTTACACC	
5hmC-spike-in control	For	GTAGATTGTATTGAGAGTGT	
	Rev	TACCCAACTTAATCGCCTTG	

e) Primers used for E-box mutagenesis

Target/construct	Orientation	Sequence (5'-3') (restriction sites are in bold, mutations are underlined)
E-box 2 mutation	For-1 (5'PstI)	**GTGCAG**CCCTAACCCTAACCCCCCAAATTTCACC
	Rev-1	ACGCCCCATGTTTTTGCCCCGCCCCTTCCTG
	For-2	GGGCGGCAAAAACATGGGGCGTGGCGGGA
	Rev-2 (5'HindIII)	**AAGCTT**GCCTTCCACCCGCCACGTGTG
E-box 3 mutation	For (5'PstI)	**GTGCAG**CCCTAACCCTAACCCCCCAAATTTCACC
	Rev (5'HindIII)	**AAGCTT**GCTTCCACCCGCCAAAAATGCCGGGGGAACC

f) Primers used for sequencing

Target/construct	Orientation	Sequence (5'-3')
pGEMT-Easy vector insert	For-M13	TGTAAAACGACGGCCATG
	Rev-M13	CAGGAAACAGCTATGAC
pCpGL-basic vector insert	For	GTTTATGTGAGCAAACAGCAG
	Rev	GCATAGGTGATGTCCACCTC

2.1.3. Results

2.1.3.1. Impact of DNA methylation on the telomerase activity and mitochondrial DNA copy number in the MSB-1 cell line

To study the impact of DNA methylation on telomerase activity in the lymphoblastoid cell line (MSB-1) latently infected and transformed by GaHV-2, telomeric repeat amplification protocol (TRAP) assay was performed. Relative telomerase activity was measured and compared between mock-treated, and 5-azacytidine treated cells at specific time points, as shown in Figure 2.8. The results showed a slight increase in telomerase activity during the first 24h after the treatment with demethylation agent, and notably more potent activity after 48h of exposure to the demethylating agent (Figure 2.8.a). In addition, significantly higher relative expression of the gene encoding the major viral capsid protein VP5, involved in a productive phase of the viral life cycle, confirmed that demethylation using 5-azacytidine induces viral reactivation from latency (Figure 2.8.b).

Due to the increasing evidence suggesting that telomere length and mitochondrial DNA (mtDNA) copy number are positively correlated in cancer, and moreover the indication that mtDNA copy number change during GaHV-2 infection *in vivo* in a specific tissue, we measured mtDNA copy number in two steps of GaHV-2 life cycle *in vitro*. Our results showed that mtDNA copy numbers did not significantly change after the viral reactivation from latency (Figure 2.8.c).

2.1.3.2. Methylation and hydroxymethylation patterns in vTR promoter after virus reactivation *in vitro* and during GaHV-2 infection

With the aim to explore the potential role of DNA methylation in the control of vTR expression, DNA methylation landscape of the vTR promoter was characterized at the critical steps of the viral infection. CpG methylation levels on the vTR promoter, stretching from 5 nucleotides (nt) downstream to 361 nt upstream of TSS (+1) were mapped using Bisulfite Genomic Sequencing

Assay (BGSA) in cell lines representing the productive or latent phase of the viral life cycle (Figure 2.9.a). During the productive phase, represented by CEFs infected with very virulent GaHV-2 RB-1B strain, overall methylation on vTR promoter was close to 15%.

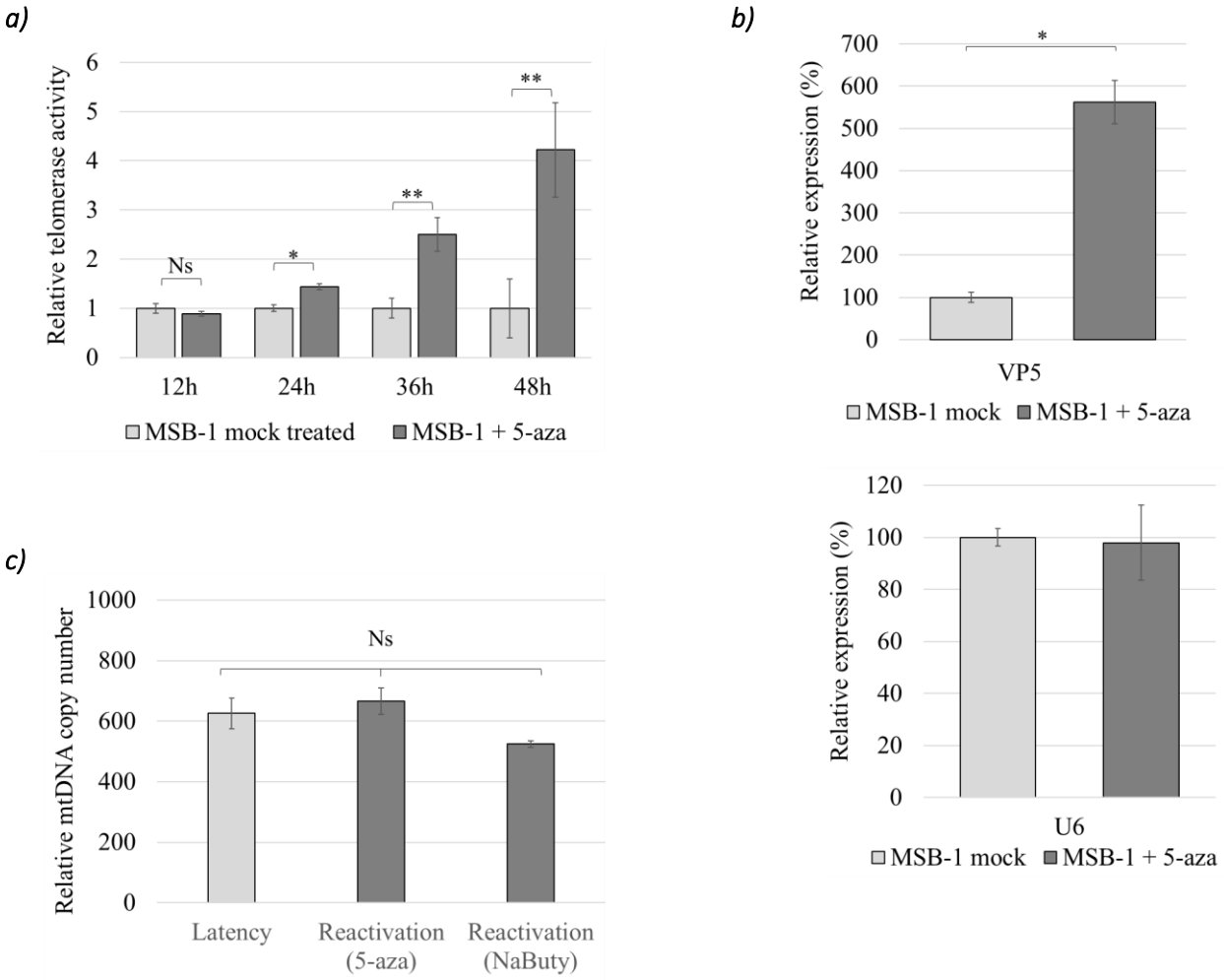

FIGURE 2.8. EFFECT OF DEMETHYLATION AGENT ON RELATIVE TELOMERASE ACTIVITY, REACTIVATION RATE AND MITOCHONDRIAL DNA COPY NUMBER.

a) Induction of viral reactivation in the lymphoblastoid MSB-1 cell line with 5 μM DNA methyltransferase inhibitor (5-azacytidine) during the period of 48 h. Significant increase in telomerase activity during the 48h after the treatment with demethylation agent was observed. **b)** Relative expression of VP5 gene was obtained using the Livak method and is shown relative to the cellular U6 control gene in MSB-1 cells (mock)treated with 5-azacytidine. Similar results were obtained for sodium butyrate treatment **c)** qPCR for mitochondrial DNA (mtDNA) amplification was performed on the gene encoding for ND2 and normalized with the nuclear gene encoding for Beclin. After viral reactivation, there was no significant change in mitochondrial DNA copy number. The determination of the significant difference was performed on triplicates using the student t-test. Comparisons for which $p \leq 0.05$ were considered statistically significant, and were marked with one asterisk, or two if $p \leq 0.005$. If there was no statistical difference, Ns was indicated.

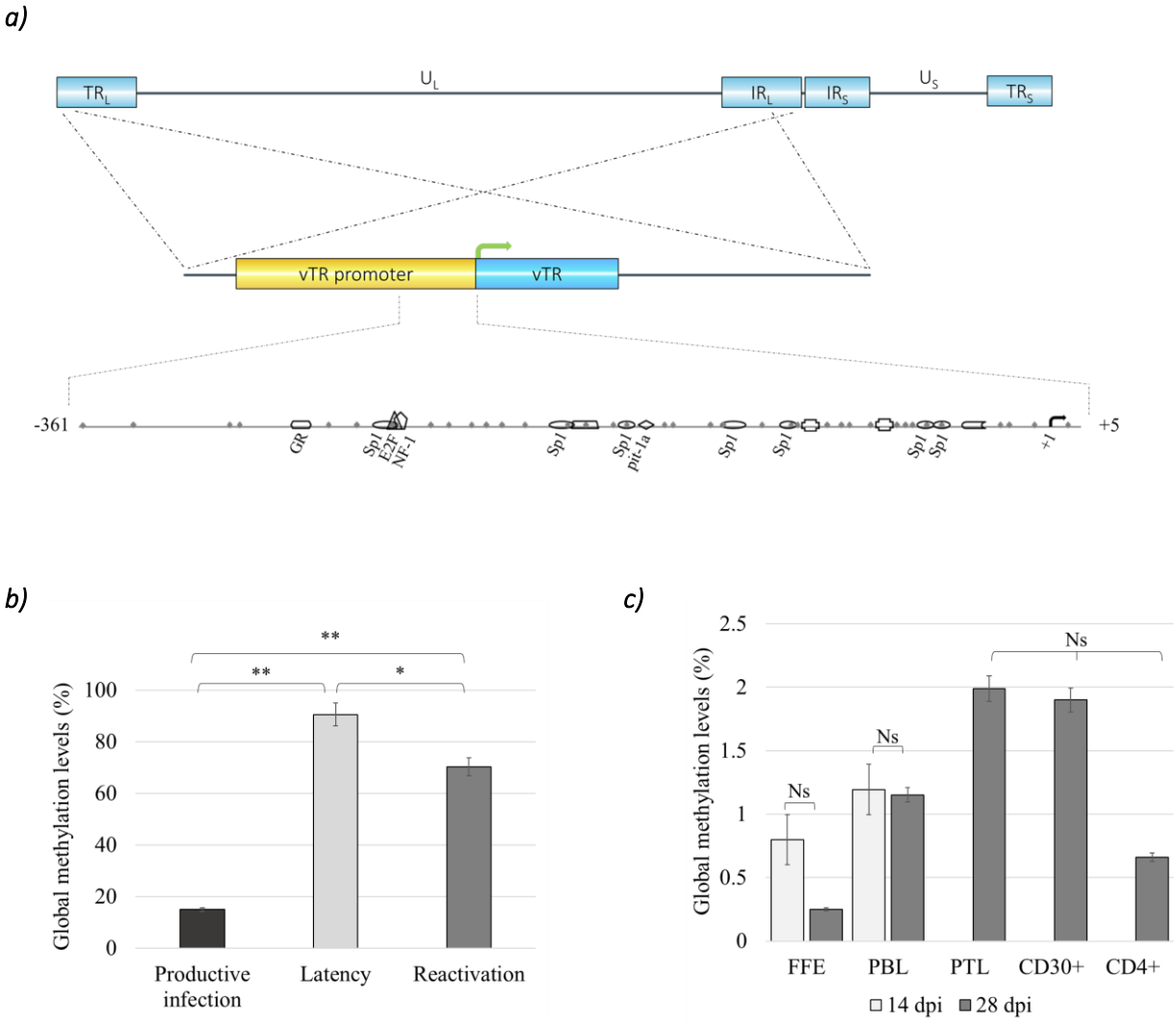

FIGURE 2.9. GLOBAL METHYLATION LEVELS OF THE VTR PROMOTER.

a) Gallid herpesvirus type 2 (GaHV-2) genome consists of unique long UL and unique short US regions flanked by the long terminal (TRL) and internal (IRL) repeats, and terminal (TRS) and internal short repeats (IRS), with the two copies of vTR located within in the TRL and IRL regions. Schematic representation of the studied region of the vTR promoter with 38 CpG positions and specific response elements obtained with Genomatix analysis software is shown. The black arrow represents the transcription start site (TSS). **b)** High 5mC levels were found *in vitro* during latency, up to 90%. Following induction of the reactivation, methylation levels significantly dropped to 70%. **c)** For *in vivo* samples, global methylation was significantly lower, depending on the cell type (feather follicle epithelium (FFE), peripheral blood leukocytes (PBL), peripheral tumour leukocytes (PTL) and sorted $CD4^+$ and $CD30^+$ lymphocytes) and the day post-infection, with no significant change between early and late infection. However, the tendency for reduction of methylation levels could be observed for feather follicle epithelium (FFE) in early and late phases of the viral life cycle. The determination of the significant difference was performed using the student t-test. The determination of the significant difference was performed on 50 randomly selected bacterial colonies using the student t-test. Comparisons for which $p \leq 0.05$ were considered statistically significant, were marked with one asterisk or two if $p \leq 0.005$. If there was no statistical difference, Ns was indicated.

Interestingly, the levels of CpG methylation throughout the vTR promoter in the quiescent virus genome in MSB-1 cells was high, up to 90%, which, upon viral reactivation, dropped down to 70% (Figure 2.9.b), with specific pattern change (Figure 2.10.a).

Methylation levels observed for *in vivo* samples were considerably lower (up to 2%) than *in vitro* and depended on the cell type and dpi. Interestingly methylation levels were higher in $CD30^+$ cells sorted from tumour tissue compared to $CD4^+$ cells. The CpG methylation levels of vTR promoter observed *in vivo* were not significant, however, the tendencies in the change of methylation status of CpG dinucleotides present in the functional c-Myc REs, during GaHV-2 life cycle, were similar to the results obtained *in vitro* (Figure 2.9.c).

To provide a more detailed view of DNA methylation, 38 CpG sites were mapped, together with the response elements within vTR promoter, *in vitro* and *in vivo* (Figure 2.10). Looking into specific methylation positions on the vTR promoter *in vitro*, CpG dinucleotides positioned in transcription start site (TSS) and neighbouring c-Myc RE (E-box 3), demonstrated a significant decrease of methylation, as well as in the area at the upstream end of the vTR promoter. Furthermore, CpG sites surrounding the second c-Myc RE (E-box 2), showed an increase in methylation, compared to the latent state. For the rest of the vTR promoter, CpG methylation levels after reactivation were lower than during latency, however, without significant change (Figure 2.10.a). For the *in vivo* samples obtained from GaHV-2-infected chicken, overall global methylation levels were significantly lower compared to *in vitro* analysis (Figure 2.10.c). DNA methylation patterns on vTR promoter in samples obtained from peripheral blood leukocytes (PBLs) at 14 days post-infection (dpi) showed an increase of methylation in CpG site present in c-Myc RE positioned two nt downstream of TSS when analysed at 28 dpi.

Moreover, for peripheral leukocytes isolated from tumour tissue (PTLs) at 28 dpi methylation in CpG sites in both TSS and upstream c-Myc RE (E-box 2) was recorded (Figure 2.10.c). The change of CpG methylation levels on vTR promoter observed at specified time points *in vivo* were not significant, however, the tendencies in the change of methylation status of CpG dinucleotides present in the functional c-Myc REs, during GaHV-2 life cycle, were similar to the results obtained *in vitro*. These findings indicated the possible role of DNA methylation in the activity of the vTR promoter at the key steps of GaHV-2 infection and were further investigated.

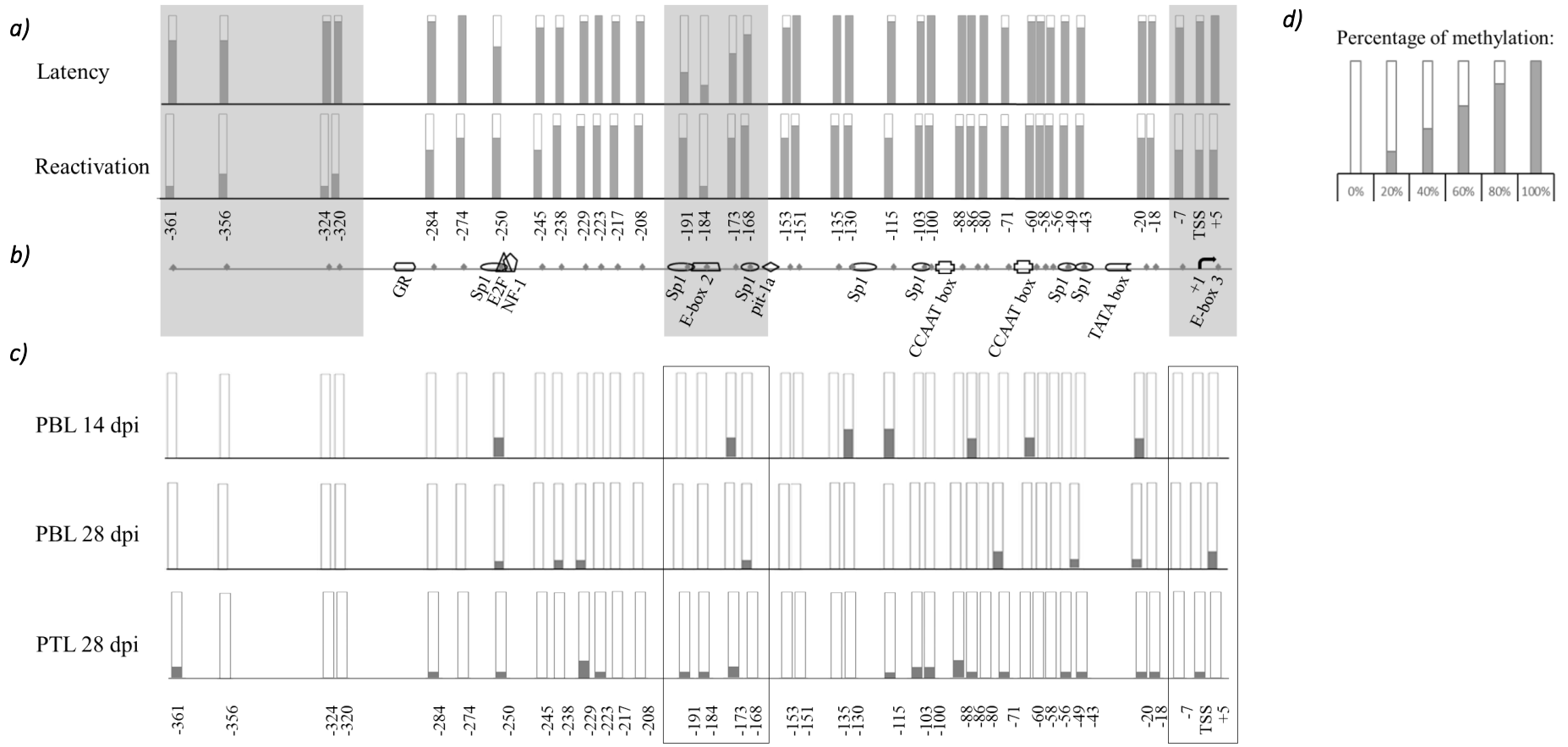

FIGURE 2.10. METHYLATION PROFILES OF VTR PROMOTER OBTAINED BY BGSA.

a) Detailed methylation mapping showed different CpG methylation patterns in latency and after viral reactivation *in vitro*, in areas upstream of transcription start site (TSS) and functional c-Myc response elements (c-Myc REs) in vTR promoter. Grey shaded areas represent sections of the vTR promoter, where significant changes of methylation patterns were observed. **b)** Schematic representation of vTR promoter with CpG positions and specific response elements obtained with Genomatix analysis software. The black arrow represents the transcription start site (TSS). Each CpG position was represented with a white bar numbered referring to the TSS. **(Figure caption continuing on the next page).**

FIGURE 2.10. METHYLATION PROFILES OF VTR PROMOTER OBTAINED BY BGSA (FIGURE ON THE PREVIOUS PAGE).

c) For the *in vivo* samples, DNA from peripheral blood leukocytes (PBLs) was isolated at day 14 and 28 post-infection, and total peripheral tumour leukocytes (PTLs) were isolated at day 28 post-infection. Significantly lower global methylation levels compared to *in vitro* samples were detected, with specific changes in methylation patterns surrounding the areas (in boxes) of c-Myc REs in the vTR promoter, however, the change was not significant. **d)** Legend for methylation percentage for the *in vitro* and *in vivo* samples. Grey colour in bars represents the percentage of methylation for a single CpG position calculated as the rate of isolated cytosines effectively converted into thymine after bisulfite treatment. The determination of the significant difference was performed on 50 randomly selected bacterial colonies using student t-test, comparisons for which $p \leq 0.05$ were considered statistically significant. Fifty randomly picked clones were used for each analysis.

Hydroxymethylation mapping on the vTR promoter measured between the latent and productive phase of GaHV-2 life cycle showed a significantly lower occurrence of 5-hydroxymethylcytosine (5hmC) on CpG dinucleotides compared to 5-methylcytosine. The levels of hydroxymethylation were at 2%, which did not change after the viral reactivation. However, the change in 5hmC positions was observed between latency and after reactivation. Absence of 5hmC was noticed around E-box 2 after reactivation (Figure 2.11.a).

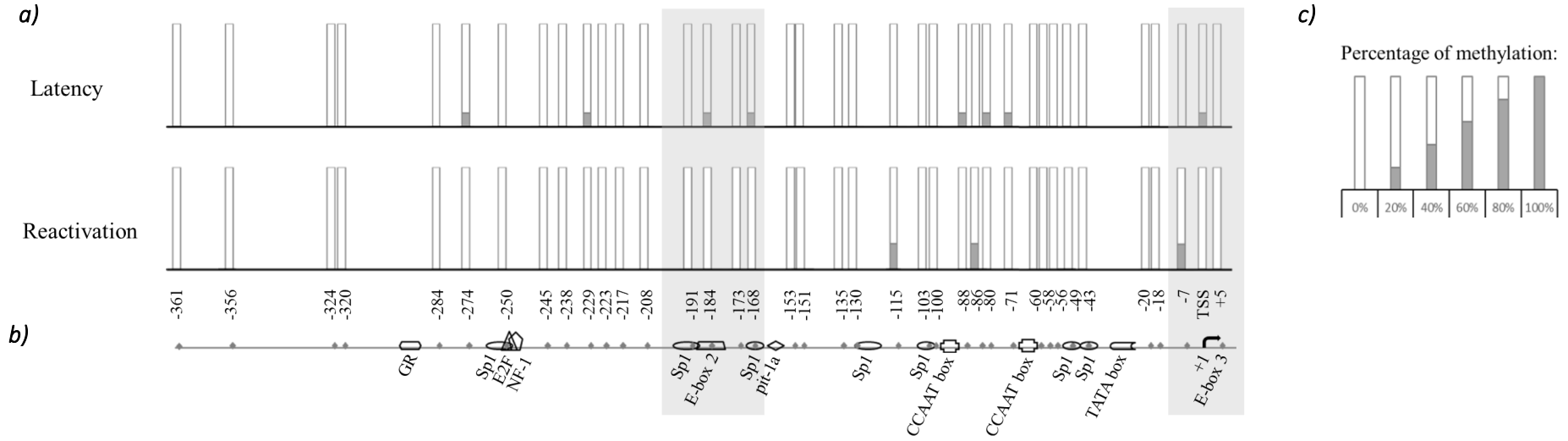

FIGURE 2.11. HYDROXYMETHYLATION PROFILES OF VTR PROMOTER OBTAINED BY 5HMC TAB-SEQ KIT.

a) Hydroxymethylation mapping showed different CpG methylation patterns in latency and after viral reactivation in the MSB-1 cell line. **b)** Schematic representation of vTR promoter with cytosine/guanine (CpG) positions and specific response elements obtained with Genomatix analysis software. The black arrow represents TSS (+1). Each CpG position was represented with a white bar numbered referring to the TSS. **c)** Legend for hydroxymethylation percentage for *in vitro* samples. Grey colour in bars represents the percentage of methylation for a single CpG position calculated as the rate of isolated cytosines effectively converted into thymine after bisulfite treatment. Grey shaded areas represent sections of the promoter, where significant changes of hydroxymethylation patterns were observed. The determination of the significant difference was performed on 35 randomly selected bacterial colonies using student t-test, comparisons for which $p \leq 0.05$ were considered statistically significant.

2.1.3.3. Genome-wide methylation patterns

To compare the genome-wide methylation landscape between key steps of GaHV-2 life cycle, the latently infected and transformed MSB-1 cell line was used. After 48h treatment with DNA methyltransferase inhibitor, reactivation of the quiescent virus genome in MSB-1 cells was monitored by measuring relative expression of the VP5 gene encoding the major viral capsid protein that is involved in a productive phase of the viral life cycle. The RT-qPCR results confirmed that demethylation using 5-azacytidine (5-aza) induces viral reactivation from latency, as presented before.

After extraction of genomic DNA from mock and 5-azacytidine treated MSB-1 cells, bisulfite-treated libraries were submitted to sequencing. The average global DNA methylation levels for quiescent viral genome in mock-treated MSB-1 cell were up to 65%. Interestingly, higher levels of CpG methylation during latency were observed in the region of the GaHV-2 genome encoding for the lytic phase protein pp38, and viral telomerase RNA subunit (vTR) compared to the region encoding for latency-associated transcripts (LATs) and major viral oncogene (Meq) (Figure 2.12.a).

After induction of viral reactivation, total average genome-wide DNA methylation dropped down to 29%. Moreover, specific methylation landscapes were observed between the latent and productive phase of the viral life cycle. The significant CpG hypomethylation was recorded on the promoters thought the viral genome after reactivation (Figure 2.12.b). Interestingly, only single locus showed hypermethylation after reactivation compared to latency. For the promoter of the gene coding for viral interleukin-8 (vIL-8), an average increase of DNA methylation of 58% was observed.

In addition, the promoters of the viral structural genes located in the unique long (UL) and short (US) segments of the genome were hypomethylated after viral reactivation compared to latency (Figure 2.12).

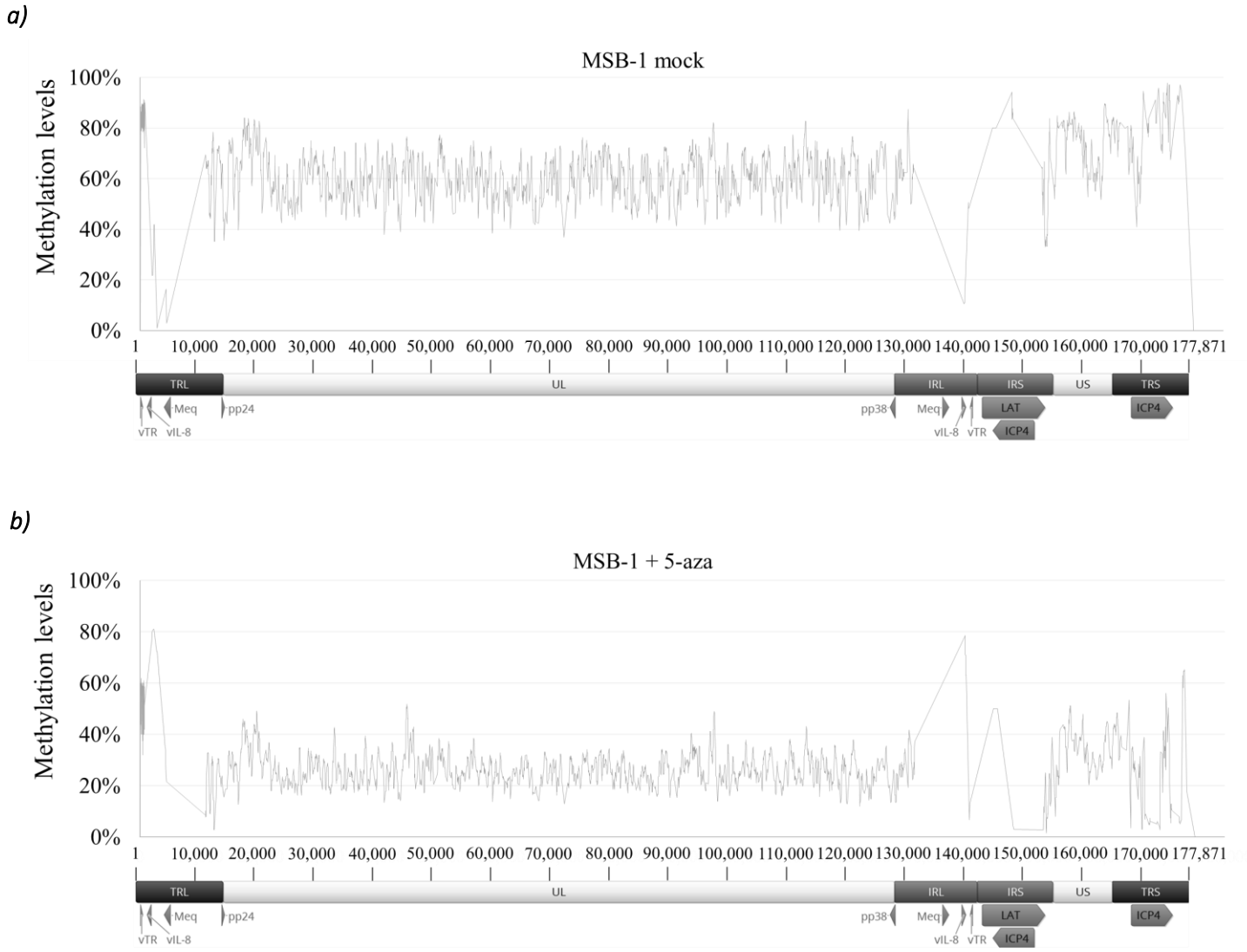

FIGURE 2.12. COMPARISON OF DNA METHYLATION LANDSCAPES BETWEEN TWO KEY STEPS OF GALLID HERPESVIRUS 2 LIFE CYCLE (FIGURE CAPTIONS ON THE NEXT PAGE).

FIGURE 2.12. COMPARISON OF DNA METHYLATION LANDSCAPES BETWEEN TWO KEY STEPS OF GALLID HERPESVIRUS 2 LIFE CYCLE (FIGURE ON THE PREVIOUS PAGE).

a) Genome-wide methylation percentages were recorded on the quiescent viral genome in mock-treated MSB-1 cells. **b)** Treatment with the inhibitor of the DNA methyltransferase 5-azacytidine (5-aza) induced viral reactivation and a significant decrease in overall DNA methylation landscape, highlighting the hypermethylation in a single locus of the viral interleukin-8 (vIL-8) promoter. Representation of the Gallid herpesvirus 2 genome organisation in base pairs (obtained with Geneious software) is indicated below each DNA methylation profile.

2.1.3.4. Impact of methylation on the activity of the vTR promoter

To address the effect of methylation on the activity of vTR promoter, luciferase promoter-reporter assay was performed. A plasmid backbone was used that lacks CpG sites except for the ones present in the target promoters. After M.SssI CpG methyltransferase hypermethylation, the relative activity of vTR promoter was measured in three chicken cell lines, MSB-1, DF-1 and LMH, as well as human HeLa cells (Figure 2.13). Hypermethylation of vTR promoter showed a significant decrease in relative activity in all cell lines, compared to non-methylated ones. In the case of the control promoter, EF1/CMV devoided of CpG dinucleotides and thus insensitive to methylation, no significant change in the activity between methylated and non-methylated promoters were observed (Figure 2.13.a). The efficiency of M.SssI treatment for vTR promoter was confirmed by digestion using methylation-sensitive restriction enzyme HpaII. Non-methylated promoters were digested with the HpaII, while no enzymatic digestion was observed in the presence of methylated promoters (Figure 2.13.b).

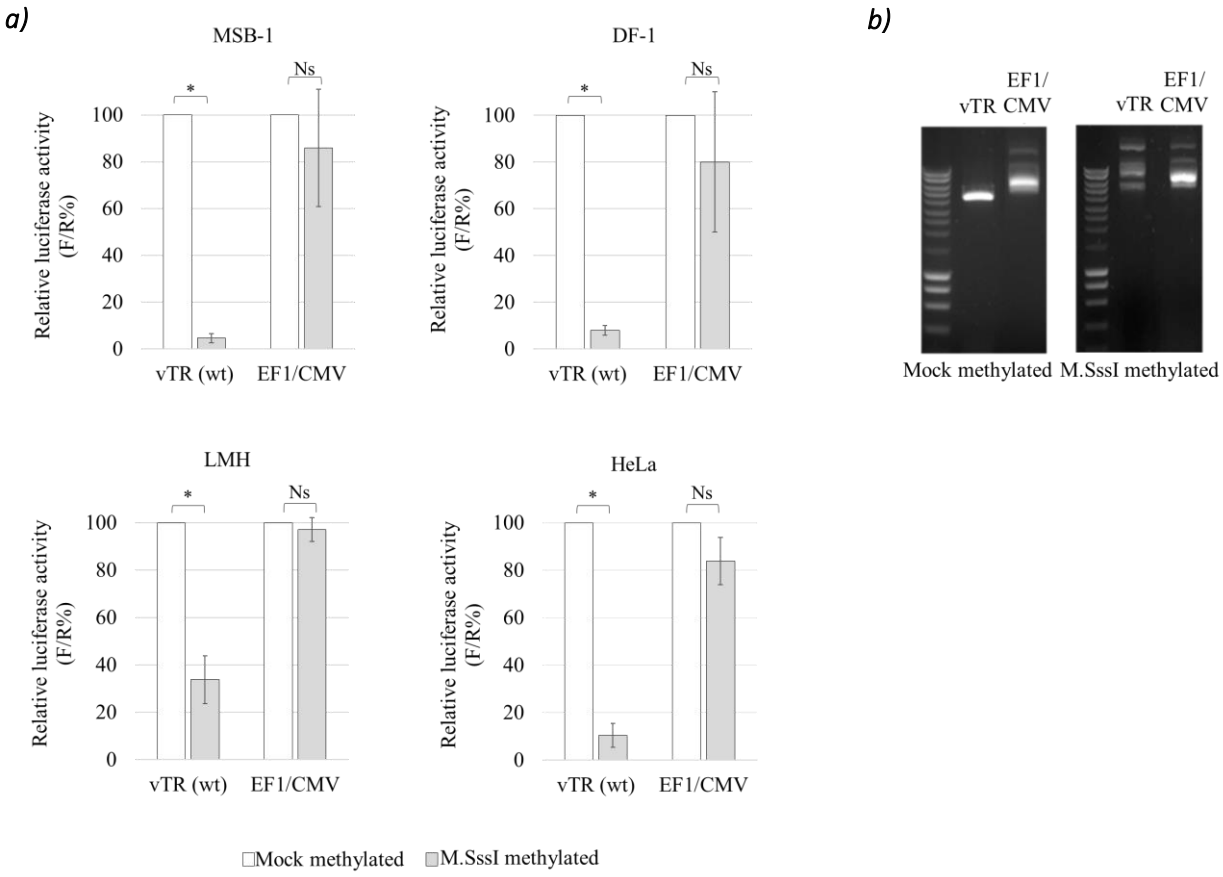

FIGURE 2.13. EFFECT OF METHYLATION ON THE VTR PROMOTER ACTIVITY.

a) After hypermethylation with DNA methyltransferase (M.SssI), the relative activity of vTR promoter was measured in different cell lines: MSB-1, DF-1, LMH, HeLa. Luciferase activity was quantified with the Dual-Luciferase Reporter Assay System, and the results are presented as Firefly/Renilla percentage (F/R%). The hybrid promoter EF1/CMV known to be insensitive to the methylation was used as a control. **b)** Methylation sensitive restriction enzyme (HpaII) was used to confirm the success of hypermethylation on vTR plasmid constructs. Effect of restriction digestion with the HpaII was shown for mock methylated plasmids and M.SssI methylated plasmids. The significantly different values obtained with student t-test (n = 3) for the vTR promoter are indicated by one asterisk if the value of $p \leq 0.05$. If there was no significant difference, Ns was indicated

2.1.3.5. Methylation process masks the effect of site-directed mutagenesis of the c-Myc binding sites

Previously presented CpG methylation mapping revealed specific methylation changes in functional c-Myc REs of the vTR promoter. To further characterize the effect of methylation on c-Myc transcription factor binding sites, functional c-Myc REs of vTR promoter were mutated using site-directed mutagenesis, obtaining E-box 2 (E2), E-box 3 (E3) and double E2E3 mutants (Figure 2.14). The luciferase reporter promoter assay was used to study the activity of mutated versus wild-type vTR promoters (Figure 2.14.a). In chicken cell lines MSB-1, DF-1 and LMH in unmethylated conditions, luciferase activity showed that the E2 mutation alone did not affect vTR expression. On the other hand, E3 and E2E3 mutations showed repression of vTR promoter activity, compared to the wild-type promoter (Figure 2.14.b). There was no significant difference between the relative luciferase activities measured for the E3 mutated vTR promoter and double mutant E2E3. The significant difference between the relative luciferase activity of the mutated E2 promoter and that of the double mutant E2E3 suggests that the E3 box is more functional in LMH, MSB-1 and DF-1. For the HeLa cell line, used as a non-homologous GaHV-2 control, only E2 mutation in the vTR promoted did not follow the same activity pattern, highlighting the difference between human and chicken cell lines and response elements (Figure 2.14.b).

More intriguingly, the luciferase activity measured for methylated and mutated E2, E3 and E2E3 promoters in MSB-1 and DF-1 as well as in LMH cells was significantly higher compared to methylated wild-type promoter (Figure 2.14.c). In the HeLa cell line, there was no significant change in the activity of the methylated promoters, except in double E-box mutant (Figure 2.14.c). The efficiency of M.SssI treatment for mutated vTR promoters was confirmed by digestion using methylation-sensitive restriction enzyme HpaII. Non-methylated promoters were digested with the HpaII. However, in the presence of methylated promoters, lack of the enzymatic digestion was observed (Figure 2.14.d). These results showed that the E3 box is involved in regulating transcription of the vTR and indicated that the methylation process masked the effect of site-directed mutagenesis of the c-Myc binding site.

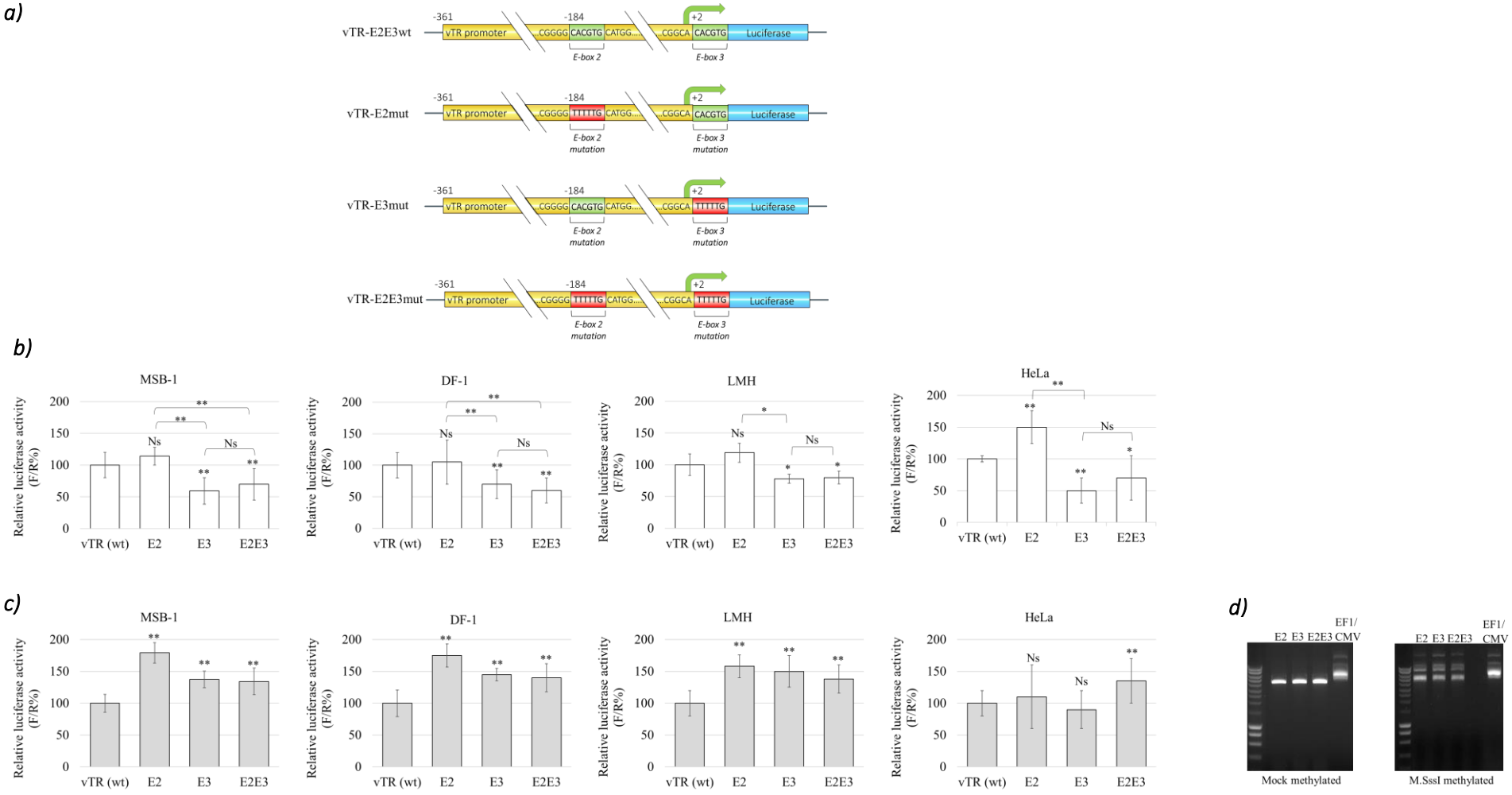

FIGURE 2.14. EFFECT OF METHYLATION ON MUTATED VTR PROMOTER CONSTRUCTS (FIGURE CAPTIONS ON THE NEXT PAGE).

FIGURE 2.14. EFFECT OF METHYLATION ON MUTATED VTR PROMOTER CONSTRUCTS (FIGURE ON THE PREVIOUS PAGE).

a) Specific response elements of the vTR promoter were studied through the mutagenesis of the E-boxes binding sites for the c-Myc transcription factor. E-box mutations were generated by PCR site-directed mutagenesis obtaining E2, E3 and E2E3 mutants. Luciferase activity was quantified with the Dual-Luciferase Reporter Assay System, and the results are presented as Firefly/Renilla percentage (F/R%). The relative activity mutated vTR promoters was measured in MSB-1 and DF-1 cell lines in **(b)** non-methylated conditions and **(c)** after hypermethylation with DNA methyltransferase (M.SssI). **d)** The hybrid promoter EF1/CMV known to be insensitive to the methylation was used as a control. Methylation sensitive restriction enzyme (HpaII) was used to confirm the success of hypermethylation on vTR plasmid constructs. Effect of restriction digestion with the HpaII was shown for mock methylated constructs and M.SssI methylated constructs. The significantly different values obtained with student t-test ($n = 3$) for the vTR promoter are indicated by two asterisks ($p \leq 0.005$). If there was no significant difference, Ns was indicated.

2.1.4. Acknowledgements

I am grateful to André Claude Mbouombouo Mfossa (Integrated Veterinary Research Unit, University of Namur) for construction and analysis of vectors bearing site-directed mutation on the vTR promoter. I thank Catherine Demazy (Department of Biology, University of Namur) and Jonathan Marescaux (Research Unit in Environmental and Evolutionary Biology, University of Namur) for the help with capillary electrophoresis analysis and Applied Biosystems 3130xl Genetic Analyzer used for TRAP assay. I especially thank Morgane Canonne (Laboratory of Cellular Biochemistry and Biology, University of Namur) for the help with mitochondrial DNA copy number assay. I am thankful for the support on the coordination of the genome-wide methylation mapping project (Benelux Team) from the Novogene Co., LTD.

2.2. Functional evaluation of c-Myc response elements in the promoter of the viral telomerase RNA subunit in a model of herpesvirus induced lymphomagenesis

2.2.1. Introduction

Previously obtained results presented in section 2.1., showed discriminative DNA methylation signatures of the vTR promoter at crucial steps of GaHV-2 infection and substantial impact of demethylation on telomerase activity. Two specific areas surrounding two functional c-Myc response elements (E-box 2 and E-box 3) showed a significant change in DNA methylation after viral reactivation. Thus, to study the importance of the c-Myc binding sites in virus-induced tumorigenesis, a recombinant virus bearing mutations in c-Myc response elements (c-Myc REs), as well as revertant, were generated using the bacterial artificial chromosome of a highly oncogenic strain (pRB-1BΔIRL) using two-step Red-mediated mutagenesis. In this study, the impact of c-Myc REs mutations was tested *in vivo* by infecting a susceptible $B^{13}B^{13}$ chicken line with prepared viral inoculums. To assess GaHV-2 replication and telomerase activity over time, blood and feather follicle epithelium (FFE) were collected at specific time points in infected chickens. Animals were daily monitored for the clinical symptoms of the disease, and euthanized 55 days-post infections, to assess the number of tumours developed in visceral organs.

Introduced mutation in vTR promoter did not affect the replication properties of the recombinant viruses *in vitro*. In contrast, replication and of the mutant virus in infected chickens was severely impaired and tumour formation completely abrogated. Our data provided a more in-depth characterisation of c-Myc oncoprotein REs and DNA methylation involvement in transcriptional regulation of vTR.

2.2.2. Materials and Methods

2.2.2.1. Cell lines

Embryonic stem cell-derived line-1 (ESCDL-1), a mesenchymal cell line used as a GaHV-2 productive infection model [397] was cultured in a supplemented Dulbecco's Modified Eagle Medium (DMEM F12 1:1) supplemented with 10% foetal bovine serum (FBS), 1% penicillin (50 units/mL) and streptomycin (50 µg/mL), 1% non-essential amino acids and 1% sodium pyruvate. Cells were maintained at 37 °C under 5% CO_2. This cell line was generously provided by Dr. Caroline Denesvre (INRA-Tours).

The chicken embryo fibroblasts (CEFs) were obtained from eleven days old chicken embryos treated by trypsinization (Lonza). The primary CEFs were cultured in Dulbecco's modified eagle medium (DMEM) (Lonza) supplemented with 2.5% FBS, 1.25% chicken serum (CS), 1% penicillin (50 units/mL) and streptomycin (50 µg/mL), 1% fungizone (GIBCO), 1.475 g/L tryptose phosphate (Sigma). Four days after primary CEFs culture, cells were passaged to produce secondary CEFs. The CEF cell cultures were maintained at 41 °C under 5% CO_2.

2.2.2.2. Bacterial artificial chromosomes and plasmids

The backbone for recombinant GaHV-2 virus construction, carrying E-box 2 and E-box 3 mutations in the vTR promoter (vTR-E2E3mut), was the bacterial artificial chromosome (BAC) of highly oncogenic RB-1B strain. BAC used was lacking part of the internal repeat long region that is restored upon virus reconstitution (pRB-1BΔIR_L) [315]. Initially, approximately 10 kbp of the long internal repeat (IR_L) of pRB-1B was deleted, leaving 0.5 kbp at the left end and 1.5 kbp at the right end of the IR_L intact to allow restoration of the sequence *via* homologous recombination during GaHV-2 replication.

Thus, the BAC containing one copy of vTR region was subjected to two-step Red-mediated mutagenesis, resulting in reconstituted recombinant virus containing the desired mutation in both vTR loci as previously described [398].

The pEP-Kan-S2 plasmid was developed [398] by addition of AphaI gene conferring kanamycin resistance surrounded by two restriction sites of I-SceI enzyme into pcDNA3 plasmid (Figure 2.15). In order to introduce the desired modifications into the BACs, a resistant marker AphaI was used as a starting point for the PCR amplification.

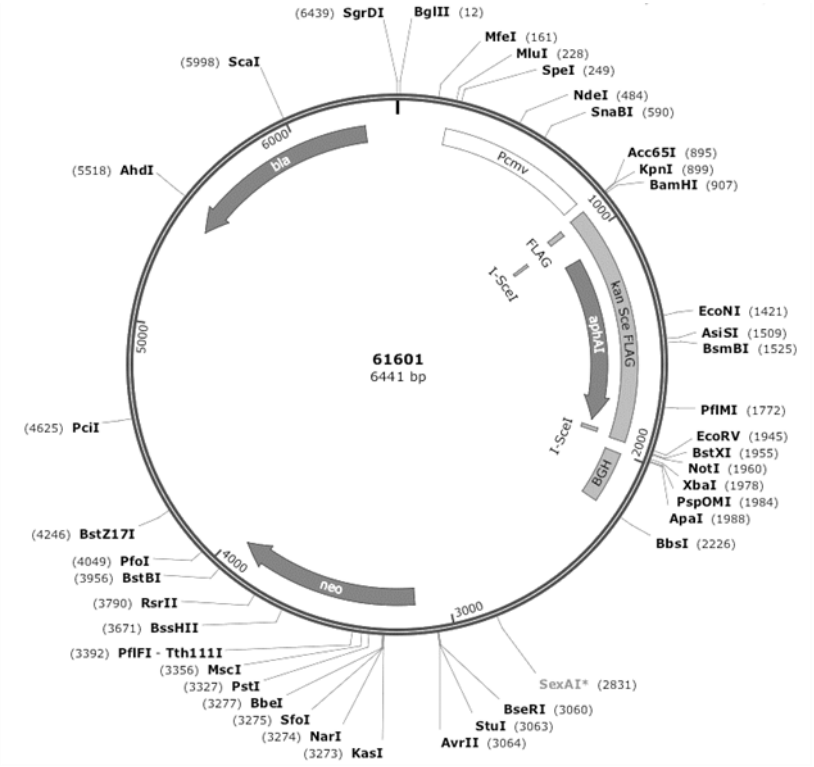

FIGURE 2.15. PEP-KAN-S2 PLASMID MAP.

6441 bp long pEPkan-S2 plasmid contains the AphaI gene giving a resistance to kanamycin surrounded by I-SceI restriction sites. In addition, the plasmid contains a neomycin resistance gene (neo) and a beta-lactamase gene (bla), giving resistance to ampicillin (Tischer et al., 2006).

2.2.2.3. Preparation of electro-competent *E. coli* strain GS1783

The *E.coli* strain GS1783, derived from DH10B strain, is genetically modified and harbours in its genome two inducible promoters. First, this strain expresses the Red-recombination system under a temperature-inducible promoter that is activated at 42 °C [398]. In addition, GS1783 strain expresses I-SceI, which expression is regulated under an arabinose inducible promoter. I-SceI is an endonuclease originated from *Saccharomyces cerevisiae* and contains 18 bp restriction site, which is rarely present in genomic sequences.

The electro-competent bacteria were grown O/N at 32 °C in 1 ml LB medium with chloramphenicol (Chl, 34 mg/ml). The following day, 200 ml of warm fresh LB medium (supplemented with Chl) were inoculated with the primary O/N culture and incubated at 32 °C under shaking (220 rpm), until the logarithmic growth phase (OD_{600}) reached between 0.5 - 0.7. Next, the heat shock was performed to activate the Red-recombination system. First, bacteria were heated for 15 minutes at 42 °C under shaking (220 rpm) followed by rapid cooling for 20 minutes in the water-ice bath while shaking (220 rpm). Following the activation step, the bacterial culture was centrifuged (5,000 rpm, 15 minutes at 4 °C), and the pellet was washed three times, with 10 ml of ice-cold 10% glycerol. Finally, the bacteria were re-suspended in 1 ml of 10% glycerol, aliquoted in 1.5 ml Eppendorf tubes (50 µl/tube) and snap-frozen for storage at -80 °C.

In this study, Red-recombination system was used to generate recombinant GaHV-2 virus that carries mutations of c-Myc binding sites in vTR promoter.

2.2.2.4. Construction of GaHV-2 recombinant viruses using two-step Red-mediated mutagenesis

The recombinant viruses were generated using a two-step Red-mediated mutagenesis system expressed by electro-competent GS1783 strain of *E.coli* as previously described [398]. The Red system from λ phage allows the insertion of linear double-stranded DNA molecules using a homologous recombination system (Figure 2.16).

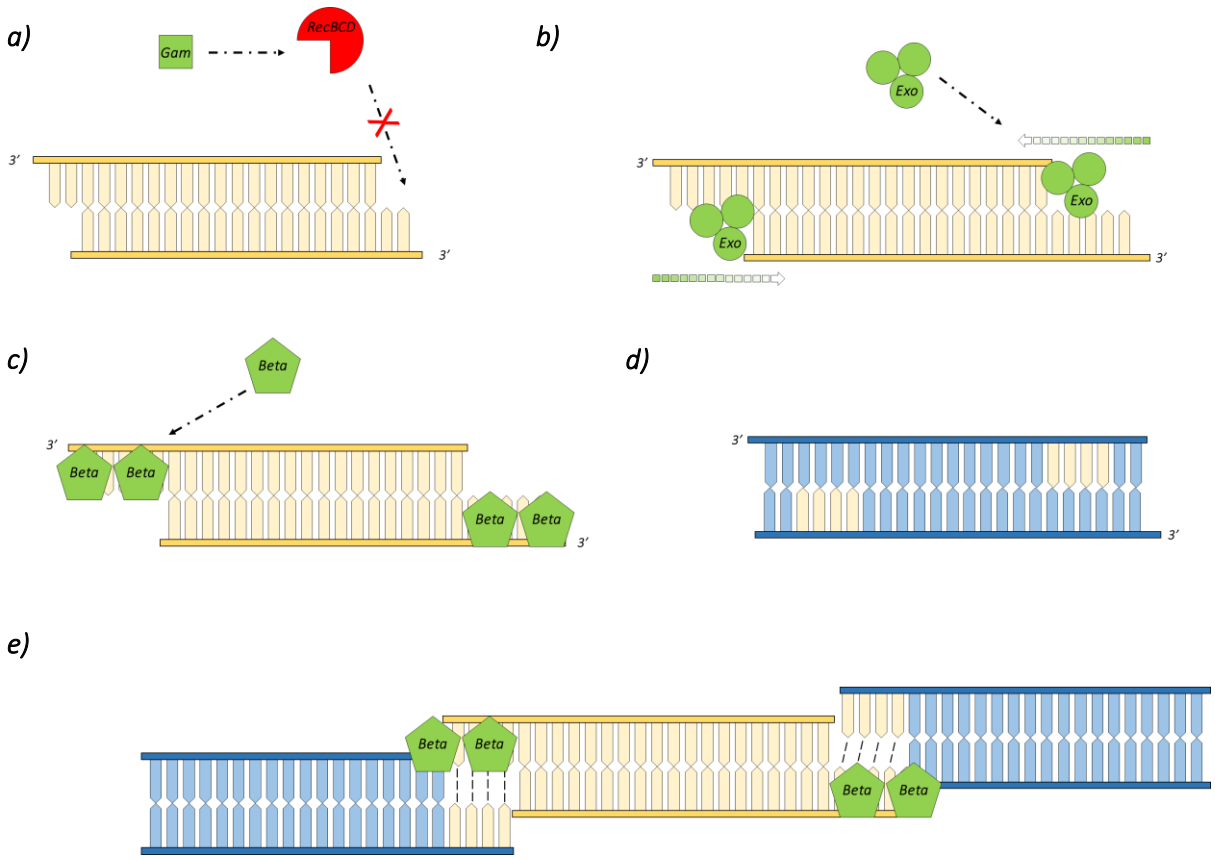

FIGURE 2.16. SCHEMATIC PREVIEW OF λ RED RECOMBINATION SYSTEM.

Double-stranded DNA serves as the backbone for the Red recombination system. **a)** *Gam* protein inhibits bacterial RecBCD helicase-nuclease complex and linear DNA degradation. **b)** *Exo* protein in the form of homotrimer possesses 5' - 3' exonuclease activity and produces 3' single-strand extensions. **c)** The *Beta* protein binds to the newly synthesized single-stranded DNA and anneals it to **(d)** complementary sequences of the target DNA, finalising the integration **(e)**.

There are three main actors of the Red system named Exo, Beta, and Gam. Firstly, the Gam protein blocks the RecBCD helicase–nuclease complex of the *E. coli* that degrades linear DNA from both free ends (Figure 2.16.a). Secondly, the 5'–3' exonuclease homotrimer Exo, produces a 3' single-strand extension (Figure 2.16.b), which is protected by the single-strand binding activity of the Beta protein. In addition, Beta anneals the single strand end obtained from the linear DNA with complementary sequences of the target DNA (Figure 2.16.c). This results in the integration into replicating DNA (Figure 2.16.d and e). However, in order to successfully introduce non-selectable sequences, such as promoters or reporter genes, and to produce "scarless" mutations or deletions, a positive selection marker should be used. These groups of markers

guarantee high efficiency of the Red recombination. One of the most commonly used selection markers is I-SceI.

In this study, the Red-recombination system was used to construct recombinant GaHV-2 mutant that carries a mutation in the CpG dinucleotides of the c-Myc oncoprotein binding sites, namely E-box 2 and E-box 3 (Figure 2.17).

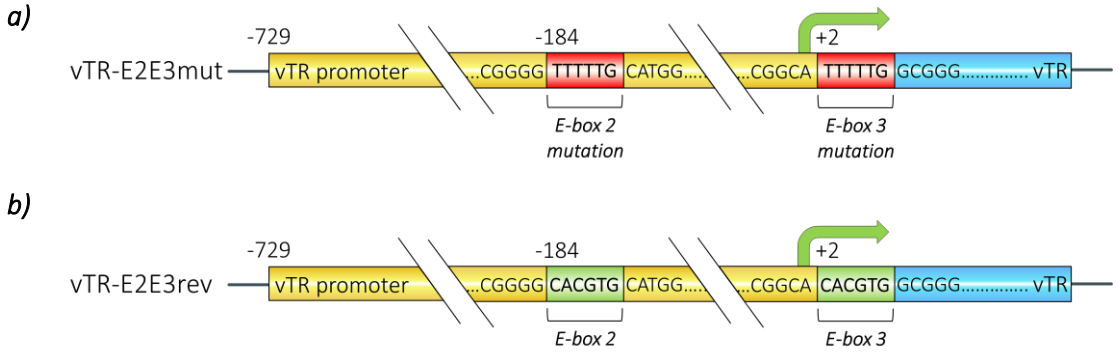

FIGURE 2.17. INTRODUCTION OF THE MUTATIONS IN THE E-BOX 2 AND E-BOX 3 IN THE VTR PROMOTER.

a) Mutations in the E-boxes of the vTR promoter were introduced by two-step Red-mediated mutagenesis using a bacterial artificial chromosome (BAC) of highly oncogenic RB-1B strain lacking part of the internal repeat long region (pRB-1BΔIRL). **b)** Using the produced mutated BAC as a backbone, revertant bacmid was produced containing the wild-type sequence.

In detail, the sequence of interest was flanked by homologous extensions and PCR-amplified using a kanamycin-resistance (AphaI) cassette from a pEP-Kan-S2 plasmid (Figure 2.18.a and b) with primers presented in Table 2.2.a. The point-mutagenesis of the E-box 2 was finalised first, followed by the E-box 3 mutation. To ensure fidelity of the PCR products, amplification with the Q5®Hot start High-Fidelity DNA polymerase (NEB) was used. High-fidelity PCR was performed as follows: 98 °C for 3 minutes, followed by 35 cycles of denaturation (98 °C, 1 minute), annealing (55 °C, 1 minute), and extension (72 °C, 1 minute) (Figure 2.18.b). PCR products were analyzed by agarose gel electrophoresis.

In the next step, electro-competent GS1783 strain of *E.coli*, prepared as previously described, were electroporated with 100 ng of purified PCR product (2500 V, 5 milliseconds), seeded on kanamycin/chloramphenicol rich LB agar plates in different dilutions and incubated at 32 °C for 48 hours. Here, a first Red mediated recombination step resulted in the integration of the whole resistance cassette into the target site, and correct intermediates were identified as viable bacterial colonies on kanamycin/chloramphenicol LB agar plates (Figure 2.18.c). The kanamycin-resistant colonies were isolated and confirmed by screen PCR targeting kanamycin cassette. Screen PCR was performed using the GoTaq® DNA Polymerase (Promega) and primer pairs targeting inserted construct (Table 2.2.b) and was performed as follows: 94 °C for 5 minutes, followed by 35 cycles of denaturation (94 °C, 1 minute), annealing (30 °C, 1 minute) and extension (55 °C, 1 minute). Clones positive for amplicon of interest were re-confirmed using Sanger sequencing on targeted vTR locus and restriction fragment length polymorphism (RFLP) method.

After that, in the second recombination step, the expression of I-SceI was induced in the presence of 1% L-(+)-arabinose that resulted in the cleavage of the I-SceI site and complete removal of the kanamycin-resistant cassette (Figure 2.18.d). Briefly, an O/N culture of positive clones obtained after the first Red mediated recombination were grown at 37 °C under shaking (220 rpm). Next, 2 ml of warm fresh LB medium with chloramphenicol (but without selection for the kanamycin) were inoculated with 100 μl of a fresh overnight culture and grown for four hours at 32 °C and shaking (220 rpm) until early logarithmic growth phase. Following first incubation, 2 ml of warm LB medium with chloramphenicol and 1% arabinose were added to the culture and incubated for 60 minutes at 32 °C under shaking (220 rpm) to induce I-SceI expression. The produced double-stranded DNA end with the adjoining duplex sequence now serves as a substrate for second Red recombination, which removes the complete marker cassette (Figure 2.18.e). In order to induce the expression of the Red recombination system, the bacterial culture was transferred into 42 °C water bath under shaking (220 rpm) for 30 minutes. After the heat shock, bacterial culture was returned to 32 °C under shaking (220 rpm) for another 4 hours followed by the bacterial seeding on chloramphenicol and 1% arabinose LB agar plates in different dilutions and incubated at 32 °C for 48 hours.

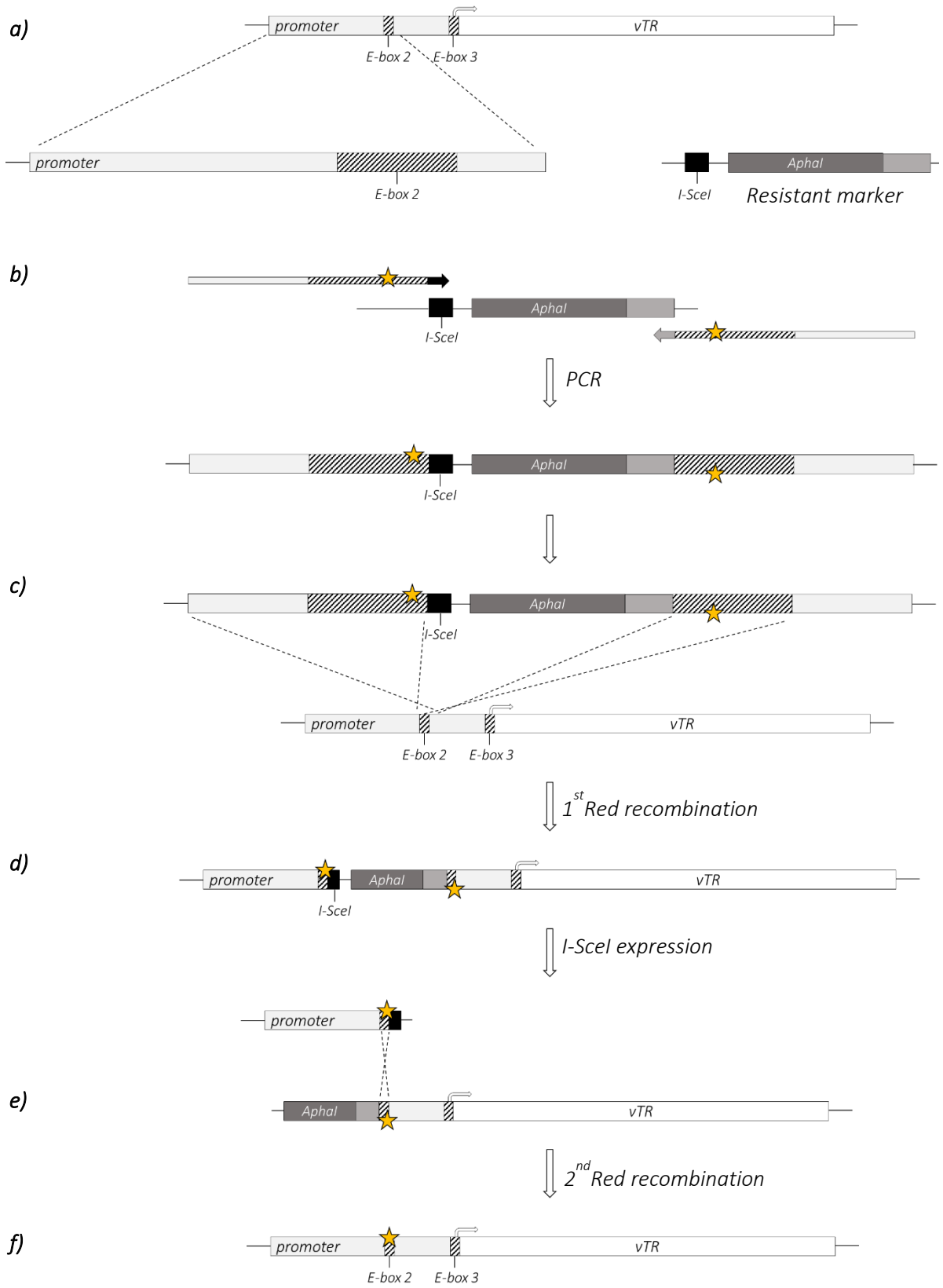

FIGURE 2.18. EXAMPLE OF STEP-BY-STEP POINT-MUTANT GENERATION BY THE RED RECOMBINATION PROTOCOL USED TO INSERT MUTATION IN THE E-BOX 2 OF VTR PROMOTER (FIGURE CAPTIONS ON THE NEXT PAGE).

FIGURE 2.18. EXAMPLE OF STEP-BY-STEP POINT-MUTANT GENERATION BY THE RED RECOMBINATION PROTOCOL USED TO INSERT MUTATION IN THE E-BOX 2 OF VTR PROMOTER (FIGURE ON THE PREVIOUS PAGE).

a) The vTR promoter E-box 2 (pattern rectangle) was a target for the introduction of the desired mutation in the CpG dinucleotide presented in the E-box 2 sequence. The I-SceI-AphaI cassette from the pEP-Kan-S2 plasmid was used to introduce the kanamycin resistance marker into the backbone bacmid. **b)** Primer pair bearing E-box 2 mutation (yellow star) was constructed containing the overlapping sequences for both I-SceI-AphaI cassette (black and grey arrow) and vTR promoter (grey rectangles). The primers were used for the I-SceI-AphaI cassette PCR amplification. **c)** During the first recombination step, the complete resistance cassette construct was integrated into the target site, and the intermediates were identified using kanamycin antibiotic resistance. **d)** The expression of the inducible I-SceI lead to the cleavage of the I-SceI site and resulted in the double-stranded DNA end with the adjoining duplex sequences. **e)** Finally, the second Red recombination removes the entire resistance marker cassette finalising the mutagenesis **(f)**.

Finally, the random clones were tested for the kanamycin resistance cassette excision on LB double replica plates with chloramphenicol and kanamycin/chloramphenicol (Figure 2.18.f) followed by screen PCR, Sanger sequencing of the targeted vTR locus and RFLP screening analysis using multiple restriction enzymes. The same principle was used to revert the mutated bacmid into the original wild-type sequence. In addition, to ensure the genome integrity after several mutagenesis steps, the mutant and revertant BAC constructs were analyzed using pair-end high throughput sequencing on Illumina® sequencing platform (Novogene).

2.2.2.5. High-throughput bacmid DNA sequencing

DNA samples were qualified by agarose gel electrophoresis analysis for DNA purity and integrity, and Qubit® 3.0 fluorometer quantitation for accurate measurement of DNA concentration.

Sample DNA with a total amount of more than 500 ng was qualified for library preparation. The genomic DNA of each sample was randomly sheared into short fragments around 350 bp, and the obtained fragments were subjected to library construction using the NEBNext® DNA Library Prep Kit. Briefly, as followed by end repairing, dA-tailing, and further ligation with

NEBNext adapter, the required fragments (in 300-500 bp size) were PCR enriched by P5 and indexed P7 oligonucleotides, followed by purification and subsequent quality check.

To check the prepared DNA libraries, Qubit® 2.0 fluorometer was firstly used to determine the concentration of the library. After dilution to 1 ng/μl, the Agilent® 2100 bioanalyzer was used to assess the insert size.

Finally, the qPCR was performed to detect the effective concentration of each library. The qualified DNA libraries were submitted to the pair-end sequencing were performed on Illumina® sequencing platform, with the read length of PE150 bp at each end (Novogene).

2.2.2.6. ESCDL-1 cell transfection with constructed bacmids

ESCDL-1 cells were transfected by using the Amaxa™ Nucleofector™ technology (Lonza) with the Basic Fibroblast kit (ref VPI-1002) and program F024 as described before [397]. Briefly, 800,000 ESCDL-1 cells were transfected with 2 μg of constructed bacmids and seeded in 6-well plates coated with 2% gelatine using. Six hours after transfection, the medium was changed to DMEM F12 (1:1) supplemented with 1.5% CS and 1% FBS and the cells were incubated overnight (O/N) at 37 °C under 5% CO_2. The next day, the medium was changed to full DMEM F12 (1:1) supplemented with 10% FBS, 1% penicillin (50 units/mL) and streptomycin (50 μg/mL), 1% non-essential amino acids and 1% sodium pyruvate. The transfected cells were passed three times together with the fresh ESCDL-1 cells followed by stock preparation in DMEM F12 (1:1) with 10% dimethyl sulfoxide (DMSO) that was stored first at -80 °C and finally in liquid nitrogen.

2.2.2.7. Bacmid titration on ESCDL-1 cells

The bacmid constructs generated expressed Green Fluorescent Protein (GFP) signal, and because of that, the recombinant BAC constructs were used to assess viral replication properties *in vitro* and were fully reconstituted afterwards for animal experiment.

In order to assess the replication properties of the constructs, a titration was performed in order to obtain plaque-forming units (PFUs). PFUs represent the number of units capable of

initiating the formation of a plaque in cell culture. These units are a fraction of the previously infected cells in which GaHV-2 was replicating. This fraction of cells is the one in which the ratio between cell viability and virus content is adapted to transfer the viral infection *via* cell-to-cell spreading, thereby initiating the formation of a plaque. Briefly, 24 well plates were seeded with 100, 000 non-infected ESCDL-1 cells per well. 24h. After seeding, the cells were inoculated with serial dilutions (1/10, 1/50, 1/250, 1/1250 and 1/6250) of previously stocked bacmid-transfected ESCDL-l. Co-infected cells were then incubated for 48h to allow plaques (foci) to form by cell-to-cell spread. To reveal the plaque formation, cells were fixed with 4% paraformaldehyde. The number of plaques was determined by using an epifluorescence microscope and counted at dilutions where individual foci were observed. The titers were expressed as the mean PFU/ml according to the formula:

$$\text{PFU/ml} = [(\text{average n° of plaques} \times \text{dilution}) / \text{volume per well}] \times 1000$$

2.2.2.8. Plaque size assay

To assess the viral spread in cell culture, plaque size assay was used as described previously [399]. 1×10^6 ESCDL-1 cells were infected with 100 PFU of the corresponding mutated and revertant BAC constructs. After 6 days post-infection (dpi), viral growth was detected, and images of minimum 50 random plaques from each recombinant virus were taken. The plaque areas were measured using Image J software (NIH) and normalized to the wild-type virus. Three independent experiments were performed, and the difference in plaque areas was evaluated using ANOVA One-way analysis of variance.

2.2.2.9. Multi-step growth kinetics assay

To assess further the replication properties of the recombinant viruses, multi-step growth kinetics assay was performed as described previously [399]. 1×10^6 ESCDL-1 cells were infected with 100 PFU of the corresponding BAC constructs. Cells were trypsinized and titrated on uninfected ESCDL-1 every 24 hours for six days. The plaques were counted under an

epifluorescence microscope. Three independent experiments were performed, and the difference in plaque areas was evaluated using ANOVA One-way analysis of variance.

2.2.2.10. Reconstitution and propagation of GaHV-2 recombinant viruses

In order to fully restore the U_S2 gene and excise GFP, reconstitution of the viruses was performed using ESCDL-1 cell line. Cells were transfected with the recombinant BAC constructs and co-transfected with a plasmid that encodes for Cre-recombinase using calcium phosphate transfection protocol as described before [400]. Briefly, 24h before transfection 500,000 secondary CEFs were seeded in a six-well plate, and three different concentrations of constructed BACs (0.5, 1 and 2 μg) and plasmid Cre/Lox (1, 2 and 5 μg) were diluted in 50 μl of sterile 10mM Tris-HCl (pH. 7.5). Next, 388 μl of sterile autoclaved Millipore water were added to the DNA mix and incubate for 4 hours at room temperature. Following the incubation, 62 μl of 2M ice-cold $CaCl_2$ were added drop by drop to the DNA mix while gentle vortexing and incubated O/N at 4 °C in Eppendorf tubes sealed with parafilm. The next day, 500 μl of cold 2× HEPES buffered saline (HBS) (Sigma-Aldrich) were added drop by drop to the transfection mix while gentle vortexing and incubated for 15 minutes in the dark at room temperature. During the incubation, CEFs were washed with phosphate-buffered saline (PBS) (Lonza) and supplemented with fresh DMEM medium (10% FBS). Next 500 μl of transfection mixture were added to the cells drop by drop while gently vortexing and incubated at 37 °C for 4 hours. Following the incubation, the cells were gently washed with PBS, and 1.5 ml of 1× HBS supplemented with 15% glycerol were added. The mixture was incubated for a maximum of 2.5 minutes, and 1x HBS + 15% glycerol was immediately removed followed by PBS washing Finally, fresh DMEM (10% FBS) was added and cells were incubated O/N at 37 °C, 5% CO_2. In following two days, the medium was changed first to DMEM (5% FBS) and then DMEM (0.5% FBS), respectively, followed by 48h incubation at 37 °C at 5% CO_2.

Following reconstitution, viruses were propagated in secondary CEF cells. CEFs were co-infected with ESCDL-1 cells containing reconstituted recombinant GaHV-2 virus and propagated for three passages before preparing the vial stocks. Viral stocks were made from highly infected cells in 10% FBS DMEM supplemented with 10% DMSO. The aliquots were firstly kept at -80 °C before the storage in liquid nitrogen.

2.2.2.11. Viral titration on CEF cells

In order to prepare viral inoculums for *in vivo* experiment, viral titration was performed as described below. Briefly, 24 well plates were seeded with 100,000 non-infected CEFs per well. 24 hours after seeding, these CEFs were inoculated with serial dilutions (1/10, 1/50, 1/250, 1/1250 and 1/6250) of infected ESCDL-1 cultures containing previously reconstituted recombinant viruses. Co-infected cells were then incubated for 48 hours to allow plaques (foci) to form by cell-to-cell spread. To reveal the plaque formation, cells were fixed with 4% paraformaldehyde.

Following the fixation, the immunostaining was realized as follows. Cells were washed four times with 500 µl of buffer I (Tris-HCl 20mM, ph8, NaCl 250 mM). Next, cells were permeabilized by incubation for 30 minutes at 4 °C with 500 µl of buffer II (buffer I + 0.5% de Triton-X100). Following incubation, cells were washed four times with 500 µl of buffer I. Saturation of the cell was realised by incubation for 1 hour with 500 µl of buffer III at room temperature (buffer II + 1% BSA, Bovine Serum Albumin), followed by four washes with 500 µl of buffer I. Following washing, the cells were first incubated with the primary antibody VP5 diluted 200 fold (generously provided by Dr. Caroline Denesvre, INRA-Tours) for one hour at room temperature. VP5 primary antibody is a monoclonal antibody produced by mouse hybridomas that target the major GaHV-2 capsid protein. The initial staining was followed by four washes with buffer II. Next, the cells were incubated with the secondary antibodies Goat anti-mouse-Alexa Fluor-488 diluted 1,000-fold for one hour at room temperature. Four washes with 500 µl of buffer II were performed, and nucleus staining was done by 4-6-diamidino-2-phenylindole (DAPI) incubation for one minute. Finally, cells were washed with 500 µl of PBS, and the number of plaques was determined by using an epifluorescence microscope. The titers were expressed as the mean PFU/ml according to the formula described in section 2.2.2.7.

2.2.2.12. Animal experiment, cell isolation, DNA and RNA extraction

White Leghorn specific pathogen-free $B^{13}B^{13}$ chickens, highly susceptible to GaHV-2, were obtained from INRA-Tours, France, and were used for the animal experiment. The animals were housed in isolated biosecurity level 3 facilities at Avian Virology and Immunology Service of Sciensano (Brussels, Belgium). Chickens were injected intramuscularly at the age of 2 days with

2,000 PFU of either mutant vTR-E2E3mut (n = 26) or revertant vTR-E2E3rev (n = 26) recombinant virus. To assess viral loads and telomerase activity in infected animals, blood samples and feather follicle epithelium (FFE) were collected at 6, 13, 20, 28, 35, 41, 48 and 55 days post-infection (dpi) and weight progression was recorded. Animals were assessed daily for the onset of common Marek's disease symptoms. At 55 dpi, chickens were euthanized and examined for tumour growth.

Genomic DNA from blood samples and FFE were isolated using the DNeasy®blood and tissue kit (Qiagen) as described by the manufacturer.

Peripheral blood leukocytes (PBLs) from anticoagulated blood were isolated using Histopaque-1077 density gradient (Sigma-Aldrich) according to the manufacturer's recommendations.

RNA from isolated PBLs was extracted using by Guanidium thiocyanate-phenol-chloroform extraction (Tri-Reagent®, Ambion). Briefly, cells were resuspended in 100 µl of PBS and lysed using 1 ml of Tri-Reagent. Following the centrifugation, the supernatant was recovered, and 200 µl of chloroform/ml of Tri-Reagent were added. Second centrifugation allowed to obtain an aqueous phase containing the RNA. In order to precipitate total RNA, 500 µl of isopropanol were added to the collected aqueous phase and centrifuged. After centrifugation, the pellet was washed with 1 ml of 70% ethanol, dried and resuspended in 18 µl of DNase/RNase free water (GIBCO). After that, a DNaseI (NEB) treatment was included. The mix contained DNase buffer 1x, RNase inhibitor, DNaseI (20U), the total RNA extracted and water in a total volume of 25 µl. The reaction was performed at 37 °C for 1 hour. The solution was extracted with phenol-chloroform and isoamyl alcohol, followed by chloroform and isoamyl alcohol extraction. The RNA was precipitated from the aqueous phase with three volumes of ethanol and 1/10 volume of sodium acetate (3M) and washed with 70% ethanol. The RNA was quantified, and purity was confirmed by measurement of the A260/A280 ratio with a Nanodrop™1000 (Thermo Scientific).

2.2.2.13. Ethics statement

The animal study was conducted following Belgian law for animal protection and the European Directive, 2010/63/EU. The Ethics committee of Sciensano (file n°. 20191016-03) approved all animal experiments.

2.2.2.14. GaHV-2 viral loads during the course of infection

GaHV-2 genome copies during viral infection were quantified using qPCR to determine the replication properties of the recombinant viruses. One μg of the DNA extracted from blood collected from eight random animals in each group were used for qPCR analysis. Virus genome copies were assessed by qPCR No Rox Probe MasterMix dTTP (Takyon) using primers and probe specific for major capsid protein VP5 of GaHV-2, according to manufacturer's recommendations. Primers used in qPCR assays are shown in Table 2.2.c. Virus genome copies were normalized against the chicken inducible nitric oxide synthase (iNOS) gene, as previously described [290]. Briefly, for the generation of standard curves in qPCR assays, PCR products of the ICP4 or iNOS gene were used. Serial 10-fold dilutions of each target were used for generating standard curves, starting with 100 ng of DNA. Total copy numbers were determined using the formula:

$$[(6.02 \times 10^{23}) \times (g/\mu l \text{ of input})] / (\text{amplicon length in bp} \times 660)$$

The standard curves were generated by plotting the cycle threshold (CT) value at each dilution with the total copy numbers.

2.2.2.15. vTR expression in recombinant virus-infected cells

vTR expression levels were determined *in vivo* by RT-qPCR from total RNA extracted from infected PBLs. DNaseI (NEB) treatment was performed on all the samples, and cDNA was generated using the SuperScript™ IV (SSIV) Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendations. Briefly, a premix was prepared containing the total RNA, dNTP 10mM and reverse gene-specific primers (2 μM) followed by the incubation at 65 °C for 5 minutes to remove the RNA secondary structure. Next, a mix containing buffer 5x, DTT 0,1 M, RNase inhibitor (40U) and the reverse transcriptase SSIV (200U) was added to the premix to perform the reverse transcription (RT). The mix was incubated at 55 °C for 15 minutes. After incubation, the samples were incubated at 80 °C for 10 minutes to inactivate the enzyme. Finally, in order to remove RNA complementary to cDNA 1 μl of RNase H (2U) were added to the samples and incubated at 37 °C for 20 minutes. In the end, newly synthesized cDNA was stored at -20 °C.

Following the RT, qPCR was performed to measure expressions of chicken TR (chTR) and vTR. The expression levels were normalized against the cellular GAPDH gene. In addition, relative vTR expression was normalised to the expression of the viral gene coding for immediate-early protein ICP4. Primers and probes used for RT-qPCR are shown in Table 2.2.d.

2.2.2.16. Telomeric repeat amplification protocol assay

Telomerase activity of 1 μg of protein extracted from the 1 x 10^6 PBLs was quantified using the semiquantitative fluorescence-based telomeric repeat amplification protocol (TRAP) assay, as previously described in section 2.1.2.3. Briefly, full protein extracts were isolated at specific time points from PBLs of infected and control chicken. Protein concentrations were determined with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and adjusted to 500 ng/μl. The telomerase amplification PCR was performed with primers shown in Table 2.1.a, as described before [217]. PCR products were analyzed by capillary electrophoresis (Applied Biosystems 3130xl Genetic Analyzer). The relative telomerase activity level of each protein extract was calculated by adding values of fluorescence intensity for all telomerase products containing 5 and up to 15 telomeric repeats and normalized to the fluorescence intensity value of the ITAS as previously described [390]. Additionally, the relative telomerase amplification measured *in vivo* was normalised using the relative expression of a major viral oncoprotein Meq in order to have a more precise way of interpreting the data in the infected cell population.

2.2.2.17. TABLE 2.2. PRIMERS USED FOR THE RED-MEDIATED E-BOX MUTAGENESIS AND C-MYC FUNCTIONAL EVALUATION.

a) primers used for the construction of recombinant viruses

Target/construct	Sequence (5'-3') (mutations are shown in bold and are underlined)	
vTR-E-box_2-mutant	Fwd	GATCCGATCCCGCAGACCCCGGCCCACAGGAAGGGGCGGGG**TTTTT**GCATGGGGCGTGGTAGGGATAACAGGGTAATCGATTT
	Rev	GGAACTCCGCGGTCATTCATCTCCCGCCACGCCCCATGC**AAAAA**CCCCGCCCCTTCCGCCAGTGTTACAACCAATTAACC
vTR-E-box_3-mutant	Fwd	GGAGGAAGCTACAAGAGCCCCACGCGGGGTTCCCCCGGCA**TTTTT**GGCGGGTGGAAGTAGGGATAACAGGGTAATCGATTT
	Rev	CCTCCGATTAGGGTTAGACACAGCGGAGCCTTCCACCCGCC**AAAAA**TGCCGGGGGGAACCGCCAGTGTTACAACCAATTAACC
vTR-E-box_2-revertant	Fwd	CCGGATCCGATCCCGCAGACCCCGGCCCACAGGAAGGGGCGGGG**CACGT**GCATGGGGCGTGGTAGGGATAACAGGGTAATCGATTT
	Rev	GAGTTTGGAACTCCGCGGTCATTCATCTCCCGCCACGCCCCATGC**ACGTG**CCCCGCCCCTTCCGCCAGTGTTACAACCAATTAACC
vTR-E-box_3-revertant	Fwd	GGAGGAAGCTACAAGAGCCCCACGCGGGGTTCCCCCGGCA**CACGT**GGCGGGTGGAAGTAGGGATAACAGGGTAATCGATTT
	Rev	CCTCCGATTAGGGTTAGACACAGCGGAGCCTTCCACCCGCC**ACGTG**TGCCGGGGGGAACCGCCAGTGTTACAACCAATTAACC

b) primers used for screen PCR and Sanger sequencing

Target/construct	Sequence (5'-3')	
vTR promoter	Fwd	GTACACCTGCCTGCACTACT
	Rev	GCGAGGACCCCAGGG

c) primers used for qPCR

Target/construct	Sequence (5'-3')		Modification	Reference
GaHV-2-VP5	Fwd	CGTGTTTTCCGGCATGTG		
	Rev	TCCCATACCAATCCTCATCCA		
	Probe	CCCCCACCAGGTGCAGGCA	5'-FAM, 3'-TAMRA	
iNOS	Fwd	GAGTGGTTTAAGGAGTTGGATCTGA		
	Rev	TTCCAGACCTCCCACCTCAA		[401]
	Probe	CTCTGCCTGCTGTTGCCAACATGC	5'-FAM, 3'-TAMRA	

d) primers used for RT-qPCR

Target/construct	Sequence (5'-3') (vTR/chTR mismatches are underlined)		Modification	Reference
GaHV-2-vTR	Fwd	CCTAATCGGAGGTATTGATGGTACTG		
	Rev	CCCTAGCCCGCTGAAAGTC		
	Probe	CCCTCCGCCCGCTGTTTTACTCG	5'-FAM, 3'-TAMRA	
GAPDH	Fwd	GAAGCTTACTGGAATGGCTTTCC		
	Rev	GGCAGGTCAGGTGAACAACA		[401]
	Probe	TGTGCCAACCCCCAAT	5'-FAM, 3'-TAMRA	
GaHV-2-ICP4	Fwd	CGTGTTTTCCGGCATGTG		
	Rev	TCCCATACCAATCCTCATCCA		
	Probe	CCCCCACCAGGTGCAGGCA	5'-FAM, 3'-TAMRA	

2.2.3. Results

2.2.3.1. Generation and replication properties of the recombinant viruses

Specific epigenetic patterns, previously observed on the vTR promoter during the switch between latent and reactivation phase of GaHV-2 life cycle showed a significant change of methylation in the CpG dinucleotides present around functional c-Myc REs. To further investigate the importance of E-boxes in vTR expression, and tumour formation during the progression of GaHV-2 infection *in vivo*, recombinant virus containing a mutation in the E-boxes was constructed. Targeted mutation of vTR promoter E-box 2 and E-box 3 (vTR-E2E3mut) was accomplished by two-step Red-mediated mutagenesis using highly oncogenic RB-1B strain lacking part of the internal repeat long region (pRB-1BΔIRL) as a backbone. In addition, the original vTR promoter sequence was restored by obtaining a revertant virus (vTR-E2E3rev). Constructed recombinant viruses were verified by RFLP using *BamHI* and *KpnI* restriction enzymes, PCR and sequencing of the vTR promoter locus (Figure 2.19).

Furthermore, to confirm the integrity of the recombinant viral genome after several mutagenesis steps, final mutant and revertant bacmid clones were analysed with high-throughput sequencing. The analysis revealed that mutagenesis of functional c-Myc response elements (E-box 2 and E-box 3) was successful, and confirmed that revertant construct had the same sequence as the wild-type reference.

Two mutations were detected in both mutant, and revertant viruses compared the reference strain BB2573\RB-1B\pvT. One mutation was localised within eGFP located in the mini-F sequence that was removed in downstream viral reconstitution in ESCDL-1 cells prior to the *in vivo* experiment. The second mutation detected was a silent point mutation in U_L32 ORF (MDV046), which is a DNA packaging protein (Table 2.3).

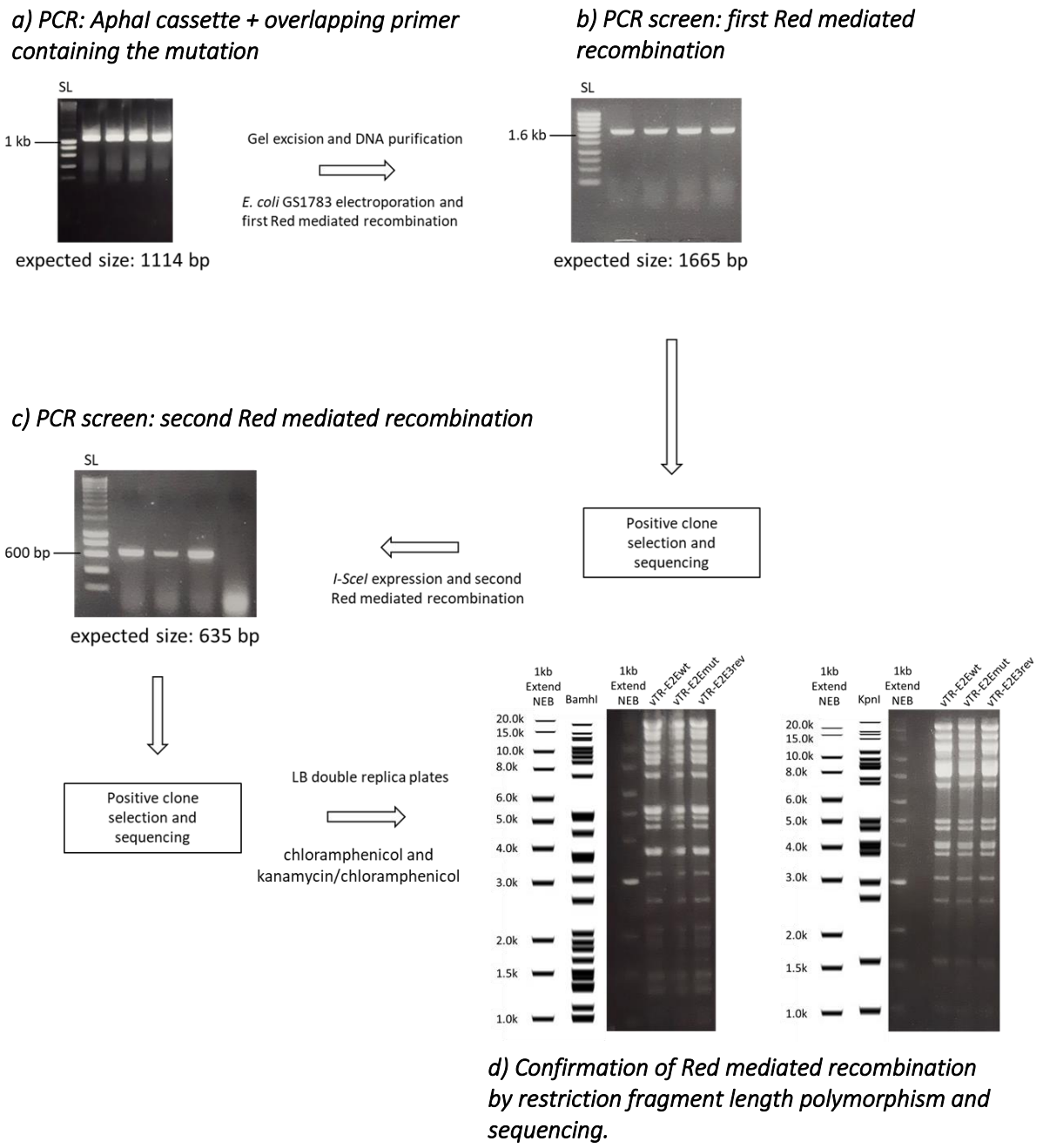

FIGURE 2.19. INTRODUCTION OF THE DESIRED MUTATION IN THE BACMID BACKBONE USING TWO-STEP RED MEDIATED MUTAGENESIS.

a) PCR targeting resistance cassette (AphaI) with specific overlapping primers containing the desired mutation was performed. **b)** First Red recombination resulted in the insertion of resistance cassette amplicon with the desired mutation in the bacmid backbone. **c)** Positive bacterial clones were selected for second Red recombination resulting in *I-SceI* expression under arabinose and excision of the AphaI cassette. **d)** Positive clones were selected on chloramphenicol and kanamycin/chloramphenicol replica plates and confirmed with restriction fragment length polymorphism and sequencing of the vTR promoter locus.

TABLE 2.3. HIGH-THROUGHPUT SEQUENCING ANALYSIS OF THE MUTANT AND REVERTANT BACMID CONSTRUCTS.

a) Mutated construct

Position	Reference	Altered	Type	Site
546	C	T	introduced point mutation	vTR-E-box 2
547	A	T	introduced point mutation	
548	C	T	introduced point mutation	
549	G	T	introduced point mutation	
730	C	T	introduced point mutation	vTR-E-box 3
731	A	T	introduced point mutation	
732	C	T	introduced point mutation	
733	G	T	introduced point mutation	
74714	A	G	silent point mutation	UL32
134507	A	G	transitional point mutation	eGFP

b) Reverted construct

Position	Reference	Altered	Type	Site
74714	A	G	silent point mutation	UL32
134507	A	G	transitional point mutation	eGFP

2.2.3.2. The mutation of CpG sites within c-Myc response elements does not affect GaHV-2 replication *in vitro*

To assess if the mutation of E-box 2 and E-box 3 influenced virus replication, BACs replication properties were investigated by plaque size assay (Figure 2.20.b) and confirmed with multi-step growth kinetics (Figure 2.20.c). Both assays indicated that replication of mutant construct was as efficient as for revertant and wild-type and was not altered by the E-box mutations *in vitro*. Thus, only mutant (vTR-E2E3mut) and revertant (vTR-E2E3rev) recombinant viruses were used for animal experiment.

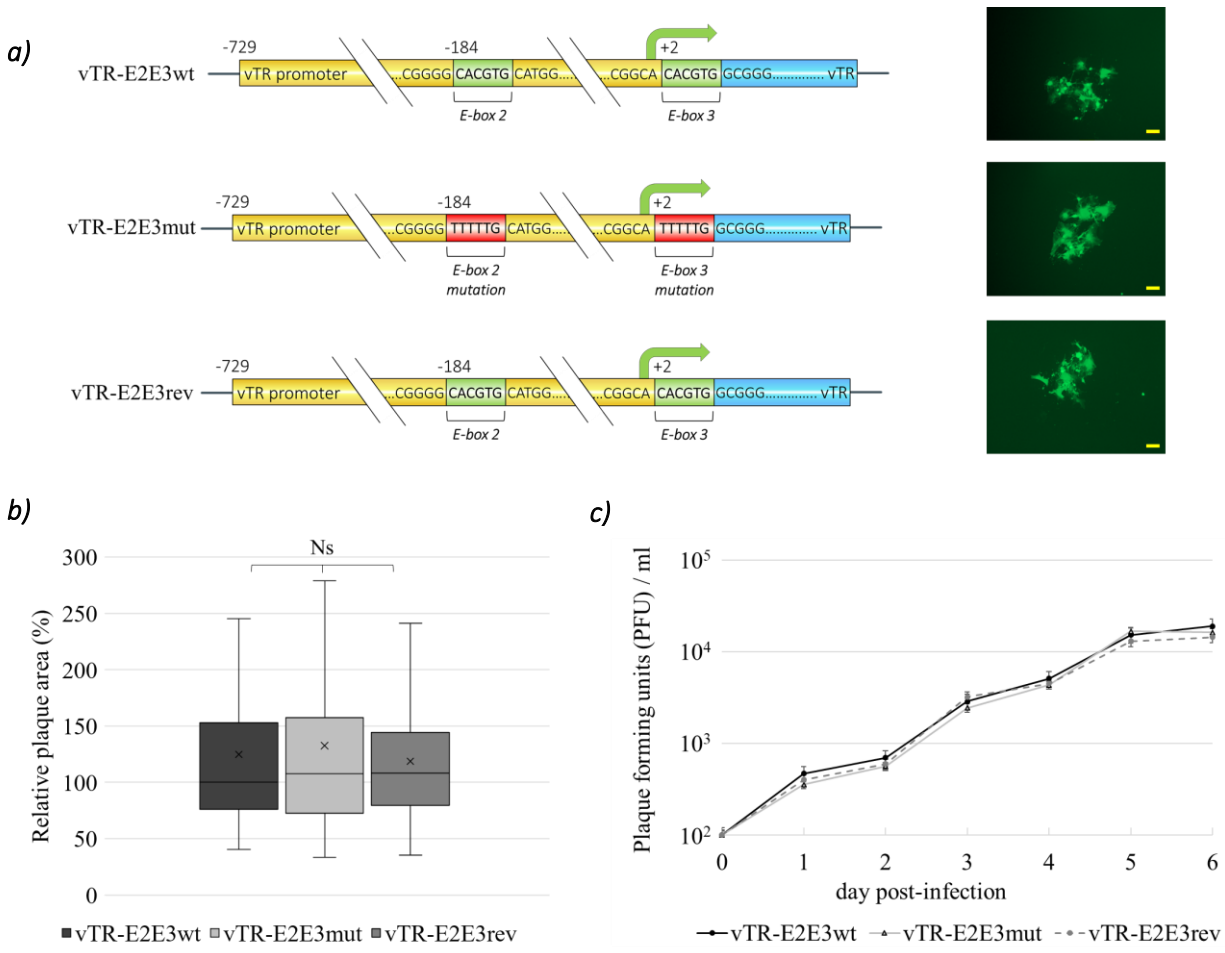

FIGURE 2.20. REPLICATION PROPERTIES OF RECOMBINANT BACS SHOWED NO DIFFERENCE BETWEEN MUTATED AND REVERTANT RECOMBINANT VIRUSES.

a) Mutations in the E-boxes of the vTR promoter were introduced by two-step Red-mediated mutagenesis using a bacterial artificial chromosome (BAC) of highly oncogenic RB-1B strain lacking part of the internal repeat long region (pRB-1BΔIRL). Using the produced mutated BAC as a backbone, revertant bacmid was produced containing the wild-type sequence. Next to each recombinant virus, a representative image of viral plaques are shown (scale bar -100 μm, yellow line). **b)** Relative plaque areas were calculated using Image J software and are shown as box plots with minimums and maximums. Results are shown as means of three independent experiments revealing no significant difference in viral replication properties between wild-type (vTR-E2E3wt), mutant (vTR-E2E3mut) or revertant (vTR-E2E3rev) viruses (one-way ANOVA). **c)** Multi-step growth analysis assay confirmed that introduced E-box mutations did not affect replication of the constructs. Average titers of an independent experiment performed in triplicates are shown with standard deviations ($p > 0.05$, Kruskal–Wallis test).

2.2.3.3. The mutation of CpG sites within c-Myc response elements results in specific phenotype with severely impaired tumour formation

To investigate the involvement of c-Myc oncoprotein and the importance of its binding on the E-box sites for the tumour development, 2-day-old $B^{13}B^{13}$ chicken were injected intramuscularly with 2000 PFU of mutant (vTR-E2E3mut) or revertant (vTR-E2E3rev) recombinant viruses. Throughout the *in vivo* experiment, the weight progression of animals was recorded at the specific time points (Figure 2.21), and the onsets of typical clinical symptoms were monitored. Interestingly, the c-Myc binding sites mutations presented a specific weight phenotype starting from 13-day post-infection (dpi) (Figure 2.21). The disease incidence in the vTR-E2E3rev was first recorded at 35 dpi while it was completely reduced upon mutation of E-box 2 and E-box 3.

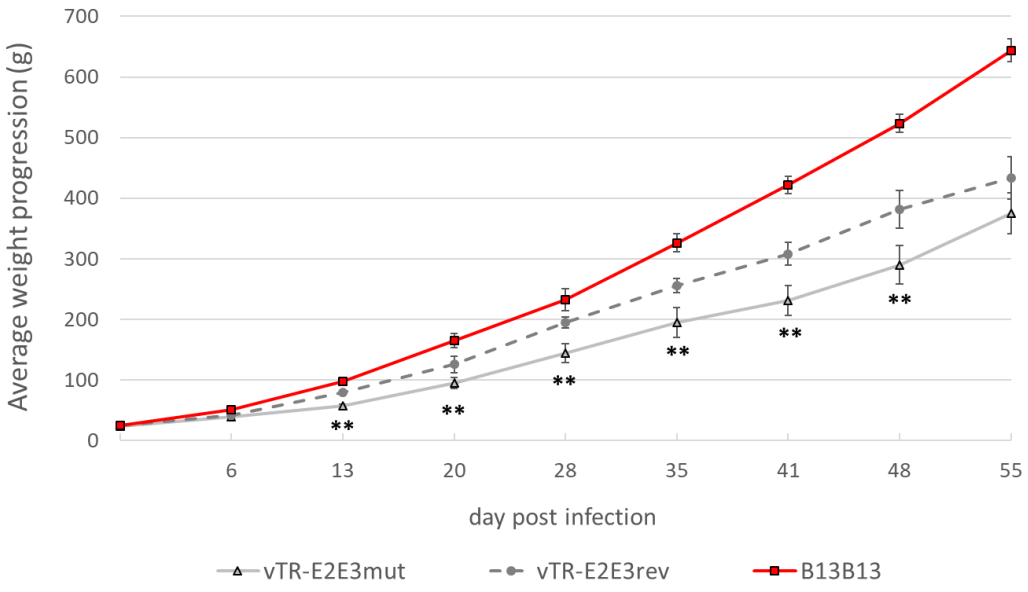

FIGURE 2.21. WEIGHT PROGRESSION OF CHICKEN INFECTED WITH RECOMBINANT VIRUSES.

Weight measurements were taken for eight random animals of each group at specific time points during the GaHV-2 infection. Weight comparison in chicken challenged with mutant (vTR-E2E3mut) or revertant (vTR-E2E3rev) recombinant virus showed significant difference starting from 13 days post-infection ($p < 0.005$, student t-test). In addition, the weight evolution of non-infected $B^{13}B^{13}$ chicken line is shown for the reference (data provided by INRA-Tours, France).

Furthermore, to assess the effect of introduced mutations on tumour propagation, the number of visceral organs with visible tumour lesions and number of tumours were recorded at 55 dpi. Strikingly, for the vTR-E2E3mut virus, no animals developed visible tumours, while 50% of animals challenged with the revertant virus did (Figure 2.22.a).

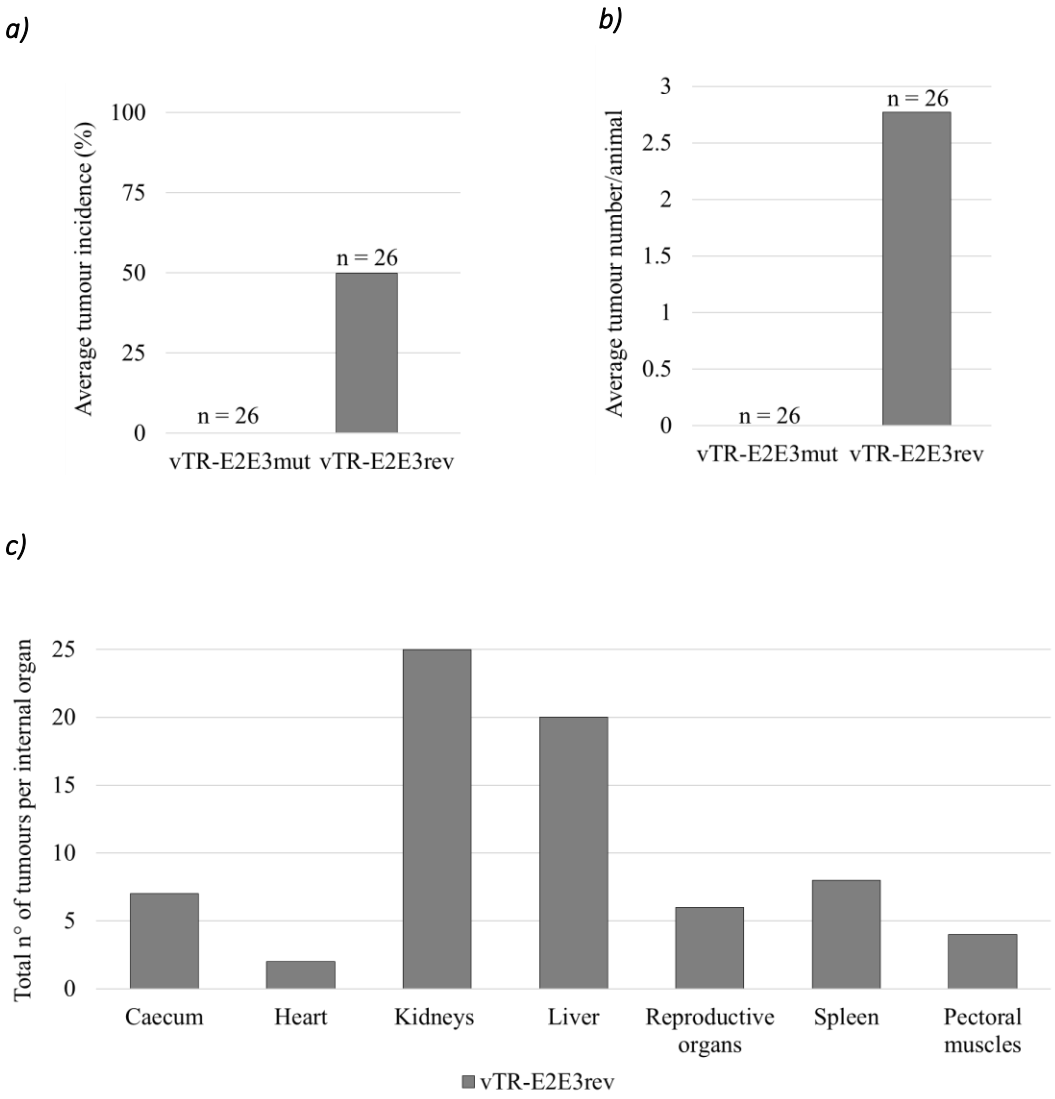

FIGURE 2.22. TUMOUR INCIDENCE AND THE AVERAGE NUMBER OF GROSS TUMOURS IN CHICKEN INFECTED WITH RECOMBINANT VIRUSES.

55 days post-infection, all animals were euthanized, and the autopsy was performed to determine tumour formation in internal organs. **a)** While 50% of animals in the revertant group (vTR-E2Erev) developed visible tumours, in the animals challenged with the mutated recombinant virus (vTR-E2E3mut), no tumours were recorded. **b)** The average number of gross tumours per animal infected with recombinant viruses was calculated. Average of 2.77 tumours were recorded for the chicken challenged with the revertant virus (vTR-E2E3rev). **c)** A total number of gross tumours developed in each observed internal organ of the chickens challenged with the revertant virus (vTR-E2E3rev).

The average of 2.77 tumours per animal were recorded for the group infected with the revertant virus (Figure 2.22.b). In addition, the total number of tumours per screened internal organs indicated that the majority of tumours were developed in the kidneys and the livers of the vTR-E2E3rev infected animals (Figure 2.22.c). In addition, the accumulation of adipose tissue was visible around the hearth and the liver of the animals that had developed visible gross tumours.

2.2.3.4. The mutation of CpG sites within c-Myc response elements results in lower viral loads in infected animals

To determine if the recombinant viruses efficiently replicated in infected chickens, viral genome copies were quantified from the whole blood and feather follicle epithelium (FFE) at specific time points. Monitoring the viral load evolution throughout GaHV-2 infection demonstrated that the introduced mutation in functional c-Myc REs affect total viral loads levels, which were significantly lower compared to the revertant virus. Furthermore, the quantification of viral copy numbers in FFE, starting at 20 dpi, showed decreased viral loads compared to the revertant virus, as well, indicating a reduction in mature virion release in the FFE (Figure 2.23.a).

2.2.3.5. The mutations of c-Myc binding sites affect relative vTR expression

To investigate the effect of the inserted mutation on vTR expression, we performed RT-qPCR assays on total RNA extracted from infected PBLs at 55 dpi, from the animals infected with the corresponding recombinant viruses. Interestingly, relative vTR expression was reduced by 2.5 fold in the animals infected with the vTR-E2E3mut compared to vTR-E2E3rev (Figure 2.23.b). GAPDH was used to normalize the data, and the expression of GAPDH was comparable between the two groups (Figure 2.23.b). In addition, relative vTR expression was normalised relative to the viral ICP4 control gene, resulting in the loss of the significant reduction in vTR expression between the conditions. However, the tendency of vTR expression reduction was preserved, as shown for results obtained with GAPDH as a control (Figure 2.23.c).

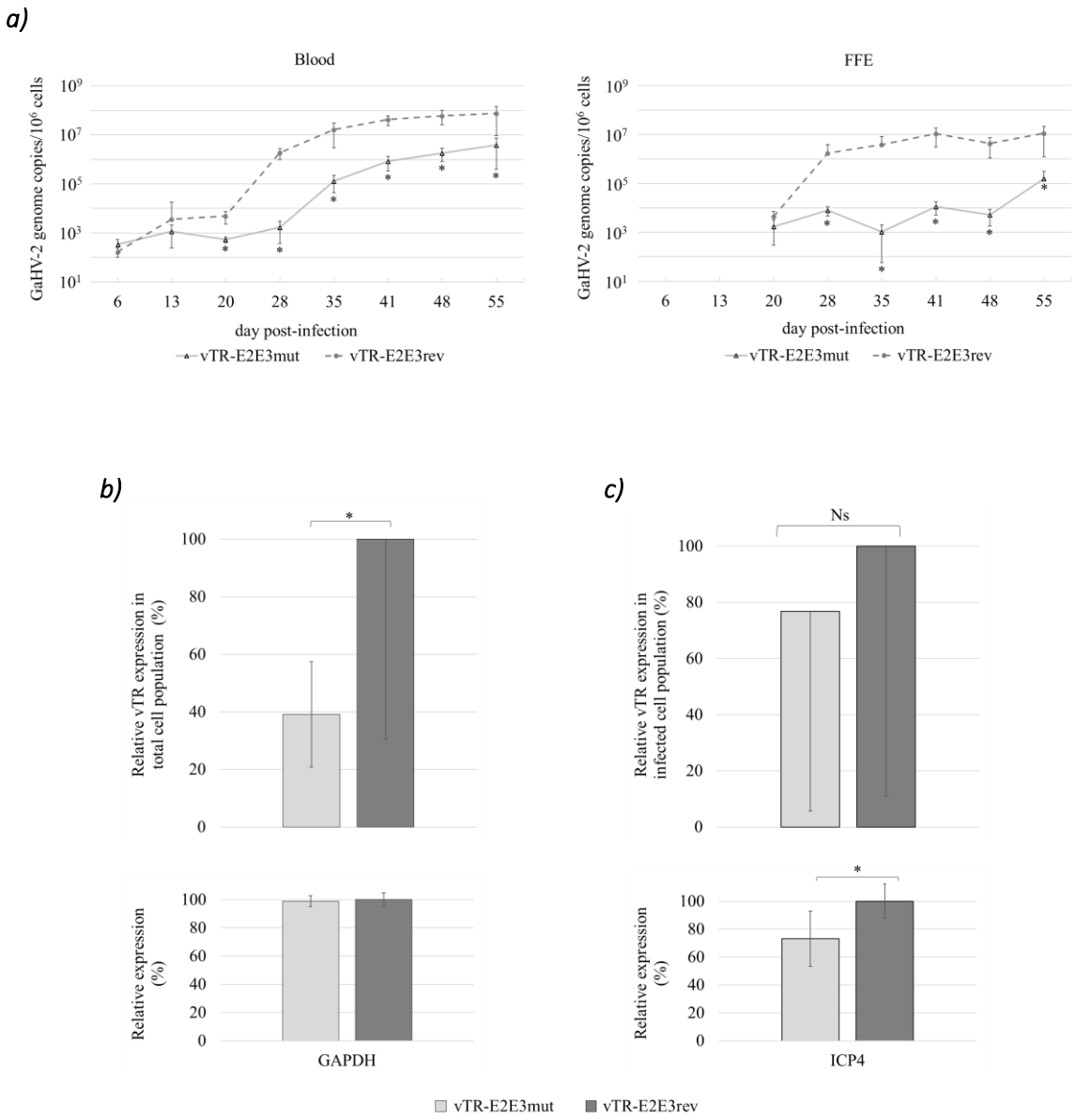

FIGURE 2.23. THE MUTATION OF THE CPG SITES WITHIN C-MYC RESPONSE ELEMENTS IN THE VTR PROMOTER RESULTS IN THE SPECIFIC DISEASE PHENOTYPE.

a) qPCR detecting GaHV-2 genome copies in the blood and feather follicle epithelium (FFE) of chicken infected with mutant (vTR-E2E3mut, n=26) or revertant (vTR-E2E3wt, n=26) viruses. The means of GaHV-2 genome copies per million cells are shown for the indicated time point. **b)** Total RNA was isolated from peripheral blood leukocytes (PBLs) at 55 dpi, and RT-qPCR was performed. Relative vTR expression was obtained using the Livak method and is shown relative to the cellular GAPDH control gene ($p \leq 0.05$, student t-test). Relative expression of control gene GAPDH in PBLs infected with the different viruses was not statistically different ($p > 0.05$, student t-test). **c)** In addition, relative vTR expression was shown as well as relative to the viral ICP4 control gene ($p \geq 0.05$, student t-test) with the similar tendency in vTR expression reduction as shown for results obtained with GAPDH as a control. Relative expression of control ICP4 gene in PBLs infected with the different viruses was statistically different ($p < 0.05$, student t-test). The means of three independent experiments with standard deviations are shown.

2.2.3.6. The mutations of c-Myc binding sites affect telomerase activity

In order to assess the effect of the c-Myc binding sited mutation on the vTR involvement in the regulation of telomerase activity, peripheral blood leukocytes (PBLs) were extracted from $B^{13}B^{13}$ chicken infected with either the revertant or mutant recombinant virus, at 35, 42 and 48 dpi. Full protein extract of 1 x 106 PBLs was analysed using semiquantitative fluorescence-based telomeric repeat amplification protocol (TRAP) assay (Figure 2.24). Introduced c-Myc REs mutations in the vTR promoter resulted in 1.9, 3.2 and 1.8 fold decrease in telomerase activity, respectively, compared to internal amplification standard (Figure 2.24.a). The relative telomerase activity was additionally normalised to the gene coding for major viral oncoprotein Meq that allowed more precise readout due to the differences in the number of viral genomes observed between the animals infected with mutant or revertant viruses. The normalisation with viral gene resulted in 1.5, 2.4 and 1.4 fold decrease in relative telomerase activity measured at 32, 42 and 48 dpi, respectively (Figure 2.24.b). The relative telomerase activity in non-infected control animals, measured from 9 to 30 days post-hatching was assessed for a basal activity reference. The highest basal telomerase activity was recorded at day 9 compared to the activity measured at 16, 23 and 30 days post-hatching. Starting from day 16, the telomerase activity measured in non-infected chicken stabilised and did not significantly change, and moreover, was significantly reduced compared to the telomerase activity measured in infected chicken (Figure 2.24.c).

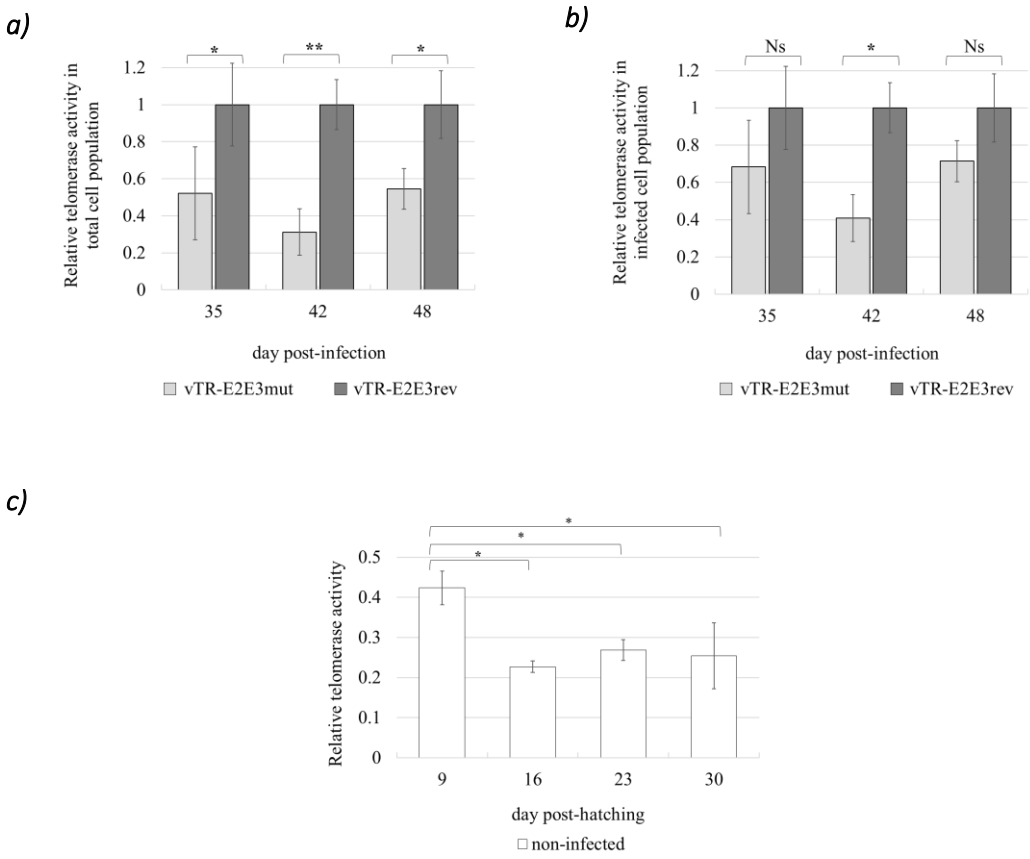

FIGURE 2.24. THE MUTATION OF THE CPG SITES WITHIN C-MYC RESPONSE ELEMENTS IN THE VTR PROMOTER RESULTS IN THE DECREASE OF RELATIVE TELOMERASE ACTIVITY.

a) The PBLs were extracted from the blood of eight random animals infected either with mutated or revertant recombinant viruses. Extracted PBLs from each group were pulled together, and total protein extract was submitted to semiquantitative fluorescence-based telomeric repeat amplification protocol assay. **b)** The relative telomerase activity was additionally normalised to the gene coding for major viral oncoprotein Meq. The relative telomerase activity was significantly decreased at day 42 post-infection in the animals infected with the virus bearing mutations in the functional c-Myc response elements of the vTR promoter ($p \leq 0.05$, student t-test), and demonstrated similar tendency at days 35 and 48 post-infection. **c)** The relative telomerase activity of non-infected chicken, highest at day 9 post-hatching, stabilised after and did not significantly change ($p > 0.05$, student t-test). The means of three independent experiments with standard deviations are shown.

2.2.4. Acknowledgements

I thank Prof. Benedikt Kaufer and Dr. Ahmed Kheimar (Institut für Virologie, Freie Universität Berlin) for the constructive discussion and help during recombinant virus preparation. I am thankful to Dr. Sébastien Boulanger (Institut de Pathologie et de Génétique ASBL, Gosselies) for the help with capillary electrophoresis analysis and Applied Biosystems 3130xl Genetic Analyzer used for TRAP assay. I am especially grateful to Alexis Chasseur and Gabrielle Trozzi (Integrated Veterinary Research Unit, University of Namur) for the help with RNA samples extraction and preparation. I am thankful for the support on the coordination of the high-throughput bacmid DNA sequencing project (Benelux Team) from the Novogene Co., LTD.

CHAPTER 3. DISCUSSION, CONCLUSIONS AND PERSPECTIVES

3.1. Discussion

Gallid herpesvirus type 2 (GaHV-2) is a highly oncogenic alphaherpesvirus that infects chickens causing paralysis, immunosuppression and fatal lymphoma in susceptible animals [299]. GaHV-2 establishes latent infection in $CD4^+$ T-lymphocytes, in which it integrates the viral genomes into the telomeres of host chromosomes [289,402,403]. The integrated virus genome is maintained in the telomeres and mobilized during reactivation. Aside from latency, telomere integration also plays an essential role in tumour formation [291].

One of the main characteristic features of herpesviruses is their ability to establish a latent infection during which most of the viral genes are silenced, resulting in no viral progeny production and viable host cells. However, it can re-enter the productive cycle and produce new virions if conditions in the cell change [404]. The crucial factors in the regulation of gene expression associated with different phases of the viral cycle in herpesviruses are epigenetic modifications [289,404]. In this context, we have studied the effect of the epigenetic changes on the promoter of the vTR gene encoding for the telomerase RNA subunit. We performed methylation mapping on the vTR promoter and demonstrated a reduction of methylation signatures after viral reactivation *in vitro*. These results follow previous data published on GaHV-2 methylation changes [405]. Epigenetic modifications regulate DNA replication and virus reactivation in other herpesviruses. DNA methylation and histone post-translational modifications (HPTMs) were observed during EBV and Kaposi Sarcoma-associated herpesvirus (KSHV) infection [404,406] while only HPTMs were found during infection with the *Alphaherpesviruses* HSV and HHV-3 [407].

In our studies, we observed an increase of the telomerase activity after viral reactivation *in vitro*. This resulted in DNA demethylation of the GaHV-2 genome and indicated that DNA methylation status is associated with telomeric transcription in GaHV-2 transformed cell line. This result is in agreement with previous studies observing high telomerase activity in tumour cell lines in comparison to the normal lymphocyte cells [390]. Telomere maintenance is necessary for unlimited cancer cell proliferation, however, recent studies suggested that telomerase could promote tumorigenesis independently of telomere elongation [408,409]. Increased telomerase activity was detected in cells infected with a variety of herpesviruses [218,409–411]; however, none of these viruses harbours any of the telomerase components except GaHV-2. Cancer-associated human herpesviruses have also been found to upregulate telomerase activity upon cells infection. In EBV-immortalized B-lymphocytes, telomerase activity was variable [412]. Similar results were observed in a nasopharyngeal carcinoma cell line expressing LMP1. Its expression was correlated

with increased hTERT promoter activity and protein levels, suggesting enhanced hTERT transcription as a mechanism for telomerase upregulation [413] and a similar effect was identified in cells expressing LANA gene (latency-associated nuclear antigen) of Kaposi's Sarcoma Herpesvirus (KSHV/HHV-8) [216,225]. These data implicate telomerase activation as a common mechanism for herpesvirus tumorigenesis and increases in telomerase activity during herpesvirus infections [414].

A previously demonstrated, telomere length and the mtDNA copy number are associated with numerous specific types of cancer [378–381] and positively correlated, that could have an early and essential effect on carcinogenesis [382]. Even no previous studies were referring to telomere length during GaHV-2 infection, a recent *in vivo* study revealed that in bursa Fabricius and in the thymus, the mtDNA copy number remained relatively constant in resistant and susceptible animals infected with GaHV-2. However, a significant difference in mtDNA copy numbers was observed in the spleen at 21 dpi, due to a continuous decrease of mtDNA contents in the susceptible birds and an increased recovery in the resistant birds, which implied that 21 dpi presents an important stage for the mitochondria changes after GaHV-2 infection [415]. To connect to the previous study, we measured mtDNA copy number in two stages of the viral cycle, latency and reactivation. Our analysis of mitochondrial a DNA (mtDNA) copy number in the MSB-1 cell line showed that there was no significant change during latency and after viral reactivation.

In vitro replication properties of recombinant viruses supported previous studies showing that vTR is dispensable for lytic GaHV-2 replication [218,347]. Furthermore, we assessed the impact of c-Myc response elements (c-Myc REs) mutations on virus-induced tumour development. In addition, numerous studies indicated that wild-type and revertant viruses replicated in similar ways *in vivo*, and resulted in similar output in tumorigenesis [218,347,402], strengthening our approach to use only revertant virus as a control. Strikingly, animals infected with the mutant virus showed abrogation of tumorigenesis, while half of the animals infected with revertant virus developed gross tumours in visceral organs. Our study presents the first report on the intriguing weight phenotype observed in the animals infected with the vTR-E2E3mut virus, which indicates further c-Myc roles in sustaining the changes that occur with transformation resulting in activation of different signalling pathways [416]. Numerous studies demonstrated c-Myc important involvement in the regulation of glycolysis and cancer metabolism [417–419], as well as mitochondrial biogenesis stimulation [420–422], however, to fully understand these processes during GaHV-2-induces tumorigenesis further studies are needed. However, this result needs to be taken with caution, since proper controls were not set in place, for example, measuring the precise amount of feed intake.

It was previously demonstrated that the vTR contributes to GaHV-2-induced lymphomagenesis, where complete deletion of vTR resulted in significantly reduced tumour incidences without affecting virus replication *in vivo* [218]. However, no previous studies reported the differences in the distribution of the tumours in tissues. Surprisingly, viral loads in susceptible $B^{13}B^{13}$ chicken infected with recombinant virus bearing c-Myc REs mutation was significantly lower compared to the revertant virus, starting from 20 dpi. This observation could be explained due to the fact that viral loads were detected in the total number of GaHV-2 transformed T-lymphocytes rather than the complete viral replication, what is supported by the lack of visible tumours in the same group. The similar tendency, however, not significant, was previously observed by Trapp et al. [218]. In addition, the significantly lower levels of viral loads observed in the feather follicle epithelium (FFE), back up previous statement, underlining a lower number of transformed T-lymphocytes circulating in the blood and thus establishing weaker secondary productive infection and viral shedding from FFE.

Moreover, even the complete deletion of vTR severely impaired tumour formation, no total abrogation was previously demonstrated [218,347]. However, the total lack of visible tumours we observed could be explained due to the differences in the major histocompatibility complex-B (MHC-B) of susceptible chicken lines previously used and the duration of the animal experiment. As demonstrated by Kheimar et al. [347], the first tumour incidence in the animals infected with a virus lacking both vTR copies was recorded around 56 dpi. Only 30% of infected animals developed tumours until 91 dpi, while wild type RB-1B caused tumours in around 90% of the animals. Moreover, a study using the same $B^{13}B^{13}$ chicken line demonstrated that 83% of animals infected with wild-type RB-1B strain develop tumours after 90 days of infection [423]. Thus, there is a high probability that our animals infected with the recombinant virus bearing c-Myc REs mutations in the vTR promoter would eventually develop visible tumours over time.

Furthermore, our findings indicated that mutations of c-Myc binding sites in the vTR promoter have a significant repression effect on vTR expression during GaHV-2 infection compared to the revertant virus normalised with the chicken control gene, and the tendency of vTR expression inhibition when normalised to viral gene, indicating that functional c-Myc REs are involved in the regulation of the vTR expression. These results support our previous observation of 50% of animals developing gross tumours in the revertant group. In addition, this observation is consistent with previous reports that demonstrated that vTR expression is not only crucial for GaHV-2 lymphomagenesis, but expression levels are necessary for GaHV-2 tumorigenic function. It was shown that vTR expression through its promoter is essential for GaHV-2 lymphomagenesis, revealing that tumour formation induced by recombinant viruses expressing vTR at lower levels

was significantly inhibited [340]. Furthermore, it was indicated that overexpression of chicken cellular TR (chTR) promoted tumour formation as efficient as vTR indicating that expression levels of chTR/vTR are of most importance for tumour development [347].

Additionally, we demonstrated that the relative telomerase activity is significantly lower in the animals infected with the virus containing mutations in the c-Myc REs of the vTR promoter compared to the revertant virus, consistent with the significantly lower vTR expression. Moreover, animals infected with both GaHV-2 recombinant viruses demonstrated significantly higher relative telomerase activity that in the non-infected chicken. Our findings correlate with previously published data that showed vTR enhance telomerase activity compared to the cellular TR [217]. (9). In addition, it was previously demonstrated that vTR-mediated telomerase activity contributes to the rapid onset of disease, but not tumour formation [219]. Therefore, the observed lack of Marek's disease incidence in the animals infected with the vTR-E2E3mut virus is likely due to the observed lower relative telomerase activity. However, the relative telomerase activity measured in infected cell population resulted in the loss of significant change at days 35 and 48 post-infection, keeping the similar tendencies observed in the total cell population. These results indicate that the observed reduction in telomerase activity could be due to the different viral loads and thus viral activity during the infection induced with the mutant or revertant virus, or the consequence of reduced vTR expression and thus lower interaction with TERT. Furthermore, the extreme phenotype observed in our study could indicate other possible regulatory events involved. Since this implication was not studied before in the same context, further studies are needed in order to obtain a full mechanistic picture of the vTR promoter regulation.

Furthermore, we showed that DNA methylation could play a role in the restriction of specific genes, such as vTR, during the GaHV-2 replication/latent cycle. Specific changes in the methylation patterns were observed throughout the vTR promoter region *in vitro*, especially in the areas surrounding functional c-Myc REs (E-box 2 and E-box 3), that serve as binding sites for proteins of the Myc/Mad/Max transcription factors family and act as crucial positive regulators of cell proliferation and death [359,424]. Previous studies have shown that c-Myc can induce telomerase activity through the transcriptional activation of hTERT [222,425]. In 2007, Shkreli et al. [357], showed that c-Myc activates transcription of the vTR gene, and binds to the vTR promoter sequence in GaHV-2-transformed cell line. The interaction of c-Myc with the vTR promoter E-boxes is involved in the higher levels of vTR expression observed during GaHV-2-induced lymphomagenesis, and EBS and E-box 2 act together with E-box 3 to regulate vTR expression in MSB-1 cell line. Moreover, the results for DNA methylation patterns *in vivo* were obtained from a total population of isolated peripheral blood leukocytes and showed random

changes in methylation patterns. Even we observed tendencies in methylation pattern change in and surrounding functional c-Myc response elements. This result indicated that cell sorting could overcome these misleading observations, highlighting the limits of our approach.

Secondly, we have explored whether another, recently described cytosine modification, 5-hydroxymethylcytosine (5hmC), has different signatures between the latent and productive phase on the vTR promoter. The 5hmC modification occurs as an intermediate during active demethylation of cytosines *in vivo* [426]. Higher levels of 5hmC on the cellular genome are usually associated with gene activation and protection from *de novo* methylation [144]. For vTR promoter, measured 5hmC was significantly lower than 5mC, both, before and after reactivation. However, the absence of hydroxymethylation around E-box 2 was observed. This reduction could explain the increase in E-box 2 methylation after viral reactivation. A study concerning EBV showed that TET-mediated 5hmC modification of lytic EBV promoters inhibits their activation. The results revealed that TET-mediated 5hmC modification of lytic EBV promoters regulates viral reactivation and suggest that decreased 5hmC modification of both cellular and viral genes may contribute to tumour development. In addition, it was confirmed that global 5hmC-modified DNA is very low or undetectable in EBV infected cells [186].

Furthermore, we demonstrated that DNA hypermethylation actively represses the transcriptional activity of the vTR promoter in the recombinant plasmids. DNA methylation is one of the epigenetic marks that are associated with repression of gene expression. It was suggested that during herpesvirus infection, the viral genome is subjected to a biphasic methylation cycle. Widely methylated during the viral latency, it returns to an unmethylated state during lytic viral replication [188,189,191]. The data obtained here corroborate with previous studies showing that DNA methylation represses the specific transcription of promoter during the latent phase [427]. The same effect has also been observed for the EBV [428]. However, in some cases, it was shown that the DNA methylation could have an opposite effect and thus be associated with transcriptional activation. For example, the reactivation of the EBV virus *via* the overexpression of the Zta viral protein, which binds preferentially to methylated sites [429].

As mentioned before, the c-Myc transcription factor plays a role in the expression of the vTR gene during the latency phase of GaHV-2. The vTR promoter has three c-Myc binding sites, namely E-box 1, E-box 2, and E-box 3, where it was confirmed that E-box 1 was not functional [24]. For this reason, the study of the effect of methylation on c-Myc transcription factor binding sites was conducted on E-boxes 2 and 3. The results obtained show that the mutation of the E-box 2 does not affect the transcriptional activity of the unmethylated vTR promoter, contrasting the E-box 3 and double E2E3 mutation that is associated with a decrease in the expression of the

vTR promoter. Similar results were obtained for LMH cells [357]. These results confirm that the E-box 3 is a *cis*-regulatory element involved in the vTR transcription. However, the double E2E3 mutation did not induce complete inhibition of the transcription, which suggests the involvement of other transcription factors regulating the activity of the vTR promoter. Indeed, the bioinformatic analysis of the vTR promoter showed a multitude of binding sites of different transcription factors, among which the binding site for the Ets transcription factor is also involved in the regulation of vTR transcription in the GaHV-2 transformed MSB-1 cell line [357].

Finally, for the methylated promoters, the mutation of E-boxes had an opposite effect. The activity of the mutated and methylated vTR promoter was significantly higher than that of the methylated wild-type promoter. These surprising results suggest that, contrary to observations made in an unmethylated situation, methylation would have masked the effect of the mutation. The high activity of the methylated mutated promoters could be explained by the fact that in the absence of E-box mutation, the c-Myc would be the main transcription factor for the activation of the vTR transcription. However, the mutation of the c-Myc binding sites could induce the recruitment of other transcription factors that are insensitive to the methylation. Indeed, a series of factors were described as being insensitive to methylation, Sp1, FCT, YY1 and C/EBP alpha-factor [429–431]. According to the results obtained in our study, it was expected to observe that the activity of the vTR promoter is negatively influenced by the methylation. The opposite effect obtained could be explained by the fact that during GaHV-2 infection, the regulation of the vTR promoter is not only dictated by the c-Myc but the result of the association of several factors, among which the Meq viral protein could have an important role. Indeed, the Meq protein has a central role in the regulation of the expression of many genes during the different phases of the GaHV-2 life cycle and could positively regulate the expression of the vTR. The Meq protein, *via* the Pro-Leu-Asp-Leu-Ser motif, could bind the C-terminal-binding protein (CtBP), which is a known co-repressor involved in the regulation of cell proliferation cell growth and apoptosis. The binding of Meq to CtBP will raise the inhibition of CtBP on the E2F protein that will bind to its consensus sequence present on the vTR gene and induce its transcription [235,326]. The viral oncoprotein Meq also has the leucine zipper binding site (B-ZIP) for the c-Jun protein. The Meq/Jun heterodimer could induce transcription of vTR by binding to the AP-1 site present at the vTR gene promoter [432].

Furthermore, the noticeable differences of this approach compared to *in vivo* observations regarding the effects of mutations in c-Myc response elements must be addressed. The effect of mutations introduced in the vTR promoter was established in an isolated context that was highly controlled in the cell lines. On the other hand, the *in vivo* conditions, due to their complex

interaction with viral and cellular machinery present unique environment that is less comparable to the *in vitro* situation, making it difficult to predict what would be the results of highly controlled *in vitro* assay when introduced in the animal model.

Moreover, an interesting increase in the promoter CpG-methylation was observed following reporter vector electroporation (prof. Thierry Arnould, personal communication, 2020). Therefore, this report indicates possible limits of the vector reporter approach in studying promoter activity. Additionally, it highlights the need for proper future strategies in assessing the negative consequences of electroporation on the DNA methylation and finding new ways how to avoid these effects.

3.2. Conclusions

In conclusion, we presented the first report that demethylation significantly increased telomerase activity *in vitro*. Moreover, we showed a significant change in methylation and hydroxymethylation levels on the vTR promoter between the latent and productive phase of GaHV-2 life cycle. In addition, the shift in methylation patterns was observed surrounding c-Myc transcription factor response elements located in the studied area of the vTR promoter. A more in-depth analysis of the methylation effect showed a significant decrease in the activity of methylated promoter compared to non-methylated ones. Additionally, the results obtained from the reporter study of methylation impact on the c-Myc transcription factor binding sites proved that the E-box 3 is a *cis*-regulatory element involved in the vTR transcription and revealed the involvement of other transcription factors regulating the activity of the vTR promoter. Contrary to observations made in an unmethylated situation, methylation seems to have masked the effect of the E-boxes mutations (Figure 2.25).

Furthermore, the importance of the functional c-Myc response elements in virus-induced tumorigenesis was studied using recombinant virus bearing mutations in E-box 2 and E-box 3, as well as revertant. Using the small homologous animal model for GaHV-2-induced lymphomagenesis, we demonstrated, for the first time, the regulatory function of the c-Myc oncoprotein during lymphomagenesis, emphasised with its binding to the E-box 2 and E-box 3 present in the vTR promoter. Mutated c-Myc response elements resulted in complete abrogation of tumour formation at 55 days post-infection and reduced viral loads detected in blood and feather follicle epithelium, implicating higher vTR expression levels in the transformation of the T-lymphocytes. Moreover, this observation was further supported by significantly reduced levels of vTR expression in mutated virus compared to the revertant control, what confirmed previous studies demonstrating c-Myc involvement in regulating vTR expression *in vitro*. Additionally, measuring relative telomerase activity, signified that lower vTR expression levels correlate with feeble telomerase activity in E-box 2 and E-box 3 mutated virus, the observation that supports the absence of visible Marek's disease symptoms in the infected birds. Our study provides further characterisation of the c-Myc response elements within the vTR promoter and their importance in the regulation of vTR expression, and moreover, vTR involvement in GaHV-2-induced tumorigenesis (Figure 2.25). To our knowledge, this is the first study to report on the attenuation of a herpesvirus following the mutation of functional response element driving the expression of a key virulence factor.

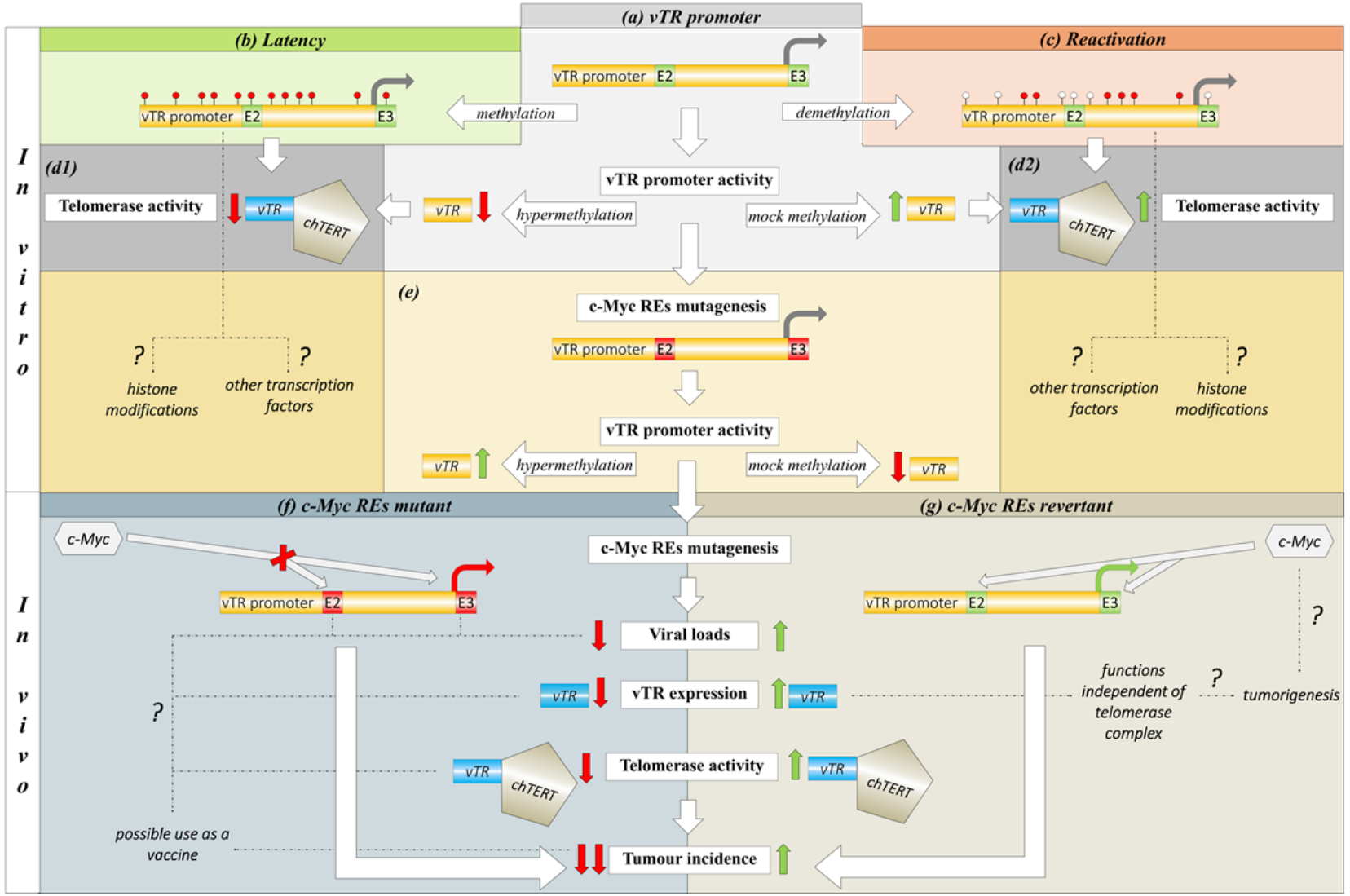

FIGURE 2.25. OVERVIEW OF THE STUDY REALISED ON THE VTR PROMOTER AND THE RESULTS OBTAINED FOR *IN VITRO* AND *IN VIVO* APPROACHES. (FIGURE CAPTIONS ON THE NEXT PAGE).

FIGURE 2.25. OVERVIEW OF THE STUDY REALISED ON THE VTR PROMOTER AND THE RESULTS OBTAINED FOR *IN VITRO* AND *IN VIVO* APPROACHES. (FIGURE ON THE PREVIOUS PAGE).

a) The primary goals of the study were to investigate *in vitro* changes in epigenetic modification on the vTR promoter between two stages of the viral life cycle, latency and reactivation. Our results showed that DNA methylation levels and patterns differ between **(b)** latency and **(c)** after viral reactivation, with the specific areas showing a significant drop in DNA methylation. **d)** Additionally, we demonstrated that viral reactivation increases telomerase activity compared to latent phase *in vitro*. **e)** Moreover, specific DNA methylation profiles and changes observed in the E-box 2 and E-box 3 during latency and after reactivation indicated their potential importance in the vTR promoter regulation. In order to investigate the importance of functional c-Myc response elements (E-box 2 and E-box 3), these sites were mutated. We showed that the mutation of the c-Myc response elements (c-Myc REs) in non-methylated conditions results in the significant reduction of vTR promoter activity compared to the wild-type. Surprisingly, the hypermethylation of the mutated vTR promoters increased promoter activity, suggesting that methylation masked the effects of c-Myc REs mutation. Furthermore, we investigated the impact of c-Myc mutagenesis *in vivo*, by infecting susceptible chicken line with either **(f)** mutant or **(g)** revertant virus. The results obtained demonstrated that c-Myc REs mutation resulted in lower viral loads in animals, compared to the animals infected with the revertant virus. In addition, the consequence of the c-Myc REs mutation was evident in the reduction of vTR expression as well as telomerase activity. Finally, the c-Myc REs mutation resulted in the abrogation of tumour development in the infected chicken. All the results obtained from *in vitro* and *in vivo* studies demonstrated the importance of functional c-Myc REs in the vTR promoter activity, and thus in regulation of vTR expression. However, more questions were raised that would need to be addressed in order to fully understand the mechanisms involved in vTR regulation and further on in the establishment of tumorigenesis in GaHV-2 context (indicated by dotted lines and question marks).

3.3. Perspectives

As discussed in the introduction, histones are subjected to the enormous number of post-translational modifications, including acetylation and methylation of lysines and arginines, phosphorylation of serines and threonines. The majority of these post-translational marks occur on the amino-terminal and carboxy-terminal histone tail domain, although some modifications occur in the central domains. Each lysine can accept one or more methyl groups and arginine residue can be mono- or di-methylated. All of these modifications are likely to control the structure and the function of the chromatin fibre, with different modifications yielding distinct functional consequences. For example, trimethylation of histone 3 at lysine 27 (H3K27me3) and lysine 9 (H3K9me3) is often associated with transcriptional repression. Also, trimethylation of histone 3 at lysine 4 (H3K4me3) and acetylation of histone 3 at lysine 9 (H3K9ac) correlates with transcription. However, there are clear exceptions, a particular mark or set of marks can have different or even opposite biological consequences. For instance, generally inhibitory H3K9 methylation in some cases can be associated with actively transcribed genes depending on the gene being studied and the cellular content.

Concerning GaHV-2, Brown et al. [188] showed that repressive marks H3K9me3 and H3K27me3 were both associated with the part of the repeat region around OryLyt from which gene expression is absent during latency. In contrast, the active marks H3K4me3 and H3K9ac are restricted to the core region that contains the MiR clusters, meq, vTR region and LAT. It is suspected that Meq is involved in histone modifications since it is one of the few proteins expressed during latency, has a nuclear distribution, binds DNA and regulates transcription. GaHV-2 ability to maintain active transcription during latency probably relates to the ability of Meq/c-Jun heterodimers to recruit histone acetyltransferases such as p300/CBP. In contrast, the ability to repress transcription as a homodimer probably relates to interaction with the cellular corepressor CtBP, which provides a potential link to the polycomb complexes responsible for H3K27me3. It is also possible that DNA methylation is involved in the recruitment of enzymes involved in histone modifications. The methylated CpG are indeed recognised by proteins such as methyl CpG binding domain proteins (MBD) which recruit chromatin repressors like histone deacetylase (NuRD) and histone methyltransferase. As well as for DNA methylation, there is no data available regarding histone modifications localised outside the repeated inverted regions of the GaHV-2 genome.

Thus, the deeper investigation whether the patterns of transcriptional repression and activation are related to the histones modifications located at the promoters of targeted vTR gene should be established. To accomplish this task, the chromatin immunoprecipitation (ChIP) assay followed by qPCR will be established. Antibodies against trimethylation modification of H3K27me3 and lysine 9 H3K9me will be used to identify silent regions, while antibodies against trimethylation of histone H3K4me3 and H3K9ac will be used to identify active regions. ChIP assay will also be performed before and after treatment of latently infected cells with histone methylase and/or histone acetylase inhibitor. This study aims to determine if histone modifications are associated with the reactivation of the virus *in vitro*. In addition, it would be interesting to investigate the patterns of histone methylation and acetylation after treating latently infected cells with DNA methyltransferase inhibitor such as 5-azacytidine. This approach will allow determining if DNA methylation is involved in the recruitment of enzymes involved in histone modifications.

Furthermore, the gene expression regulation is a complex mechanism intertwining multiple levels of control, including epigenetic, post-transcriptional and post-translational modifications. This control depends on interactions with numerous proteins and regulatory non-coding RNAs. Thus, the understanding and the identification of the regulatory proteins that control DNA transcription as well as RNA translation represent a crucial step in the comprehension in deciphering the fundamental processes in cellular biology [433]. The recently developed method of an open DNA mass spectrometry-based identification of proteins interacting with DNA [434] could be implemented in order to identify transcription factors and co-regulators interaction with the vTR promoter according to its methylation status. This type of DNA affinity capture method was shown to be useful in experiments performed on short [435] and long DNA sequence [434]. Moreover, the rate of DNA/protein complex recovery after the downstream reactions was estimated to around 100%, stipulating that all the capture proteins are likely to be involved in the next steps of analysis resulting in protein identification [434]. In the case of vTR, the core promoter will be amplified by high fidelity PCR. To allow a reversible immobilization of the capture probe to streptavidin-coated magnetic beads, the promoter will be amplified with one modified primer. Hypermethylation of vTR promoter will be performed using CpG methyltransferase M.SssI. Proteins identified by mass spectrometry in methylated versus unmethylated conditions will be compared to identify differential recruitment of transcriptional regulators in tested conditions.

However, this approach could be deemed as highly artificial that does not investigate chromatinized DNA. In order to avoid the limitations of this approach, reverse chromatin immunoprecipitation (rev-ChIP) assay could be implemented [436]. *In vitro* samples treated with inhibitors of DNA methyltransferases and histone deacetylases, as well as *in vivo* samples obtained

during different stages of the viral life cycle could be submitted to this approach named proteomics of isolated chromatin segments This strategy allows purification of an endogenous segment of chromatin in sufficient quantity and purity to identify the associated proteins. It relies on nucleic acid hybridization as the basis for purification, that allows isolation of specific formaldehyde cross-linked chromatin regions and identification of the proteins bound to those loci using mass spectrometric analysis [436].

Additionally, the discoveries revealed in this study open different possibilities in the exploration of potential new approaches in the future development of Marek's disease (MD) vaccines. MD vaccines are widely used in the poultry industry to protect chickens from GaHV-2-induced tumorigenesis. The current "gold standard" in vaccination is the live-attenuated GaHV-2 strain CVI988/Rispens, which efficiently protects chickens against very virulent field strains; however, it is unable to inhibit the horizontal viral spread, resulting in the need for total animal vaccination.

To further test the applicability of our recombinant virus bearing mutations in the functional c-Myc response elements, as a possible vaccine, a novel *in vivo* study is necessary. In the future study, *in vivo* characterisation of the recombinant virus should be analysed for 13+ weeks in order to have the detailed picture of lymphomagenesis establishment in the animals infected with the mutated recombinant virus, as well as to be in concordance with timelines used in other *in vivo* studies tracking GaHV-2 infection. Secondly, contact animals should be included in the experiment to refer to the question of horizontal transmission efficiency. The future results obtained should be compared with the results for the control groups consisting of the animals infected with the GaHV-2 very virulent field strain RB-1B, as well as infected with CVI988/Rispens and their respective contact animals. This novel *in vivo* study could potentially answer the question is the virus baring mutation in functional c-Myc response elements promising and usable future vaccine.

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CHAPTER 5. BRIEF PUBLICATION OVERVIEW

5.1. Written publications

5.1.1. Published papers – as a first author

- Pejaković S., Mbouombouo Mfossa A.C, Wiggers L., Kheimar A., Coupeau D., Kaufer B.B., Muylkens B., 2020. Role of DNA methylation and CpG sites in the viral telomerase RNA promoter during Gallid herpesvirus 2 pathogenesis. Journal of Virology, (Manuscript accepted for publication).

- Pejaković S., Wiggers L., Coupeau D., Kirschvink N., Mason J., Muylkens B., 2018. Test selection for antibody detection according to the seroprevalence level of Schmallenberg virus in sheep. PLoS ONE 13. doi:10.1371/journal.pone.0196532

5.1.2. Published papers – as a co-author

- Coupeau D., Burton N., Lejeune N., Loret S., Petit A., Pejakovic S., Poulain F., Bonil L., Trozzi G., Wiggers L., Willemart K., André E., Laenen L., Cuypers L., Bogaerts P., van Ranst M., Muylkens B., Gillet N., 2020. SARS-CoV-2 detection for diagnosis purposes in the setting of a molecular biology research lab. Methods and Protocols, 3, 59. doi:10.3390/mps3030059

- Boumart I., Figueroa T., Dambrine G., Muylkens B., Pejakovic S., Rasschaert D., Dupuy C., 2018. GaHV-2 ICP22 protein is expressed from a bicistronic transcript regulated by three GaHV-2 microRNAs. Journal of General Virology. doi:10.1099/jgv.0.001124

- Gennart I., Coupeau D., Pejaković S., Laurent S., Rasschaert D., Muylkens B., 2015. Marek's disease: Genetic regulation of gallid herpesvirus 2 infection and latency. Veterinary Journal 205. doi:10.1016/j.tvjl.2015.04.038

5.1.3. Papers in preparation

- Pejaković S., Poulain F., Muylkens B., 2020. Genome-wide DNA methylation profiles change after viral reactivation from latency in Gallid herpesvirus type 2 transformed cell line.

- Pejaković S., Muylkens B., 2020 (Review). Viral oncogenesis: Lessons from homologous animal models.

5.2. Publications related to the conferences and meetings

5.2.1. Oral communications

- 31st August to 3rd September 2015: The Xth International Congress of Veterinary Virology of ESV, Montpellier, France
  "Epigenetic regulation of the viral RNA telomerase subunit promoter over-expressed in lymphoma induced by Marek's Disease Virus"

- 29th July to 2nd August 2018: The 12th International Symposium on Marek's Disease and Avian Herpesviruses, Yangzhou, China
  "Epigenetic regulation of the viral telomerase RNA subunit promoter over-expressed during Marek's Disease Virus induced oncogenesis"

- 14th June to 17th June 2020: The 13th International Symposium on Marek's Disease and Avian Herpesviruses, Guelph, Canada (talk accepted, postponed due to Covid-19 outbreak):
  "Functional evaluation of c-Myc response elements in the promoter of the viral telomerase RNA subunit during Marek's Disease virus infection"

- 8th September 2020: Non-coding RNA in health and disease, Namur, Belgium (talk accepted, postponed due to Covid-19 outbreak):
  "Functional evaluation of c-Myc response elements in the promoter of the viral telomerase RNA subunit during Marek's Disease virus infection"

5.2.2. Poster presentations

- 3rd and 4th March 2016: 6th Clinical Epigenetics Society (CLEPSO) International Meeting, Düsseldorf, Germany
  "Discriminative methylation patterns in the promoters of viral genes in a model of Marek's Disease Virus induced oncogenesis"

- 6th to 9th July 2016: 11th International Symposium on Marek's Disease and Avian Herpesviruses, Tours, France
  "Epigenetic regulation of the viral RNA telomerase subunit over-expressed in lymphoma induced by Marek's Disease Virus"

- 24th and 25th March 2017: NARILIS Cancer Research Pole, Namur, Belgium
  "DNA methylation and hydroxymethylation on the viral telomerase RNA subunit promoter in a model of herpesvirus induced oncogenesis

- 11th May 2017: Interuniversity PhD Student Day, Liege, Belgium
  "DNA methylation and hydroxymethylation on the viral telomerase RNA subunit promoter in a model of herpesvirus induced oncogenesis"

- 24th November 2017: 1st NARILIS Infectiology research Pole Annual Meeting, Namur, Belgium,
  "Epigenetic regulation of the viral telomerase RNA subunit promoter over-expressed during Marek's Disease Virus induced oncogenesis"

- 30th November 2018: 2nd NARILIS Infectiology research Pole Annual Meeting, Namur, Belgium,
  "Epigenetic regulation of the viral telomerase RNA subunit promoter over-expressed during Marek's Disease Virus induced oncogenesis

CHAPTER 6. ANNEXES

6.1. ANNEX A: Viral oncogenesis: Lessons from homologous animal models (Review)

Srđan Pejaković, Benoît Muylkens

Namur Research Institute for Life Sciences, Integrated Veterinary Research Unit, Department of Veterinary Medicine, Faculty of Sciences, University of Namur, Namur, 5000, Belgium

6.1.1. Abstract

The interactions between viruses and the hosts entail complex machinery and numerous events that, in the end, drive viral infection, which sometimes leads to oncogenesis. The vast number of information concerning viral pathogenesis and oncogenesis, as well as the host responses, have been obtained using animal models. These models are helpful for understanding virus-host interactions and better deciphering different steps of cancer development. Well-defined small animal models with the reliable kinetics of disease induction and progression, together with the fact that oncogenesis can be studied in a natural virus-host system, allows unique studies that are impossible to perform in other non-natural models of viral disease and oncogenesis. In particular, they are essential for understanding viral replicative/pathogenic/oncogenic properties in humans. To this date, animal research models are necessary to describe *in vivo* situation and are essential for developing a detailed knowledge of virus-caused infectious diseases. This review aims to present the studies on well-established homologous animal models used for translational oncogenic research for three viral families: Retroviridae, Herpesviridae and Papillomaviridae, and the examples of lessons obtained that helped in deciphering the mechanisms of major steps of oncogenesis.

Keywords**:** Animal models; Viral oncogenesis; Herpesviridae; Papillomaviridae; Retroviridae

6.1.2. Introduction

Since the early work of Greek philosopher Aristotle and others like him, animal models have been used as a comparative research tool to study human anatomy and physiology. Animal models slowly reached the level where they are used in almost all the fields of biomedical research including biology, immunology, infectious diseases and oncology (Ericsson et al., 2013). In today society, cancers cause over ten million new cases resulting in over six million human deaths per year (Parkin et al., 2005). Most cancers will inevitably lead to the death of the affected organism if the tumour acquires several biological capabilities during the multistep development process (Hanahan and Weinberg, 2017). These general capabilities of cancers in humans and other vertebrates are similar. Therefore, animal models of oncogenesis can be used as relevant tools in studying the molecular biology of cancer.

Research done in the past three decades showed that several viruses play a significant role in the development of animal and human cancers, with 10% of cancers associated with viral infections (Schiller and Lowy, 2014). Contribution of the oncogenic viruses to different steps of the carcinogenesis and the association of a virus with a given cancer can vary from 15-100% (Parkin, 2006). These viruses are widespread among animals and can cause economic losses in animal husbandry and serve as valuable models to study viruses affecting humans (Truyen and Lochelt, 2006). Seven different viruses have been linked to oncogenesis for 12% of human cancers: Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), and human papillomavirus (HPV), human T-cell lymphotropic virus (HTLV), Kaposi's sarcoma herpesvirus (HHV-8), and Merkel cell polyomavirus (MCPyV) (White et al., 2014).

Aim of this review is to present examples of important studies on three oncogenic viral families: Retroviridae (Table A.1), Herpesviridae and Papillomaviridae (Table A.2), as well as major lessons obtained from corresponding homologous animal models that helped to decipher biological processes involved in the hallmark steps of carcinogenesis. Two main modes of action, genome instability resulting in mutations of regulatory genes, and the infiltration of immune-inflammatory cells, play an essential part in the induction of tumorigenesis steps. Each step possesses a specific role in supporting the development, progression, and persistence of tumours in the organism (Hanahan and Weinberg, 2017).

By using animal models of virus-induced oncogenesis, a comprehensive view was obtained on these steps of cancer development. These models also opened avenues to test new therapies targeting one or several properties of the tumour cells.

TABLE A.1. EXAMPLES OF ANIMAL AND HUMAN ONCOGENIC RNA VIRUSES AND ASSOCIATED TUMOURS.

	Animal oncogenic RNA viruses		Human oncogenic RNA viruses	
	Virus	**Tumour**	**Virus**	**Tumour**
Retroviridae	RSV	sarcoma	HTLV	adult T-cell leukaemia
	AEV	sarcoma, carcinoma		
	ALV	B-cell lymphoma		
	BLV	lymphoma		
	FLV	lymphoma		
	FSV	fibrosarcoma		
	GALV	myeloid leukaemia		
	JSRV	adenocarcinoma		
	KoRV	leukaemia, osteochondroma		
	MuLV	leukaemia		
	MSV	osteosarcoma		
	MMTV	mammary tumours		

AEV, Avian erythroblastosis virus; ALV, Avian leukosis virus; BLV, Bovine leukaemia virus; FLV, Feline leukaemia virus; FSV, Feline sarcoma virus; GALV, Gibbon Ape Leukemia Virus; HTLV, Human T-cell leukaemia virus; JSRV, Jaagsiekte sheep retrovirus; KoRV, Koala retrovirus; MMTV, Mouse mammary tumour virus; MSV, Murine sarcoma virus; MuLV, Murine leukaemia virus; RSV, Rous sarcoma virus.

Many animal tumours have been associated with Retroviruses and studies on these RNA viruses have been important for the discovery of oncogenes, tumour suppressors and different regulations of cellular signal transduction pathways. On the other hand, discoveries obtained from studies on the DNA viruses ranged from mechanisms controlling cell growth and the discovery of cellular tumour suppressor genes to molecular mechanisms of virus-induced cell transformation (Damania, 2007).

The *in vitro* studies of oncogenic viruses and their interactions with the natural hosts are invaluable for understanding the key pathways that are involved in the main steps of carcinogenesis. However, to study in detail virus-host interactions and to better decipher the steps of cancer development, it is necessary to observe these viruses and their actions in the whole organism using animal models.

TABLE A.2. EXAMPLES OF ANIMAL AND HUMAN ONCOGENIC DNA VIRUSES AND ASSOCIATED TUMOURS.

	Animal oncogenic DNA viruses		Human oncogenic DNA viruses	
	Virus	**Tumour**	**Virus**	**Tumour**
Herpesviridae	GaHV-2	lymphoma, carcinoma	EBV	Burkitt's lymphoma, Hodgkin's lymphoma, B-cell lymphoma, nasopharyngeal carcinoma
	HVS		KSHV	Kaposi's sarcoma, lymphoma
Papillomaviridae	BPV [a]	papilloma, carcinoma	HPV[b]	oral, cervical and anal cancer
	CRPV	sarcoma, lymphoma		
	MmuPV1	carcinoma		

[a] types 1, 2, 4

[b] types 6, 8, 11, 16, 18, 31, 45

BPV, Bovine papillomavirus; CRPV, Cottontail rabbit papillomavirus; EBV, Epstein-Barr virus; GaHV-2, Gallid herpes virus type 2; HPV, Human papillomavirus; HVS, Herpesvirus saimiri; KSHV, Kaposi's sarcoma-associated herpesvirus; MmuPV1, Mouse papillomavirus.

6.1.3. Retroviridae

Retroviruses are characterized by their ability to integrate their genome into host-cell DNA. After infection of the cell, the viral RNA genome is reversely transcribed by the viral reverse transcriptase into a double-stranded DNA. Newly synthesised DNA integrates into the host's chromosome and is expressed under the control of viral transcriptional regulatory sequences. The main consequence of viral DNA integration is the retrovirus ability to activate or inactivate cellular genes using proviral insertion (Temin & Baltimore, 1972, Coffin, 1979). Animal retroviruses, which belong to the Alpharetrovirus and Gammaretrovirus genera, induce tumours using these two ways. The studies on Retroviruses led to the finding of RSV transforming gene, v-src, and its hybridization to cellular sequences, the discovery of proto-oncogenes and the mechanisms of insertional mutagenesis (Stehelin et al., 1976, Kurth, 1983, Bradshaw, 1987), what will be discussed below.

6.1.3.1. Sarcoma viruses and functions of oncogenes and proto-oncogenes

The idea that viruses could have a role in the development of tumours in animals originated when Peyton Rous described a filterable Rous sarcoma virus (RSV) in cell extracts of a chicken tumour that could transmit the tumour to the healthy chickens (Rous, 1911). Modern tumour virology was born with the development of *in vitro* transformation assay for RSV (Temin et al., 1958), which enabled genetic analysis of the retroviral life cycle and virus-cell interactions that lead transformation resulting in malignant cells (Varmus, 1984; Weinberg et al., 1989). This established RSV as a highly favourable model system for studying oncogenic viruses. Most importantly, studies on RSV enabled the identification of v-src as the only RSV gene that was required for triggering cell transformation (Martin et al., 1971; Lai et al., 1973). Shortly after followed the discovery that the v-Src was a phosphoprotein (Brugge and Erikson, 1977; Purchio et al., 1978) with protein kinase activity (Collett and Erikson, 1978; Levinson et al., 1978), which phosphorylates tyrosine residues (Hunter and Sefton, 1980; Collett et al., 1980). v-Src oncogene together with its animal model, played a ground-breaking role in deciphering subtle cellular mechanisms involved in oncogenesis and provided the first evidence of the association between proto-oncogene and oncogene. This research was essential in the discovery of v-src cellular homologue, the c-src (Stehelin et al., 1976), baring the difference at its carboxyl (C)-termini (Takeya and Hanafusa, 1983). This boosted the research and discovery of different cellular oncogenes, which contribute to the development of neoplasms and are the major driving force of oncogenesis (Table A.3). Furthermore, the discovery that v-Src is a constitutively activated form of c-Src led to obtaining the most important lessons of the molecular mechanisms behind Src activation.

The lessons obtained from v-Src/c-Src research provided an important connection between oncogene and proto-oncogene. More importantly, it highlighted a crucial concept in cancer biology, that the loss of a negative regulatory domain transforms the proto-oncogene into an oncogene. It has been revealed that many different processes that can alter c-Src activity, and in many cases, these or similar mechanisms of regulation are found in different members of the Src family of tyrosine kinases. These mechanisms of regulation, among many, include alterations in the phosphorylation status of Src, mediated by kinases and phosphatases (Figure A.1) and/or Src interaction with Src-binding proteins (Bjorge et al., 2000). In addition, mutations in cellular oncogenes found in human tumours originated from mutagenic errors are often similar or identical to those discovered in trans-acting oncogenic retroviruses (Parada et al., 1982).

TABLE A.3. DIFFERENT ANIMAL RETROVIRUSES WITH ASSOCIATED ONCOGENES.

Virus	Species	Oncogene	Tumour	Reference
Rous sarcoma	chicken	src	sarcoma	Stehelin et al., 1976
Avian myeloblastosis	chicken	myb	myeloblastic leukaemia	Roussel et al., 1979
Avian myelocytomatosis	chicken	myc	carcinoma, sarcoma	Sheiness and Bishop, 1979
Fujinami sarcoma	chicken	fps	sarcoma	Hanafusa et al., 1980
Reticuloendotheliosis	turkey	rel	lymphatic leukaemia	Chen et al., 1981
Simian sarcoma	monkey	sis	sarcoma	Dalla-Favera et al., 1981
Feline sarcoma (ST)	cat	fes	fibrosarcoma	Franchini et al., 1981
Moloney murine sarcoma	mouse	mos	sarcoma	Blair et al., 1981
	rat	Tpl-1	T cell lymphoma	Bear et al., 1989
		Tpl-2		Makris et al., 1993
FBJ murine osteosarcoma	mouse	fos	chondrosarcoma	Curran et al., 1982
Kirsten murine sarcoma	rat	K-ras	sarcoma	Der et al., 1982
Feline sarcoma (McD)	cat	fms	fibrosarcoma	Donner et al., 1982
Harvey murine sarcoma	rat	H-ras	sarcoma	Parada et al., 1982
Abelson murine leukaemia	mouse	abl	B cell lymphoma	Srinivasan et al., 1982
Mouse mammary tumour	mouse	int-1	carcinoma	Nusse et al., 1984
		int-2		Peters et al., 1984
Murine leukaemia	mouse	pim-1	lymphoma	Theo Cuypers et al., 1984
		bmi-1	lymphoma	van Lohuizen et al., 1991
Avian sarcoma	chicken	jun	fibrosarcoma	Maki et al., 1987

All the lessons obtained on Src activation and its role in cancer development will help to understand the processes involved in the human oncogenesis and will mark Src as an important target for the future development of new therapies (Irby and Yeatman, 2000).

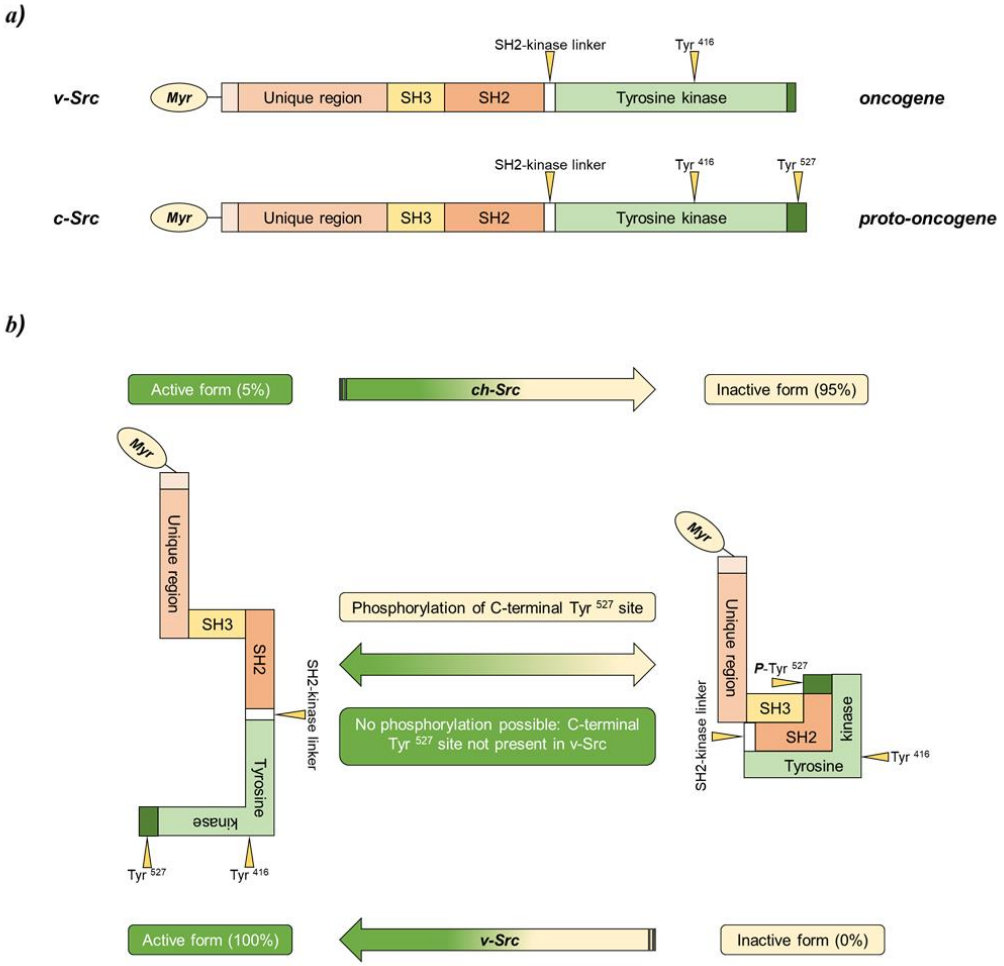

FIGURE A.1. ONE OF THE PROPOSED MECHANISMS OF CELLULAR SRC ACTIVATION INVOLVED IN PROMOTING ONCOGENESIS.

a) Src is a member of the family of structurally related protein tyrosine kinases. Src-family members at the N-terminus bear a domain that undergoes fatty acid modification (Myristoylation - Myr), a unique region, an SH3 domain, an SH2 domain, the SH2-kinase linker, a kinase domain, and a C-terminus regulatory domain. Cellular (c-Src) and viral Src (v-Src) possess a positive regulatory autophosphorylation site (Tyr 416). In addition, c-Src has a negative regulatory phosphorylation site at its C-terminus (Tyr 527). **b)** Src inactive conformation (on the right), with the SH2 domain coupled with Tyr 527, the SH3 domain coupled with the SH2-kinase linker and dephosphorylated Tyr 416. Phosphorylation of Tyr 527 (***P***-Tyr 527) by Csk leads to interaction between the SH2 domain and Tyr 527, resulting in c-Src inactivation (on the right). Dephosphorylation of Tyr 527 by protein tyrosine phosphatase disrupts the interaction between the SH2 domain and Tyr 527, Tyr 419 is auto-phosphorylated, resulting in c-Src activation (on the left).

6.1.3.2. Activation mechanisms of transactivator protein Tax deciphered using bovine leukaemia virus and human T-cell lymphotropic virus

The causative agent of enzootic bovine leukosis is bovine leukaemia virus (BLV) that belongs to the Retroviridae family and Deltaretrovirus genus (Miller et al., 1969). In addition to BLV, delta-type retroviruses also include human T-cell lymphotropic virus type 1 and 2 (HTLV-1 and HTLV-2) and simian T-cell lymphotropic virus type1 and 2 (STLV-1 and STLV-2) (El Hajj et al., 2012). Although retroviruses have been associated with many animal tumours, only HTLV-1 has been associated with human cancers (Table A.1) (McLaughlin-Drubin and Munger, 2008). The comparative virology approach is useful to characterize related pathogens and to speculate on possible shared mechanisms. Therefore, studies on BLV, which is evolutionary related to HTLV-1, may facilitate a better understanding of the mechanism of leukemogenesis induced by HTLV-1. In addition to the classical retroviral genes that are required to complete the viral cycle, BLV also encodes a series of additional accessory genes as well as microRNAs that modulate viral and cellular gene expression (Derse, 1987; Derse, 1988; Kincaid et al., 2012). These genes include Tax and G4 oncogenes that can promote the transformation of cells (Willems et al., 1992; Kerkhofs et al., 1998). Although the mechanisms of cell transformation remain to be further characterized, BLV and HTLV-1 Tax share cellular targets (Twizere et al., 2003). Furthermore, the negative strand chain of HTLV-1 genome codes for unique HBZ gene (Gaudray et al., 2002). Since the BLV genome does not code for HBZ, it has been assumed that the Tax protein plays a crucial role in the BLV and HTLV-1 oncogenesis and thus has been extensively studied (Table A.4, adapted from Aida et al., 2013). One of the best-characterized functions of Tax is the activation of viral transcription. The Tax protein acts on Tax-responsive element (TxRE) in the U3 region of the 5'LTR and stimulates transactivation of the viral genome (Figure A.2) (Derse, 1987; Willems et al., 1987; Katoh et al., 1989). It also stimulates transcription of many cellular genes by stimulating activity of nuclear factor $\varkappa$B (NF-$\varkappa$B), serum responsive factor (SRF) and cyclic AMP responsive element-binding protein (CREB) binding motifs (Kashanchi and Brady, 2005), which results in neoplastic transformation of the cells (Smith and Greene 1990; Yamaoka et al., 1996; Akagi et al., 1997). Tax activates and modulates the expression of several cellular genes (Ruben et al., 1988; Wano et al., 1988; Doi et al., 1989; Dittmer et al., 1997) that are involved in the regulation of cell growth (Figure A.2) (Tajima and Aida, 2002; Marriott and Semmes, 2005). Additionally, Tax binds and inactivates mitotic arrest-defective 1 (MAD1) protein (Jin et al., 1998), disturbing the mitotic spindle checkpoint, and suppressing DNA repair (Figure A.2). (Kashanchi and Brady, 2005). Altogether, these alternations boost the progression of the cell cycle.

TABLE A.4. LESSONS OBTAINED FROM STUDIES ON BLV AND HTLV-1 TAX PROTEIN.

TAX PROTEIN FUNCTION	
BLV	**HTLV-1**
Transcriptional activator of viral expression	Transcriptional activator of viral expression
Derse, 1987; Willems et al., 1987; Katoh et al., 1989	Kashanchi and Brady, 2005
Oncogenic potential	Oncogenic potential
Willems et al., 1990	Matsuoka and Jeang, 2011
Activator of NF-*κ*B pathway	Functional regulator of cellular proteins *via* direct binding
Szynal et al., 2003; Klener et al., 2006	Boxus et al., 2008
Involvement in host cell transcription, signaling, stress response and immune response	Involvement in of DNA damage induction, cellular senescence and apoptosis
Arainga et al., 2012	Chlichlia and Khazaie, 2010

Thus, the involvement of Tax in transcriptional regulation, cell cycle progression, chromosome instability and DNA damage repair in infected cells mark Tax as a primary initiator of tumorigenesis. In addition, Tax, similarly to the Myc oncogene, cooperates with the Ha-ras oncogene to induce full transformation of cells that form tumours, emphasizing the immortalizing potential of Tax (Gillet et al., 2007). Furthermore, Tax protein uses numerous strategies to escape apoptotic pathways of the host cell. Three main strategies of apoptotic evasion have been described: NF-kB over-expression, caspase blocking, and disruption of apoptosis regulators, such as p53 (Karimi et al., 2017).

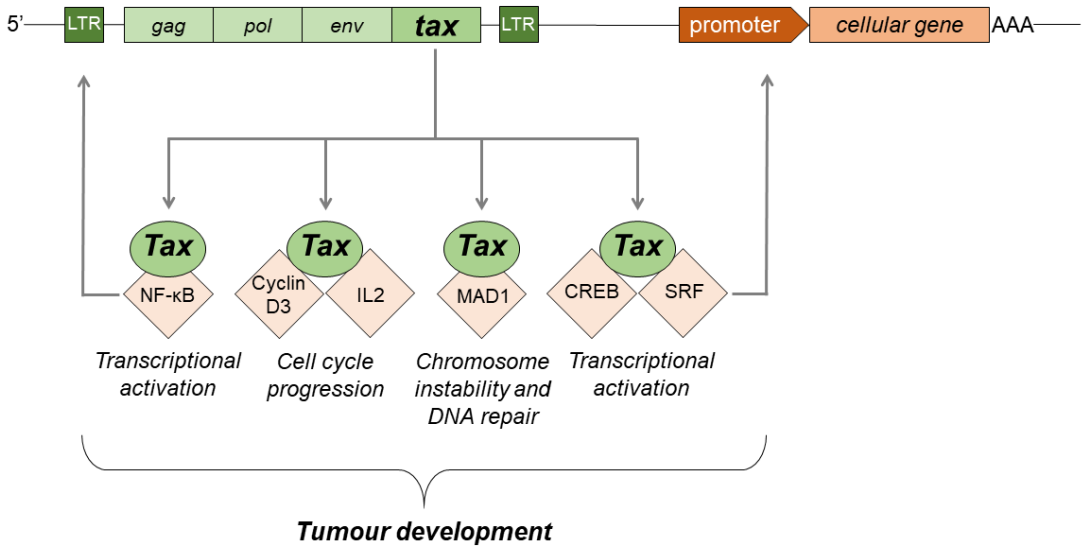

FIGURE A.2. THE MULTIFUNCTIONAL CO-REGULATION OF VIRAL AND CELLULAR PATHWAYS BY TAX TRANSACTIVATOR PROTEIN.

Tax does not directly bind to the DNA, however, it increases transcription of viral genes from the 5' long terminal repeat (5'LTR) and stimulates transcription of cellular genes by stimulating activity of nuclear factor κB (NF-κB), serum responsive factor (SRF) and cyclic AMP responsive element-binding protein (CREB) in cellular genes, promoting cell transformation. In addition, Tax activates several cellular genes, including IL-2 involved in T-cell growth. Further effects on the cell cycle are transcriptional activation of cdk2 and cdk4 and transcription repression of cyclin-dependent kinase (Cdk) inhibitors. The tax also directly binds to cyclin D3 stabilising cyclin D/Cdk4 complexes. Additionally, Tax binds and inactivates mitotic arrest-defective 1 (MAD1) protein, disturbing the mitotic spindle checkpoint and suppressing nucleotide excision repair (adapted from Maeda et al., 2008).

6.1.3.3. The mechanisms of retroviral insertional mutagenesis discovered using three retroviral homologous animal models

Retroviral replication necessitates the integration of a viral genome into the host DNA, resulting in a potential mutagenic process. This insertion may result in proto-oncogene activation, and the first evidence of this process has been demonstrated using the avian leukosis virus (ALV) (Hayward et al., 1981). Furthermore, cancer-causing insertional mutations change the transcriptional activity rather than the coding sequences of the genes (Robinson, 1983). The observation of how common the event of the activation is in virus-induced lymphoid leukosis led to the discovery of numerous common insertion sites (CIS) and the important host genes closely

involved in oncogenesis. (Neil and Cameron, 2002). Three homologous animal models, avian leukosis virus (ALV), mouse mammary tumour virus (MMTV) and murine leukaemia virus (MuLV), have been widely used for studies of insertional mutagenesis.

Main lessons obtained using these retroviral animal models revealed that provirus-mediated activation and expression of proto-oncogenes, leading to tumorigenesis, have been divided into two main mechanisms: activation of proto-oncogenes and inactivation of tumour suppressor genes (Figure A.3) (Mikkers and Berns, 2003). The most commonly observed outcome of insertional mutagenesis of CIS target genes is transcriptional activation due to the insertion of viral promoters of enhancer elements (Figure A.3.a). It occurs when the retrovirus integrates near a promoter of the target gene, which results in enhancing the activity of viral long terminal repeats (LTRs) sequences or by stabilisation of the messenger RNA when the virus integrates within the 3' untranslated region. Secondly, if the provirus integrates into the 5' region of a target gene promoter it activates gene expression through promoter sequences in LTR (Figure A.3.b). Finally, in some cases, viral integration within the cellular genomic region can disrupt the translation unit, resulting in inactivation of a gene due to interruption of the gene structure (Figure A.3.c) (Jonkers and Berns, 1996).

Retroviral CSIs and the pathways they target are frequently deregulated in human cancers, making them important actors in understanding oncogenic signalling pathways and potential targets for tumour intervention (Mikkers and Berns, 2003; Hilkens, 2006; Kim et al., 2011; Callahan et al., 2012; Klijn et al., 2013). This emphasizes the relevance of understanding and mapping distinct oncogenic pathways for a variety of different tumour types. Moreover, another contribution of these models in deciphering cancer development refers to the fate of the cells that underwent viral insertional mutagenesis. Following the activation of a single proto-oncogene or the inactivation of a single tumour suppressor gene, the cell infected with retrovirus is far from acquiring cancerous phenotype. Using these animal models of retroviral induced cancer, it has been indicated that following the initial step of viral insertion, additional events are required to initiate cancer development (Green et al., 1987). This explains why some of the retroviral infections never lead to tumorigenesis (Weinberg, 1989). Finally, this cumulative process of additional mutations required for the rise of cancer dictates the time needed between the initial retroviral infection and the initiation of tumour development (Duesberg, 1987).

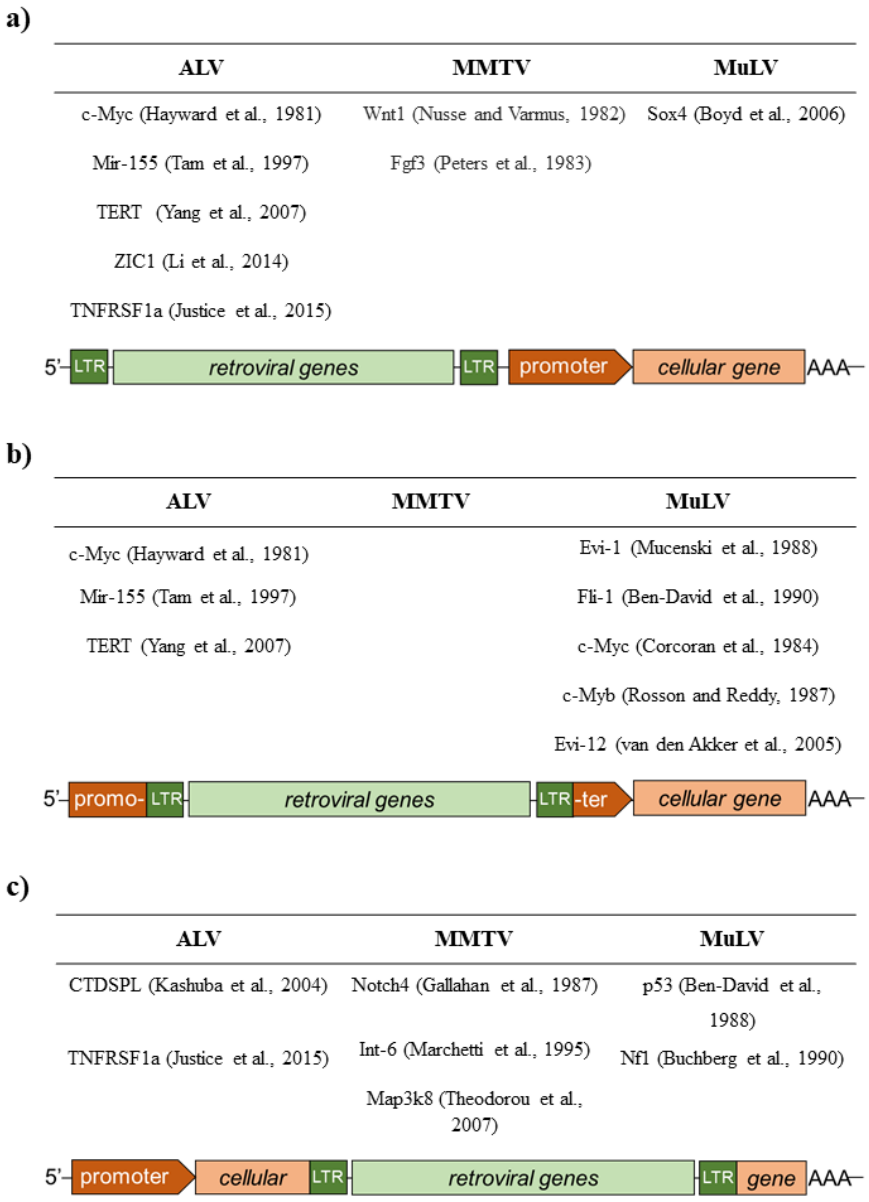

FIGURE A.3. MECHANISMS OF RETROVIRAL INSERTIONAL MUTAGENESIS.

Examples of viral integration sites and commonly targeted genes involved in oncogenesis are shown for three oncogenic retroviruses studied in corresponding homologous animal models (Avian leukosis virus - AVL; Mouse mammary tumour virus - MMTV and Murine leukaemia virus - MuLV). a) The provirus can integrate near the promoter of the cellular target gene, enhancing the gene expression controlled by the cellular promoter. b) Insertion of the retrovirus within the 5' region of the target gene promoter activates the gene expression under the control of viral promoter sequences in the long terminal repeats (LTR). c) The proviral integration within the cellular intragenic region may result in the truncated transcript *via* the internal polyA signal (-AAA) or disrupted gene structure.

6.1.4. Papillomaviridae

6.1.4.1. Animal papillomaviruses as models for HPV infection and carcinogenesis

Papillomavirus (PV) infection manifests itself in the form of hyperplasia or warts on the skin. Furthermore, some of these viruses, like the cottontail rabbit papillomavirus (CRPV), the bovine papillomavirus type 4 (BPV4) and the high-risk human papillomaviruses (HR-HPV) are strongly implicated in the occurrence of malignant lesions (zur Hausen and Schneider, 1987). Papillomaviruses show species- and tissue-specific tropisms, which, therefore, makes their study difficult in a relevant laboratory model (Spurgeon et al., 2019). Many animal models have been used over the last decades to study PV infection, such as the CRPV, the canine oral papillomavirus (COPV) and BPV. The CRPV and BPV have been used as homologous animal model systems to study the viral interaction with the natural host and with environmental co-factors (Campo, 1997). Fascinatingly, in the cattle which have been feeding on bracken fern (Pteridium aquilinum), known for its carcinogenic activity (Evans and Mason, 1965), a correlation between persistent papillomatosis and cancer was observed (Jarrett et al., 1978).

Early on, the CRPV was the first papillomavirus to be experimentally studied (Shope and Hurst, 1933) and was the first to be associated with malignant progression (Rous and Beard, 1935). Since then, CRPV animal model has been used extensively to study restricted tissue and species association of PVs (Parsons and Kidd, 1942; Harvey et al., 1998). This model has been essential for studies of viral function and structure, deciphering the importance of viral genes involved in the viral life cycle (Figure A.4) (Brandsma et al., 1992; Belnap et al., 1996; Jeckel et al., 2003; Nonnenmacher et al., 2006). Besides, latent infection and development of the vaccines were thoroughly studied using CRPV animal model (Christensen et al., 2017). In recent years, the CRPV animal model has become a significant preclinical model for testing antiviral and immunotherapeutic strategies for papillomavirus infections (Figure A.4) (Christensen, 2005).

The significant advantages and lessons obtained using animal models of PV-infection have been a deep understanding of the viral life cycle and tight regulation of viral proteins during the progression of the infection (Figure A.4) (Campo, 1998; Doorbar, 2005). The importance of PV genes, and proteins they encode, was noticed from early studies done on bovine papillomavirus type 1 (BPV1). BPV, unlike HPV, replicates in and transforms fibroblasts and epithelial cells (Campo, 1997; Munday, 2014).

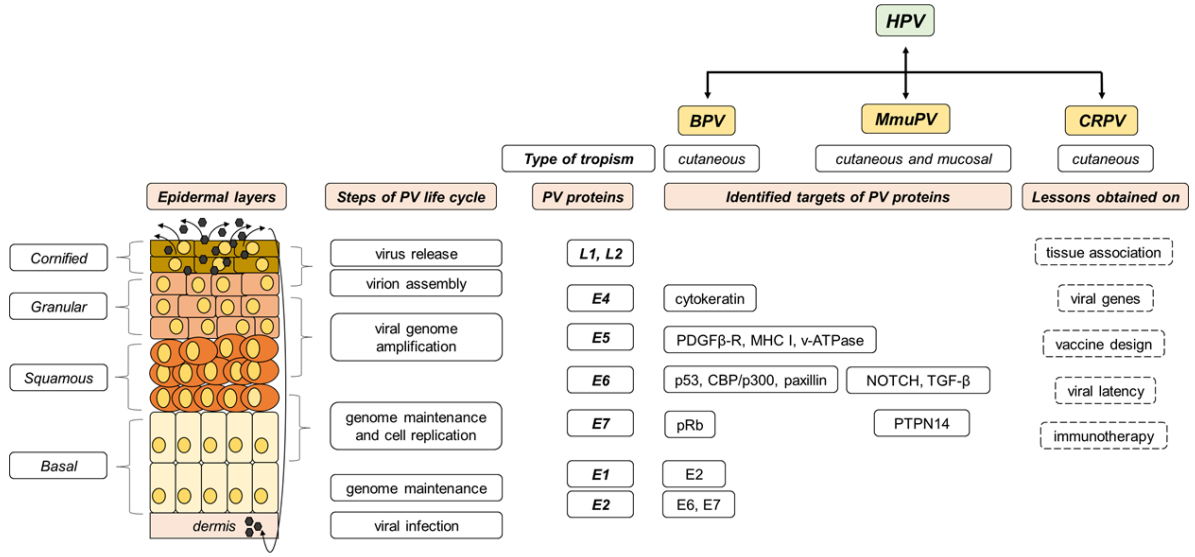

FIGURE A.4. A SCHEMATIC REPRESENTATION OF THE PAPILLOMAVIRUS LIFE CYCLE HIGHLIGHTING THE INVOLVEMENT OF VIRAL PROTEINS WITH THEIR TARGETS DURING VIRAL INFECTION AND LESSONS OBTAINED FROM THREE ANIMAL MODELS.

The action of the papillomavirus (PV) proteins in viral replication is a highly regulated process and spatially associated with the epithelial differentiation. After viral particles (black hexagons) infect dermis through the laceration, the viral genome is maintained in low copy numbers. In this stage, the viral E1 and E2 proteins are expressed in order to maintain the viral DNA as an episome. During epithelial differentiation, expression of the E6 and E7 oncogenes stimulate cell cycle progression and are associated with regulators of the cell cycle. The E7 up-regulation increases the expression of proteins involved in viral DNA replication (E1, E2, E4 and E5). Finally, the major capsid protein L1 is expressed after L2 allowing the assembly of infectious viral particles in the upper layers of the epithelium (Doorbar, 2005). Examples of identified targets of PV proteins (solid line rectangles) and research subjects (dash line rectangles) are indicated for three animal models. CRPV - Cottontail papillomavirus, BPV - Bovine papillomavirus, HPV - Human papillomavirus, MmuPV - Mouse papillomavirus.

The BPV animal model has been useful in assigning key functions to many of the papillomavirus early proteins (Doorbar, 2016). Lessons obtained from the E1 protein showed its functions in viral DNA replication (Lambert, 1991) and the viral E2 protein is an important viral transcription factor and is an accessory protein for replication (Figure A.4) (Araldi et al., 2017). Furthermore, BPV viral oncoproteins E6 and E7 have been shown to be involved in the regulation of cell-cycle entry and cell proliferation, similar to high-risk HPV types (Campo, 1997). In addition, BPV E6 protein binds paxillin, resulting in its transforming function (Tong and Howley, 1997; Pol et al., 1998), and the transcriptional co-activator CBP/p300, leading to the down-regulation of CBP/p300-mediated transactivation (Figure A.4) (Zimmermann et al., 2000).

TABLE A.5. EXAMPLES OF CURRENT RESEARCH AREAS AND TRANSLATIONAL LESSONS THAT MAY BE ACQUIRED FROM HOMOLOGOUS ANIMAL MODELS.

Oncogenic virus	Homologous animal model	Research area	Translational lessons	Reference
KoRV	koala	piRNA	host anti-viral response	Haase et al., 2019
BLV	bovine	decreasing the seroprevalence of BLV	management of HTLV infection	Rodríguez et al., 2011
		BLV vaccine development	translational knowledge for HTLV-1 vaccine	Abdala et al., 2019
JRSV	sheep	host pneumocytes transformation	role of alveolar cells in human lung cancer	Murgia et al., 2011
		multiple techniques integration	pre-clinical diagnosis and tumour pathogenesis	Humann-Ziehank et al., 2013
				Gray et al., 2019
		chemotherapeutic agents	application in human adenocarcinoma	Varela et al., 2008
		miniaturized implantable sensors	targeted therapies against radiation and chemo-resistant regions	Marland et al., 2018
MmuPV1	mouse	tissue and species-tropisms	host-restricting factors for viral infections	Hu et al., 2017
		innate immune response	HPV vaccine development	
		hormonal studies	long-term contraceptive during HPV infections	Hu et al., 2017
		viral transmission	infertility and associated childhood diseases	Mammas et al., 2014
		cutaneous and mucosal infections and cancer development	therapeutic vaccines	Assi et al., 2014
				Petrelli et al., 2014

BLV, Bovine leukaemia virus; HPV, Human papillomavirus; HTLV, Human T-cell lymphotropic virus; JSRV, Jaagsiekte sheep retrovirus; KoRV, Koala retrovirus; MmuPV1, Mouse papillomavirus.

E7 oncoprotein can bind to two conserved motifs of pRb tumour suppressor protein which negatively regulates the cell cycle (Sherr, 1994) resulting in loss of pRb functions (Figure A.4) (White et al., 1994). Also, the BPV E5 was characterised as a transmembrane oncoprotein involved in binding to the v-ATPase affecting the Golgi complex (Figure A.4) (Campo, 1997) and in inhibition of foreign peptide display on MHC-1 (Marchetti et al., 2009).

Even though CRPV and BPV models of HPV infection are extensively used (Campo, 2002), due to species and tissue tropisms understanding and research of several aspects of HPVs infection and carcinogenesis using these models have been limited (Doorbar et al., 2016; Cladel et al., 2017). However, in 2011 a mouse papillomavirus (MmuPV1) has been isolated and sequenced among a colony of nude mice. It provided the opportunity to extend our understanding of papillomavirus infection and disease progression in the small-animal model (Ingle et al., 2011; Hu et al., 2017). MmuPV1 has both cutaneous and mucosal tropism (Cladel et al., 2017) and in addition, primary infections at cutaneous sites could lead to secondary infections at mucosal sites (Cladel et al., 2013). Early studies of the MmuPV1 malicious potential highlight the implication of major viral oncogenes E6 and E7 (Joh et al., 2011). MmuPV1 E6 shares some biochemical and functional characteristics with HPV8 E6 inhibiting NOTCH and TGF-β signalling delaying infected cell differentiation and apoptosis of differentiated keratinocytes (Figure A.4) (Meyers et al., 2017).

MmuPV1 E7 seems to share oncogenic properties with the HR-HPV E7 and binds the same non-transmembrane protein tyrosine phosphate (PTPN14) (Figure A.4) (White et al., 2016). Thus, MmuPV1 offers an opportunity to study papillomaviruses in an efficient laboratory model. The animal model of MmuPV1 will most certainly advance by a significant step existing knowledge of HPV infection and carcinogenesis (Hu et al., 2017). The field of application of MmuPV1 as a model for HPV infection is summarized in Table A.5.

6.1.5. Herpesviridae

6.1.5.1. Non-coding RNAs and the lessons on the regulation of viral pathogenesis obtained using herpesvirus homologous animal model

Herpesviruses establish life-long, persistent infections in their hosts that is hallmarked by two unique phases of the viral life cycle, productive replication and latency. The switch between these two phases is a tightly regulated process, facilitated in part by viral non-coding RNAs (ncRNAs). ncRNAs include ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and regulatory RNAs such as microRNAs (miRNAs), piwiRNAs (piRNAs), small nucleolar RNAs (snoRNAs) and long ncRNAs (lncRNA) (Hancock and Skalsky, 2017). Of the known virally encoded miRNAs, 97% have been discovered in herpesviruses, suggesting that miRNA-mediated gene regulation plays an important step in herpesvirus infection (Hicks and Liu, 2013). The majority of viral

miRNAs encoded by herpesviruses, such as Epstein-Barr virus (EBV) and the avian Gallid herpesvirus 2 (GaHV-2) highlight important roles of miRNAs in viral pathogenesis in different hosts (Skalsky and Cullen, 2010). Similar to EBV, GaHV-2 is one of the few oncogenic herpesviruses that induces tumours in its natural host (Luo et al., 2010). GaHV-2 serves as a versatile small-animal model for studying different aspects of herpesvirus pathogenesis and induced oncogenesis in a natural virus-host system with remarkable reliability (Osterrieder et al., 2006). During GaHV-2 infection, numerous viral factors, proteins and transcripts contribute to lymphomagenesis and include the major oncoprotein Meq (Jones et al., 1992), the viral interleukin-8 (Parcells et al., 2001; Engel et al., 2012) and GaHV-2-encoded miRNAs (Muylkens et al., 2010; Yao et al., 2009; Zhao et al., 2011; Coupeau et al., 2012). Twenty-six mature viral miRNAs have been identified in GaHV-2 genome (Yao and Nair, 2014). GaHV-2 miRNAs function as, either, regulators of cell cycle or viral replication. Some herpesviruses, including KSHV and GaHV-2, express orthologues of oncogenic cellular miR-155 associated with several human cancers. They target the same host's mRNAs as cellular miR-155, promoting virus-induced cell transformation (Guo and Steitz, 2014). Numerous transcription factors are potentially shared targets for miR-155 and GaHV-2-miR-M4-5p (Figure A.5) (Muylkens et al., 2010; Parnas et al., 2014; Yao and Nair, 2014; Figueroa et al., 2016). It was shown that inhibition of these common cellular targets results in enhancement of tumorigenesis (Parnas et al., 2014) and suppress apoptosis in cell culture (Figure A.5) (Xu et al., 2011). In addition, GaHV-2-miRs allow the virus to escape the host immune response (Figure A.5) (Parnas et al., 2014). Recently it has been shown that GaHV-2-miRs can act as a putative tumour suppressor targeting and downregulating expression of Meq (Figure A.5) (Teng et al., 2017), target and restrict RLORF8 expression and may have a role in the regulation of lymphocyte growth (Figure A.5) (Parnas et al., 2014). Furthermore, this avian herpesvirus encodes a viral telomerase RNA subunit (vTR) of the telomerase complex. vTR, a non-coding RNA, is crucial for efficient lymphoma formation (Fragnet et al., 2003; Trapp et al., 2006; Kaufer et al., 2010). vTR interacts with the chicken telomerase reverse transcriptase subunit (TERT) and enhances telomerase activity (Fragnet et al., 2003) and also, re-localize ribosomal protein L22 (Kaufer et al., 2011) that plays an important role in T-cell development and transformation (Figure A.5) (Anderson et al., 2007). It has been shown that the c-Myc oncoprotein is involved in the regulation of vTR during GaHV-2-induced lymphomagenesis (Shkreli et al., 2007) and that increased expression of vTR is essential for the oncogenic function of GaHV-2 (Chbab et al., 2010).

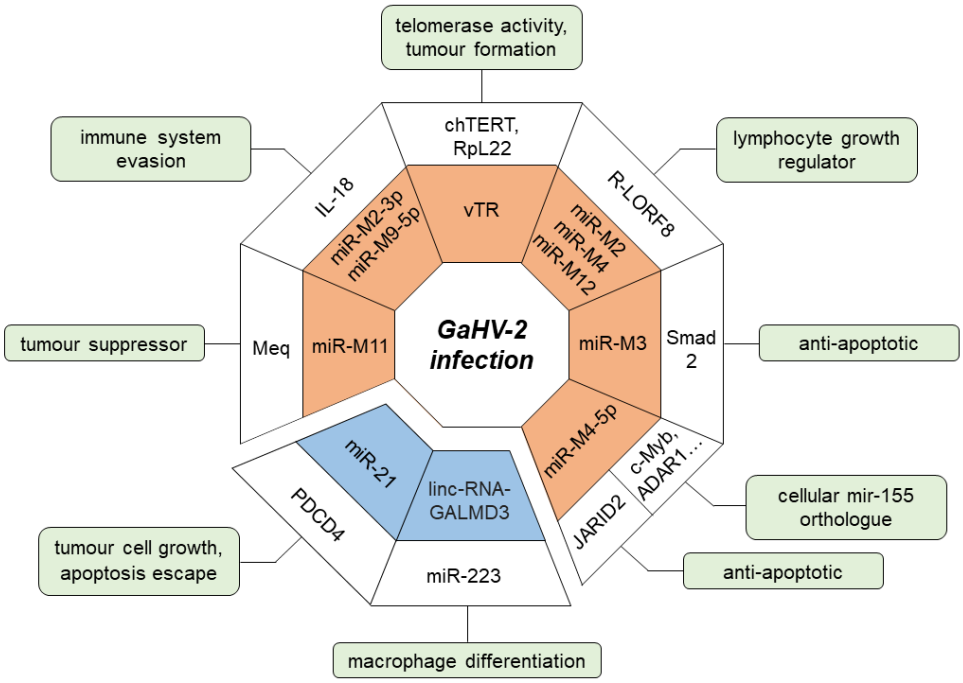

FIGURE A.5. THE REGULATION OF VIRAL AND CELLULAR GENES DURING GAHV-2 INFECTION UTILISES NON-CODING RNAS (NCRNAS).

Representation of viral (orange trapezoid) and cellular (blue trapezoid) ncRNAs with their specific targets (white trapezoid) and the effect they have that contribute to the establishment of lymphomagenesis (green rectangle).

In addition to GaHV-2-expressed miRNA, expressional regulation of cellular (gga) miRNAs (Yao et al., 2009; Tian et al., 2012; Stik et al., 2013) and lncRNA (Han et al., 2017; He et al., 2019; You et al., 2019) may contribute to the establishment of tumorigenesis (Figure A.5) (Gennart et al., 2015). Two cellular miRs are highly expressed during GaHV-2 latency and tumorigenesis, promoting cell proliferation (Lambeth et al., 2009), tumour cell growth and apoptosis escape (Figure A.5) (Stik et al., 2013). In recent years, the important role of lncRNAs has been established in numerous biological processes, cellular development and different disease state, including cancer (Gupta et al., 2010; Hou et al., 2014; Elling et al., 2016). During GaHV-2-induced disease, long intergenic non-coding RNA (lincRNA) GALMD3 has a role in *cis*-regulation of gga-miR-223 expression, which has been shown to be involved in the development of the immune organs (Hicks et al., 2009), cell proliferation (Sun et al., 2010), macrophage differentiation (Ismail et al., 2013) and *trans*-regulates gene expression on the chicken genome (Han et al., 2017). Linc-GALMD1 coordinates the expression of GaHV-2- and tumour-related genes and regulates immune responses to GaHV-2 infection, indicating its role as a viral gene regulator contributing to

tumour suppression (He at al., 2019). Furthermore, lncRNAs have been found to be involved in B-cell activation and the Wnt signalling pathway and have been strongly correlated with GaHV-2-resistant candidate genes suggesting that lncRNAs may affect GaHV-2 resistance and tumorigenesis through their target genes (You et al., 2019).

6.1.6. Conclusions

In the long history of biological and medicinal science, the use of animals for research purposes is a common practice. The astonishing similarities between animals and humans, both anatomical and physiological, allowed ever-expanding research of different biological mechanisms in animal models. Animal models have been and will be used to understand the basics of biology and to develop and novel vaccines and therapies for numerous diseases (Table A.5). Since the discovery of the Rous sarcoma virus at the beginning of the previous century, the homologous animal models of virus-induced diseases, including cancer, have been at the frontier of the oncogenesis research. For several reasons, in particular, the relative simplicity of viral genome, the impact on viral replication and especially on cell division, where the viral role is more easily controllable or identifiable, make these models unique and inevitable in understanding virus-host interactions and better deciphering different steps of cancer development.

6.1.7. References

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