DOCTOR OF SCIENCES

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Gennart, Isabelle

Award date:
2018

Awarding institution:
University of Namur

Université de Namur
Faculté de sciences
Département vétérinaire
Unité de recherche vétérinaire intégrée (URVI)

Study of the epigenetic silencing of a host microRNA in Marek's disease

Isabelle Gennart

Promoteur
Prof. Benoit Muylkens

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

Année académique 2017-2018

remerciements

Tout d'abord, j'aimerais remercier Patsy Renard et Benjamin Dewals pour m'avoir suivie durant ma thèse en faisant partie de mon comité d'accompagnement. J'aimerais aussi remercier Nicolas Gilet, Benjamin Le Calvé, Carine Van Lint, ainsi que Sylvie Laurent pour m'avoir fait l'honneur de faire partie de mon jury de thèse.

Merci à Nathalie Kirschvink pour m'avoir acceptée en URVI depuis mon master 2 et fait confiance au point de me proposer de donner des travaux pratiques de parasitologie à des vétérinaires ! Merci également pour ton aide en fin de thèse, grâce à toi, j'ai pu travailler sur mon manuscrit l'esprit beaucoup plus léger.

Cher Benoît, je ne te remercierai jamais assez pour tout ce que tu m'as appris, aussi bien scientifiquement que humainement. Merci pour ta patience, ta confiance et ton soutien dans les moments difficiles de la thèse. Merci pour la correction de ce manuscrit qui ne fut pas une mince affaire ! Merci pour tout. J'ai été très chanceuse de t'avoir eu comme promoteur et j'espère que les suivants s'en rendront compte. Tu m'as poussée pour que je donne le meilleur de moi-même et je t'en remercie. Merci pour ces six ans, merci de tout cœur. J'aimerais pouvoir exprimer tellement de choses mais, comme tu le sais, je ne suis pas très douée pour le faire sur papier. Merci !

J'aimerais aussi remercier cette fine équipe qu'est l'URVI : merci à Damien pour tout ton aide dans le labo. Merci pour tes nombreux conseils et tes « mais qu'est-ce que tu fais ? » me permettant d'éviter de nombreuses gaffes. Merci pour les sorties, les bons moments de détente autour d'une bonne bière (ou plus). Merci beaucoup à mon petit papy croate, Srdan. Depuis le début, on s'est tout de suite bien entendu. Merci pour ton amitié sincère et ton sens de l'humour. Merci pour ton soutien et ton aide tout au long de ma thèse. Je ne saurai jamais assez te remercier pour les expérimentations in vivo avec les trajets jusqu'à Tours. Ton dos en a payé le prix. Merci à Laetitia, notre histoire a commencé en tant que collègues et se poursuivra en tant qu'amies. Merci pour ton aide durant ma fin de thèse, merci pour ta disponibilité et merci pour ton écoute. Merci pour la relecture de mon manuscrit alors que tu étais en congé. Je crois ce qui me manquera le plus, ce sont nos trajets en bus le matin et le soir, nos « tu prends le bus à quelle heure ? », nos rires, nos bavardages scientifiques (et pas que) et ta bonne humeur. Tu vas me manquer Laeti. Je sais qu'on habite tout près mais cela va me faire vraiment bizarre de ne plus te voir tous les jours. Un ENORME merci pour tout ! Merci à toi Chantal, notre secrétaire d'enfer. Merci aussi aux « petits nouveaux » de l'URVI, Noémie, Florian et Nicolas. Merci à tous ces doctorants venus d'ailleurs, Jimmy, Harvey, Qaoutar, Fatima, Bilkiss, Eric, Gaston et Sarah. Merci aussi à Marianne, Wiebke, Fanny, Carine, Claire et Jean-Michel. Merci aussi aux anciens : François, Sophie et Frédérique notre tornade rousse. Merci également aux étudiants de master et de haute école qui m'ont énormément aidé durant ma thèse pour avancer. Merci également à nos amis les Français de Tours : Denis, Ginette, Sylvie, Perrine, Imane, Thomas et Adrien de m'avoir accueillie durant mon master 2 dans votre laboratoire. Merci Perrine et à Imane pour m'avoir hébergée lorsque je venais à Tours pour mes poules ou pour des défenses de thèse. Merci pour ces bons moments passés lors des pots de thèse !

Merci à tous mes amis biologistes qui n'ont pas arrêté d'entendre parler de ma thèse pendant toutes ces

années. Merci à eux pour ces sorties et ces discussions qui m'ont permis de relativiser. Merci de m'avoir si bien entourée. Merci pour votre soutien ! Merci Jessica, Donna, Anne-Laurence, Anne-Sophie (Voisin et Gathy), Yassine, Sophie, Marie, François, Claudie, Quentin, P-A, Sylvie, Vincent, Florence, Noémie et à tous ceux à que j'ai croisé durant mon parcours.

Merci à toute ma famille, à mes parents qui ont toujours cru en moi. Merci à mes nombreux frères et sœurs et à leur compagne/compagnon, Xavier, Thibault, Caroline, Marie-Sophie, Killian, Sixtine, Gilles et Pierre-Alexandre pour vos tranches de rire et les bons de moments de détente qui m'ont permis de rester un minimum saine d'esprit lors de l'écriture de la thèse. Merci aussi bien sûr à ma belle-famille, Magali, Pierre, Mathilde et Benjamin pour votre aide, votre soutien et votre écoute. Merci à tous !

A Maxime un énorme merci ! Merci à toi mon homme aux multiples casquettes. Tu as été un compagnon, un père, un cuisinier, une aide psychologique et un homme plus que patient avec moi. La fin de thèse est loin d'avoir été facile pour toi. Merci pour ton soutien, merci d'être resté, merci de de t'être occupé de notre petit garçon tant de soirées. Je vous aime tous les deux énormément ! Nous vivons une belle aventure tous les trois, merci mes deux amours.

J'aimerais juste finir par un petit mot pour mon grand-père qui est parti trop tôt et qui malheureusement ne pourra pas assister à ma défense. Tu as toujours cru en moi et as toujours été très patient. Merci pour toute ton aide durant 27 ans de ma vie. Merci pour tout Bonpapa !

En résumé, merci à tous ceux qui ont croisé mon chemin. Cette thèse a été une magnifique première expérience professionnelle. J'ai fait partie d'une équipe en or alliant travail et amusement. Que demander de plus ! J'ai appris énormément et l'idée de ne plus venir chaque jour travailler en URVI me laisse un peu mélancolique mais avec un mélange d'excitation face aux nouveaux challenges qui me feront face dans le futur. Merci à tous, merci pour tout !

Summary

During latency, herpesvirus infection results in the establishment of a dormant state in which a highly restricted set of viral genes are expressed. This is associated with extensive methylation of CpG motifs in non-expressed viral genes. Together with these alterations of the viral genome, several host genes undergo epigenetic modifications during the latent infection. In some of the human and animal herpesvirus infections, these epigenetic dysregulations of cellular genes are involved in the development of cancer. This PhD program was carried out using an animal model of virus induced lymphoma causing the Marek's disease (MD) in chicken. This lymphoproliferative disease is the ultimate consequence of chicken infection with virulent strains of *gallid herpesvirus-2* (GaHV-2). This *Alphaherpesvirinae* actually shares several properties with *Gammaherpesvirinae* (such as human herpesvirus-4 and -8) which are associated with the development of tumors under specific conditions in latently infected cells. GaHV-2 was shown to modulate the expression of several cellular miRNAs in chicken. Altered expressions of host-encoded miRNAs were analyzed *in vitro* and *in vivo* in several studies. Although only few of the cellular miRNA dysregulations triggered by GaHV-2 were analyzed in depth, these studies suggested that altered expressions of host miRNAs are involved in the molecular pathways of GaHV-2 oncogenicity.

Therefore, we decided to focus on a host miRNA, miR-126 since several pieces of evidence suggested it might be downregulated during GaHV-2 tumorigenesis. Originally described as a miRNA mediating proper angiogenesis and vascular integrity, miR-126 has been reported to impair cancer progression through signaling pathways that control tumor cell proliferation, migration, invasion and survival. MiR-126 was shown to be downregulated in several human cancers such as in oral, stomach, colon, lung, breast, cervix, bladder and prostate carcinoma. MiR-126 is an intronic miRNA integrated in the intron 7 of a cellular gene, the epidermal growth factor like domain 7 (*egfl-7*). In human, the latter gene is controlled by three promoters generating three transcripts, a long, an alternative and a short one. It was shown that epigenetic modifications have an impact on the alternative transcript expression by remodeling the alternative promoter.

The core issue of this PhD program is to know whether miR-126 downregulation is a key event of GaHV-2 oncogenicity. Three study were carried out in order to bring some insights.

The first part of this work, was to set up a thorough quantification method of miR-126 expression. This method is essential to compare, in the most accurate way, miR-126 expression level in any situation corresponding to the different stages of GaHV-2 infection. The quantification method was tested during an *in vivo* challenge with a chicken line highly sensitive to MD (B13/B13 strain). Chickens were infected with a very virulent strain of GaHV-2 (RB-1B). The method relied on the use of several reference genes (RGs): *gapdh*, β*-actin*, small nuclear RNA U6 (*U6*), *18SrRNA*, Hydroxymethylbilan synthase (*hmbs*) and succinyl dehydrogenase (*sdha*). The three RGs that showed the most stable expression were selected with an Excel-based software (GeNorm) and used to normalize the expression level of three cellular genes (miR-126, *cd4* and *cd8*) and one viral one (*meq*, the major oncogene of GaHV-2). The results showed that depending on the RG used, different conclusions have been obtained. The conclusion of this part is that combining several RGs to normalize gene expression is crucial for drawing accurate conclusions from quantification data.

The second part of this work determined miR-126 expression level at the key steps of GaHV-2 infection (*in vitro* and *in vivo*) and looked out for epigenetic modifications involved in the control of its expression. The miR-126 expression level was evaluated by using the reliable quantification method set up in the first part of the study. The cellular miRNA was found to be repressed during the viral-induced oncogenesis phase. In order to determine whether miR-126 low expression level was associated with specific epigenetic signatures and with peculiar transcription profiles, DNA methylation patterns and transcriptional isoforms were established at miR-126 genomic locus. Repression was found to be associated with hypermethylation at a CpG island located in the miR-126 host gene (*egfl-7*). Moreover, transcriptional isoforms analysis showed the existence of alternative promoters during chicken development. This analysis also permitted revealing the existence of two main initiation sites associated each with a CpG islands.
Since miR-126 repression is likely to play a pivotal role in altering gene expression patterns during cell transformation, we investigated the impact of miR-126 restoration. A strategy was developed to overexpress miR-126 and control miRNAs in transformed CD4+ T cells propagated from MD lymphoma. To this end, a conditional expression system (Tet-on inducible expression) was developed to counteract miR-126 shutdown. The preliminary functional analysis showed that miR-126 inhibition might participate to the cancerous process induced by GaHV-2 by supporting cell proliferation.

To complete the *in vitro* functional analysis, the third part of this work concentrated on the creation of a recombinant GaHV-2 that constitutively expresses the miRNA lost during lymphoma development. The aim was to interfere with miR-126 silencing during the natural course of GaHV-2 infection. Recombinant viruses expressing either wild-type or mutated versions of miR-126 were generated from a very virulent GaHV-2 strain (RB-1B) cloned as an infectious Bacterial Artificial Chromosome (BAC). Infectious viruses were amplified and characterized *in vitro* to evaluate if the recombinant viruses replicate to similar levels as the original strain and if this strategy is adapted to overexpress miR-126 together with viral infection.

Altogether, these results brought new insights on the expression and the regulation of a cellular miRNA in the context of MD.

Contents

Abbreviations

5aza = 5'azacytidine
ADAM9 = metalloproteinase domain-containing protein 9
ADP = Adenosine DiPhosphate
Ago = Argonaute protein
ADP = Adenosine DiPhosphate
Ago = Argonaute protein
ALL = Acute lymphoblastic Leukemia
AML = Acute Myeloid Leukemia
Ang-1 = Angiopoietin-1
ATF = Activating Transcription Factors
AVMA = American Veterinary Medical Association
AVY = Agouti Viable Yellow
Bcl2 = B Cell Lymphoma 2
BCR/ABL = Break Cluster Region /ABeLson murine leukemia viral oncogene homolog1
BF = Bursa of Fabricius
BGSA = Bisulfite Genomic Sequencing Analysis
Bp = Baise pair
BPA = BisPhenol A
Brd = Bearded gene
bZIP = basic-Leucine ZIPper
CAGE = Cap Analysis of Gene Expression
CCNE1 =CyCliNe E1
CCR4 = C-C chemokine Receptor type 4
CDC42 = Cell Division Cycle 42
CDH1 = E-CaDHerine
CDK = Cyclin-Dependent Kinase
CEFs = Chicken Embryo Fibroblasts
CGI shores = CpG Island shores
CGMMV = Cucumber Green Mottle Mosaic Virus
CpG = dinucleotide Cytosin/Guanine
CREB = C-AMP Response Element-Binding protein
CRK = V-crk sarcoma virus CT10 oncogene homolog
CRM1 = exportin 1
CSDC2 = Cold Shock Domain-Containing protein 2
CT = Caecal Tonsil
CtBP = COOH terminal-Binding Protein
DCP = DeCaPping enzymes
DGCR8 = DiGeaorge syndrome Critical Region 8
DHFR = DiHydroFolate Reductase
DIM5 = histone methyltransferase
DMEM = Dulbecco's Modified Eagle Medium

DNA = DeoxyriboNucleic Acid
DNMT = DNA MethyTransferase
Dpi = Days post infection
DSB = Double Strand Break
dsRNA = double stranded RNA
DSS = Dextan Sulphate Sodium
DTT = 1,4-Dithiothreitol
E = Early
EBV = Epstein Barr Virus
EBVaGC = EBV associated Gastric Cancer
ECM =ExtraCellular Matrix
EDTA = Ethylen Diamine Tetra Acid
EGFL-7 = Epidermal Growth Factor Like-7
eIF4E = eukaryotic translation Initiation Factor 4E
ElHV = Elephantid HerpesVirus
EMI = EMIlin like domain
EMT = Eptihelial-Mesenchymal Transition
ERK = Extracellular signal-Regulated Kinase
ESCC = Esophageal Squamous Cell Carcinoma
Ets = E26 transformation specific sequence
EZH2 = Enhancer of Zeste Homolog 2
FFE = Feather Follicle Epithelium
Flp/FRT = Flippase/Flippase Recognition Target
GaHV-2 = gallid HerpesVirus-2
GAPDH = Glyceraldehyde 3 Phosphate DeHydrogenase
gC = glycoprotein C
GDF8 = myostatin gene
Gga = Gallus gallus
GSP = Gene Specific Primer
GW182 = GaWky 182
H3K4me3 = Histone 3 lysine 4 trimethylation
H4K16Ac = Histone 4 lysine 16 Acetylated
HAT = Histone Acetyl Transferase
HCMV = Human CytoMegaloVirus
HDAC = Histone DeACetylase
HHV = Human HerpesVirus
HKMT = Histone lysine MethylTransferase
HMBS = HydroxyMethylBilan Synthase
HOX = HOmeoboX
HPTMs = Histone Post-Translational Modifications
ICP4 = Infected Cell Protein 4
IE = Immediate Early
IF = ImmunoFluorescence
IGF1 = Insulin-like Growth Factor 1
IGF1R = IGF1 Receptor
IKKα β = I kappaKinaseα β
IL-2 = InterLeukin 2
IPO8 = IPOrtin8
IR = Internal Repeat
IR = Insulin Receptor
IRF5 = Interferon Regulatory Factor 5

IRL = Internal Repeat Long
IRS = Internal Repeat Short
IRS-1 = Insulin Receptor Substrate-1
JARID2 = Jumonji AT Rich Interactive Domain 2
JMJD2 = JmjC domain-containing proteins
KDa = KiloDalton
KLF-2 = Krueppel-Like Factor-2
Kpb = Kilo Base Pairs
KSHV = Kaposi's Sarcoma HerpesVirus
L = Late
LADs = Lamina Associated Domains
LANA = Latency Associated Nuclear Antigen
LAT = Latency Associated Transcript
LMP = Latent Membrane Protein
LncRNA = Long non-coding RNA
LOCK = Large Organized Chromatin lysine modification
LOXL2 = Lysyl Oxidase 2
LSD-1 = Lysine Specific Demethylase 1
LTR = Left Terminal Repeat
MAF = MusculoAponeurotic Fibrosarcoma oncogene homolog
MAP = Mean Arterial blood Pressure
MAX = Myc Associated factor X
MBD3 = Methyl Binding Domain
MD = Marek's Disease
MDCC = Marek's Disease Cell Culture
MDFIC = MyoD Family Inhibitor domain
MDV-1 = Marek's Disease Virus-1
MeCP2 = Methyl CpG binding protein 2
MeHV-1 = Meleagrid HerpesVirus-1
MERE = MEq Responsive Element
MHV = Murine HerpesVirus
MICB = Major histocompatibility complex I-related Chain B
miRISC = Mature miRNA
MiRNAs = microRNAs
MMP = Matrix Metallo-Proteinase
mRNA = messenger RNA
MTA = MeTastasis-Associated proteins
MYBL1 = MYB Proto-Oncogene Like 1
Nabutyrate = Sodium butyrate
NAD+ = Nicotinamide Adenine Dinucleotide
ND = None Determined
NEK6 = NIMA related kinase 6
NF-κB = Nuclear Factor-κ light-chain-enhancer of activated B cells
NFI-A = Nuclear Factor I-A
NK = Natural Killer
NLS = Nuclear Localization Signal
NMDA = N-Methyl-D-Aspartate
NPC = NasoPharyngeal Carcinoma
NT = Non-Tested
Nt = Nucleotide
NuRD = Nucleosome Remodeling and Deacetylase

Oct1 = OCTamere DNA binding protein
PABP1 = Poly A Binding Protein 1
PAZ = Piwi-Argonaute-Zwille
PBL = Peripheral Blood Leucocytes
PBS = Phosphate Buffered Saline
PcG = Polycomb Group
PCR = Polymerase Chain Reaction
PDCD4 = Programmed Death Cell 4
PDK-1 = Phosphoinositide-Dependent Kinase 1
PFU = Plaque Forming Unit
PEL = Primary Effusion Lymphoma
PI3K = PhosphatidylInositide 3-kinase
PIK3R2 = PhosphatidylInositide-3 Kinase Regulatory subunit 2
PKB = Protein Kinase B
PLK2 = Polo-Like Kinase 2
PolII = RNA polymerase II
PP38 = PhosPhoprotein 38
pRB = RetinoBlastome protein
Pre-miRNA = PREcursor miRNA
Pri-miRNA = PRImary miRNA
PRMT = Protein arginine N-MethylTransferase 1
PVC = PeriVascular Cells
PWWP = Proline-tryptophan-tryptophan-Proline domain
qRT-PCR = quantitative Reverse Transcription-Polymerase Chain Reaction
RACE = Rapid Amplification of cDNA Ends
RbAp = histone binding proteins
RG = Reference Gene
RISC = RNA Induced Silencing Complex
RLC = RISC Loading Complex
RLORF5a = Open Reading Frame
RNA = RiboNucleic Acid
RNAi = RNA interference
RPMI-1640 = Roswell Park Memorial Institute-1640
rRNA = ribosomal RNA
RTR = Right Terminal Repeat
SDHA = Succinyl DeHydrogenAse
SET7/9 = SET-domain protein methyltransferase superfamily
siRNA = small interfering RNA
SIRT1 = NAD-dependent deacetylase sirtuin-1
SLC7A5 = SoLute Carrier family 7 member 5
SNF = Sucrose Non-Fermentable
SMAD2 = Mothers Against Decapentaplegic homolog 2
SnoRNA = Small NucleOlar RNA
SO-miRNA = Splice-site-Overlapping miRNA
SOX2 = Sex determining region Y bOX 2
SPF = Specific Pathogen Free
SPRED1 = SProuty-Related protein with EHV-1 Domain
SR = Serine/Arginine dipeptide rich proteins
SSIII = SuperScript III
STAT3 = Signal Transducer and Activator of Transcription 3
TERT = TElomerase Reverse Transcriptase

Tet = Tetracycline
TetR = Tet Repressor
TF = Transcription Factor
TFDP2 = Transcription Factor E2F Dimerization Partner 2
TGA = Transcriptional Gene Activation
TGS = Transcriptional Gene Silencing
TNRC6 = TriNucleotide Repeat-Containing gene 6A
TR = Terminal Repeat
TRBP = Transactivating Response RNA Binding Protein
TRPC6 = Transient Receptor Potential Canonical 6 TRL = Terminal Repeat Long
TRS = Terminal Repeat Short
tRNA = transfer RNA
TSA = TrichoStatin A
TSS = Transcriptional Start Site
U6 = small nuclear RNA U6
UCP2 = UnCoupling Protein 2
UHFR = Ubiquitin-like with PHD and zing Finger domain proteins
UL = Unique Long
US = Unique Short
UTR = UnTranslated Region
VEGF = Vascular Endothelial Growth Factor
VEGFR2 = Vascular Endothelial Growth Factor Receptor 2
vIL-6 = viral InterLeukin-6
VP5/16 = Viral Protein 5/16
vTR = viral Telomerase RNA

Chapter 1

Introduction

1.1 MicroRNAs

1.1.1 Introduction

MicroRNAs (miRNAs) are short, single stranded, noncoding RNA molecules of around 21-24 nucleotides length. The first miRNA (lin-4) was discovered more than 30 years ago in *Caenorhabditis elegans* and is implicated in the regulation of nematode development (Kaufman and Miska, 2010). Few years later, the interference RNA phenomenon was discovered (Fire et al., 1998; Timmons and Fire, 1998). Nowadays, miRNAs are described as key regulators of the gene expression in a very large panel of living organisms from four kingdoms: Animalia, Planta, Fungi and Protista. Interestingly, 35 viral species infecting Animals are also known to encode miRNAs (http://www.mirbase.org/; a large repository of microRNA sequence information) to regulate either their own gene expression and/or their host gene expression. MiRNAs are known to act at a post-transcriptional level in the cytoplasm but recent studies showed also an impact of miRNAs in the nucleus (Catalanotto et al., 2016). In the cytoplasm, miRNAs are associated mainly to repression of gene expression (Ambros, 2004) while in the nucleus miRNAs were shown to be associated to repression and activation of gene expression (Catalanotto et al., 2016). In miRbase more than 38 000 miRNAs are identified. MiRNAs are implicated in many different cellular processes such as development (Wang et al., 2007), cell differentiation (Hao et al., 2017), proliferation (Wang et al., 2014; Yin et al., 2015) and tissue function (Wang et al., 2014; Yin et al., 2015). Since miRNAs are crucial to maintain homeostasis in the cell, it is not surprising that their dysregulation leads to disease such as cancer (Pichler and Calin, 2015). MiRNAs are also known to have their expression deregulated during viral infection leading to virus persistence in the host and induction of virus-induced tumorigenesis (Piedade and Azevedo-pereira, 2016; Fiorucci et al., 2015).

1.1.2 Biogenesis

Transcription

MiRNAs are localized either in inter or intragenic regions in which they are either isolated or grouped into cluster (Olena and Patton, 2010). The miRNAs from intergenic region are in non-coding regions and possess their own promoter. Half of miRNAs from vertebrates are processed from introns of protein-coding genes or genes encoding other classes of non-coding RNA (snoRNAs, miRNAs and lncRNAs) (Rodriguez et al., 2004). The miRNAs from intragenic regions can be in introns, exons, untranslated regions and even overlap splicing sites (splice-site-overlapping miRNA or SO-miRNA) in non-coding or coding genes (figure 1.1) (Lagos-quintana et al., 2001; Godnic et al., 2013). These intronic miRNAs are usually in the same orientation than their host genes and they are likely to be under the control of the promoter driving the primary mRNA transcript (Rodriguez et al., 2004).

Most of the time miRNAs are transcribed by the cellular RNA polymerase II (polII) giving long primary transcript RNA (pri-miRNA) presenting one or several stem loop structure(s). This pri-miRNA possesses a cap and a poly A tail on its 5' and 3' ends, respectively (figure 1.2 A) (Lee et al., 2004; Bortolin-Cavaillé et al., 2009; Cai et al., 2004).
Some miRNAs were shown to be transcribed by the RNA polymerase III (polIII), known to be implicated more specifically in small RNAs synthesis (Pascale et al., 2018). In glioma, an oncomiR, miR-138, was demonstrated to be overexpressed impacting cell proliferation and survival. This overexpression was attributed to the binding of the leucine-zipper transcription factor CCAAT/enhancer binding protein β (C/EBPβ) recruiting the polIII initiation complex (Pascale et al., 2018).

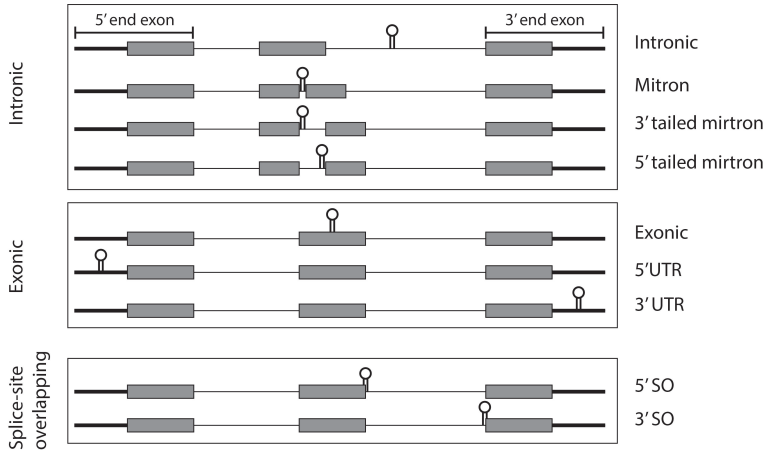

Figure 1.1: **Localization and nomination of intragenic microRNAs**. Thin black bars are introns. Bold black bars are untranslated regions (UTRs). Grey rectangles are the coding part of exons. The stem loop structure is the microRNA. SO for splice-site overlapping miRNA. (adapted from (Mattioli et al., 2014))

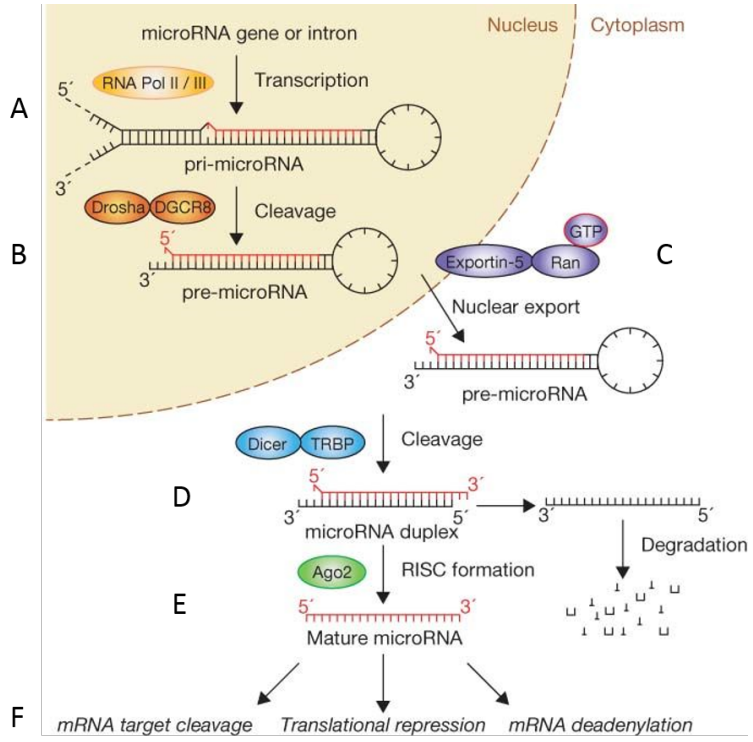

Figure 1.2: **microRNAs biogenesis**. The different steps of microRNA transcription and maturation are described from gene transcription to targeted messenger RNA regulation by interference RNA (RNAi). RNA pol II/ III stands for RNA polymerase II and III. DGCR8 stands for digeorge syndrome critical region 8. TRBP stands for human immunodeficiency virus transactivating response RNA-binding protein. RISC stands for RNA-induced silencing complex. (adapted from (Winter et al., 2009).

Processing in the nucleus

Concomitantly with transcription, the pri-miRNA is processed to give the precursor miRNA (pre-miRNA) of about 70-90 nucleotides in length from the stem loop structure (figure 1.2 B). The cleavage is performed by the microprocessor complex made of an endonuclease from RNAse III family and its cofactor, DROSHA and Digeorge syndrome critical region 8 (DGCR8) respectively (Lee et al., 2003; Han et al., 2004; Gregory et al., 2004; Denli et al., 2004). DROSHA contains two domains, RIIIDa that cleaves the 3' strand and RIIIDb which cleaves the 5' strand adjacent to the hairpin. DGCR8 helps for the identification of the cleavage site in the pri-miRNA structure.
In some cases, the processing of the pri-miRNA is independent of microprocessor complex and is done by the splicing machinery allowing the release of the pri-miRNA from the intron. These miRNAs are called mirtrons (figure 1.1). This process is not common but is found throughout the animal kingdom (Okamura et al., 2007; Ruby et al., 2007). Another miRNAs subset comes from short introns and they are name agotrons (Hansen et al., 2016). The mature agotrons are similar to the pre-miRNAs (around 80-100 nt length) but they have a biogenesis independent from the microprocessor complex and Dicer. In these specific cases, agotrons are processed by Argonaute proteins (Ago) that migrates into the nucleus. The catalytic activity of Ago is then able to cleave the agotrons to produce a structure similar to the pre-miRNA (Cheloufi et al., 2010).
The pre-miRNA is then transported from the nucleus to the cytoplasm by a RAN-GTPase dependent manner with the Exportin 5 (figure 1.2 C) (Wang et al., 2011). The Exportin 5 is a protein from the karyopherin family which has a role in structural RNA transport like tRNA (Lee et al., 2011). The complex exportin 5 and Ran GTP recognizes the 2 nucleotides in 3' overhang structure of the pre-miRNA which means that the recognition is based on structure and not on the sequence (Okada et al., 2009).

Processing in the cytoplasm

In the cytoplasm, the pre-miRNA is processed by another endonuclease and its cofactor, Dicer and human immunodeficiency virus transactivating response RNA-binding protein (TRBP) respectively (figure 1.2 D). It gives a miRNA duplex of about 21-24 nucleotides in length (Gatignol et al., 2005; Haase et al., 2005; Chendrimada et al., 2005). Both strands in the miRNA duplex have a phosphate at the 5' end and a 2 nucleotides overhang with a hydroxyl at the 3' end. Dicer is a protein of about 218 Kda with a central domain Piwi-Argonaute-Zwille (PAZ) implicated in the interaction with the pre-miRNA (Provost et al., 2002). TRBP is a protein of around 50 Kda and it is essential to mediate the association of Dicer with the RNA duplex and the recruitment of the argonaute protein 2 (Ago2) (Chendrimada et al., 2005). Dicer, TRBP and Ago2 association is called the RNA induced silencing complex (RISC) loading complex (RLC) and is the essential structure required to load dsRNA fragments. This trimeric complex provides foundation for the assembly of an active RISC through the recruitment of additional proteins such as helicases (Robb and Rana, 2007), nucleases (Caudy et al., 2003) and RNA binding proteins (Meister et al., 2005). All these proteins are associated to Ago2 in the RISC complex.

As mentioned earlier, miRNAs undergo two catalytic processes from a precursor to give the mature miRNA. The microprocessor complex and DICER cleave at precise location giving defined extremities on the mature miRNA. Sometimes heterogeneous cleavage site happens creating isomiRNAs of the mature miRNA (Lee et al., 2010; Marti et al., 2010). These isomiRNAs have differential target recognition, different biological properties and isomiRNAs preference depend on cell type (Baran-gale et al., 2013; Vickers et al., 2013).

RISC loading

Mature miRNAs are formed by selection of one strand of the miRNA duplex. If the RNA duplex has a complementarity in the central region, one strand is selected (guide strand). The other one (star strand) is

trimmed by Ago2 and is further degraded by the nuclease complex C3PO (figure 1.2 E) (Matranga et al., 2005; Shin, 2008; Liu et al., 2009). This is the mechanism for RISC loading for the related siRNA pathway. Most miRNA duplexes lack central complementarity and rely on strand unwinding due to the helicase activity of the RISC complex (Maniataki and Mourelatos, 2005; Macrae et al., 2008). The selection of the guide strand by RISC is based on thermodynamic stability. The strand with the less stable part at the 5' terminus is selected (Khvorova et al., 2003). Sometimes the star strand might be chosen and plays a role by targeting its own set of target mRNAs (Okamura et al., 2008; Muylkens et al., 2010).

1.1.3 Functions of microRNAs as post-transcriptional regulators

The mature miRNA recognizes its mRNA target mainly with its seed sequence. The seed is defined as a stretch of 7 or 8 consecutive nucleotides localized at the 5'end of the mature miRNA. This nucleotide stretch is involved in a perfect base pairing with the target mRNA in so called "seed match" sequence. The mature miRNA binds predominantly on seed match sequences localized at the 3'UTR of its target (Carthew and Sontheimer, 2009) but it may also bind on 5'UTR and coding sequence of the targeted RNA (Lee et al., 2009; Forman and Coller, 2010). Moreover, three type of interactions between the miRNA and its target are mediated through non-canonical seed pairing rules: the perfect hybridization, the 5'dominant and the 3' compensatory hybridizations (Chi et al., 2013; Seok et al., 2016; Brennecke et al., 2005).

1. A **perfect hybridization** between the mature miRNA and its mRNA target induces the cleavage of mRNA strand by Ago2 (figure 1.3 A) (Liu et al., 2004). This phenomenon happens mostly in plants and is rare in animals. An example in animals is the targeting of the *hoxb8* mRNA by miR-196a (Yekta et al., 2004). *Hoxb8* is part of a homeobox (HOX) cluster genes that are a group of related transcription factors genes crucial for development in animals (Yekta et al., 2004). MiR-196a possesses a perfect complementarity (with the exception of a single G:U wobble) with its target site located in the 3'UTR of *hoxb8*. This hybridization led to the cleavage of *hoxb8* (Yekta et al., 2004). The complementarity match sequence between miR-196a and the 3'UTR of *hoxb8* is highly conserved between human, mouse, rat, frog, zebrafish and pufferfish.

2. A second category of miRNA target sites is the **5' dominant sites** in which targets base-pair well to the 5' end of the miRNA (figure 1.3 B) (Brennecke et al., 2005). In this category it is important to distinguish two subgroups: "canonical" sites which the target pairs well at both the 5' end and the 3'end and "seed" sites which the target has little or no 3' pairing support (Brennecke et al., 2005). An example for the subgroup "canonical" sites was described in the Texel sheep (Clop et al., 2006). Its muscle mass was due to a mutation observed in the 3'UTR of the myostatin gene (*gdf8*) creating a target site for miR-1 and miR-206. These two miRNAs are highly expressed in skeletal muscle. The 5'dominant canonical hybridization caused the translational inhibition of *gdf8* contributing to the muscular hypertrophy of the Texel sheep (Clop et al., 2006). An example for the subgroup "seed" sites was studied in Drosophila melanogaster. The bearded gene (*brd*) was targeted and translationally repressed by two miRNAs, miR-4 and miR-79 (Lai, 2002). The repression *brd* gene led to phenotypic effects on adult sensory organs, like loss of commitment of extra proneural cluster cells to the sensory organ precursor fate. The *brd* 3'UTR contains 3 sequence elements, known as Brd boxes, complementary to the 5' region of the two miRNAs. The 3 box target sites consist of 7 mer seeds with little or no base-pairing to the 3'end of either miR-4 or miR-79 (Brennecke et al., 2005).

3. The last category is the **3' compensatory sites** which have weak 5' base pairing and depend on strong compensatory pairing to the 3' end of the miRNA (figure 1.3 C) (Brennecke et al., 2005). This group includes seed matches of 4 to 6 base-pairs and seeds of 7 or 8 bases that contain G:U pairs, single nucleotide bulges or mismatches. An example in Human cytomegalovirus (HCMV) is the targeting of the major histocompatibility complex I-related chain B (*micb*) gene by the viral miRNA,

HCMV-miR-UL112. *Micb* is critical for the natural killer (NK) activation to kill virus-infected cells and tumor cells. The repression of *micb* by HCMV-miR-UL112 was shown to occur during viral infection, leading to reduced killing by NK cells (Stern-ginossar et al., 2007).

Besides the alternative binding strategies, miRNAs usually bind on their targets with an incomplete complementarity leading to translation inhibition or destabilization of the mRNA leading to degradation (figure 1.2 F). In this case, the degradation is not performed directly by Ago2. The targeted mRNA is recruited to a complex containing GW182 proteins in P bodies present in the cytoplasm where translation inhibition and degradation occur (Hammond, 2015).
For translation inhibition different mechanisms have been described. In normal condition, translation begins with the binding of the cap with the polyA tail by the intermediate of eukaryotic translation initiation factor E (eIF4E) and eIF4G to form a loop. Under this circular form, translation can be initiated (figure 1.4 A). A first mechanism of translation inhibition, is to prevent the circular form of the targeted mRNA either by deadenylation and decapping leading to degradation of the mRNA (figure 1.4 B). Deadenylation and decaping are facilitated by CCR4-NOT and DCP1:DCP2 complexes promoting mRNA degradation by accelerating its destabilization (Behm-ansmant et al., 2006). The recruitment of these complexes is possible in the P bodies with the GW182 protein. This protein binds on the mRNA target by interaction with Ago1 in the RISC complex and induces mRNA degradation by decapping and deadenylation. Additionally, due to deadenylation the poly A binding protein (PABP1) cannot bind on mRNA 3' extremity preventing the circularization of the mRNA (Carthew and Sontheimer, 2009). A second mechanism is that miRISC is able to interfere with ribosomes. The mature miRNA was shown to block the association between the 60S ribosomal subunit with the preinitiation complex 40S (figure 1.4 C) (Chendrimada et al., 2007). The repression then occurs with the impossibility for the competent ribosome to assemble at the start codon. Another potential mechanism is the degradation of the nascent polypeptide chain cotranslationally (figure 1.4 D) (Eulalio et al., 2008). The protease implicated in this process is not known. The third mechanism is that miRISC enters into competition with eIF4E, EIF4G for the binding on the 5' cap structure (figure 1.4 E). In the RISC complex, Ago2 possesses a domain (Mid) which bears significant similarity to eIF4E binding site (Kiriakidou et al., 2007).

1.1.4 Transcriptional gene silencing (TGS) and transcriptional gene activation (TGA)

As mentioned earlier, the mature miRNA is able to translocate into the nucleus with the help of different transporters such as Importin 8 (IPO8) and exportin 1 (CRM1) (Weinmann et al., 2009; Wei et al., 2014; Castanotto et al., 2009). In P bodies, miRNA and Ago protein bind to a protein containing a nuclear localization signal (NLS) named TNRC6A allowing them to translocate into the nucleus (Nishi et al., 2013). Among all mature miRNAs produced in the whole cell, 25 % are exclusively present in the cytoplasm. The remaining 75 % are localized both in the nucleus and in the cytoplasm (Gagnon et al., 2014). In the nucleus, the minimum miRISC complex is smaller and has a different composition than in the cytoplasm. Moreover, miRISC may be associated with functionally different proteins in the nucleus and it is assumed that it would allow RISC to harbor different functions. Several roles have been identified for miRNAs located in the nucleus:

1. There were shown to be implicated in the post-transcriptional regulation of small RNA molecules (Tang et al., 2012; Zisoulis et al., 2012). A first example is the repression of miR-15a/16-1 maturation in mouse cells by miR-709 (Tang et al., 2012). A 19-nucleotide element on the primiRNA-15a/16-1 was targeted by miR-709 blocking the processing of this primRNA on a premiRNA leading to the biogenesis suppression of miR-15a/16-1 (Tang et al., 2012). The inhibition of miR-15a/16-1 protects from apoptosis since miR-15a/16-1 targets an anti-apoptotic gene, *bcl2* (Cimmino et al., 2005).

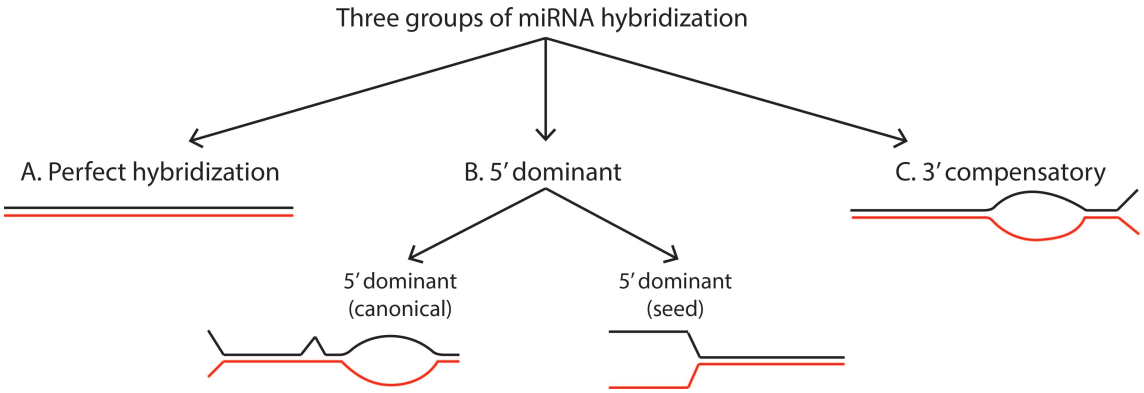

Figure 1.3: **MiRNA hybridization groups**. A. Perfect hybridization model between the entire miRNA sequence and a part of the target mRNA 3'UTR. B. 5'dominant sites in which targets base-pair well to the 5' end of the miRNA. This group is divided into two subgroups: "canonical" (in which target pairs well at both 5'end and the 3' end) and "seed" (in which the target has little or no 3' pairing support). C. 3' compensatory sites which have weak 5' base pairing and depend on strong compensatory pairing to the 3' end of the miRNA. In each groups, the upper diagram illustrates the mode of pairing between the target site (upper line) and miRNA (lower line, red) (Brennecke et al., 2005).

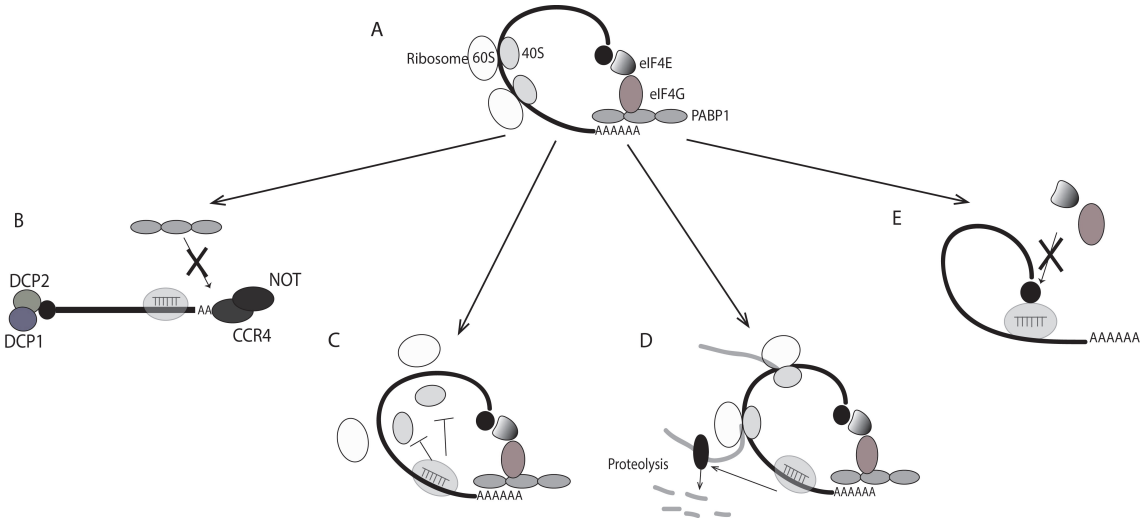

Figure 1.4: **Mechanisms of translation inhibition mediated by microRNAs**. A. Translation of a mRNA in normal condition. B. Circularization and degradation of the mRNA after deadenylation and decapping process. The deadenylation prevents the binding of the PABP1 complex. C. Inhibition of translation by preventing the association of the two subunits of ribosomes or degradation of the nascent peptide. D. miRISC enters in competition with eIF4E by binding the cap of the mRNA. E. degradation of the nascent polypeptide chain cotranslationally. eIF4E and G for eukaryotic translation initiation factor4 E and G. PABP1 for ployA binding protein1. 40S and 60S for the small and big ribosomal subunit respectively. DCP1 and 2 for decapping enzymes. CCR4 for C-C chemokine receptor type 4. CCR4-NOT for deadenylase complex.

2. Another potential function of nuclear miRNA would be the regulation of ribosomal RNA (rRNA) abundance in the nucleolus (Politz et al., 2006). Several studies showed that some miRNAs are concentrated into the nucleolus as both pre-miRNA and mature miRNA forms (Bai et al., 2014). An example is the co-localization of miR-206 observed the 28S ribosomal RNA, in the nucleolus and in some extend in the cytoplasm (Bai et al., 2014). The impact of this co-localization is not fully understood but it is though that miR-206 might have an implication in 28S ribosomal RNA abundance. Moreover, nucleoli could be a storage place allowing the release of miRNAs (precursor and mature) rapidly in the nucleoplasm and/or cytoplasm due to a stress (Li et al., 2013).

3. An additional function of nuclear miRNAs is their implication in splicing events. Liu and collaborators assumed that miRISC may bind on the nascent transcript impairing the binding of the spliceosome complex to the splice site without affecting pre-mRNA transcription level and stability (Liu et al., 2012, 2015a). An example is the deletion of the exon 51 of the dystrophin transcript due to the binding of a synthetic single stranded-siRNA on it. The action of miRISC is only possible by recognition with its seed sequence on the exon sequence leading to the deletion of the targeted exon (Liu et al., 2012, 2015a). Two other studies are in agreement to say that small non-coding RNAs (such as miRNA) are implicated in the splicing event (Ameyar-Zazoua et al., 2012; Alló et al., 2009). Alló et al. showed that small duplex RNA targets both intronic and exonic regions near alternative exon. This miRNA loading favored the inclusion of a variant exon (Alló et al., 2009). Immunoprecipitation assay for nuclear AGO1 and AGO2 proteins showed their interaction with core components of the splicing machinery and several splicing factors (Ameyar-Zazoua et al., 2012). Ameyas-Zazoua et al. showed that AGO1 and AGO2 are required for an efficient splicing event to occur (Ameyar-Zazoua et al., 2012).

4. A last and the most studied role of miRNAs in the nucleus is the transcriptional regulation of gene expression. Transcriptional gene activation (TGA) and transcriptional gene silencing (TGS) were observed to be performed by the intermediate of miRNAs. The first miRNA discovered to induce an activation of gene expression is miR-373 (Place et al., 2008). The miR-373 nuclear import is required to induce the expression of the E-cadherin (*cdh1*) and the cold-shock domain-containing protein 2 (*csdc2*) by binding on their promoter. Another example is the activation of lin-4 expression by itself (Turner et al., 2014). It was observed that complementary element recognized by lin-4 was present on its own promoter allowing transcriptional activation by triggering the recruitment of RNA polII. Many more examples exist (Majid et al., 2010; Liu et al., 2013). The miRNA binds on targeted promoter with its seed sequence and enhance transcription by recruitment of Ago2 protein (Huang et al., 2012) and an enrichment of RNA polymerase II (Huang et al., 2012; Matsui et al., 2013) as well as permissive chromatin marks such as histone 3 Lysine 4 trimethylation (H3K4me3) associated with gene expression (Huang et al., 2012). Two models exist to explain the mechanism of how miRNA induce gene expression in the nucleus. (A) In human genome, bidirectional transcription generates a sense orientated coding transcript and antisense orientated non-coding transcript (mainly long non-coding RNA (lncRNA)). The latter overlaps the sense promoter. Seventy percent of gene promoters are overlapped by non-coding RNA transcript (Gingeras, 2007). This transcript binds on the sense promoter and induces repression of gene expression through recruitment of repressive complexes (chromatin effectors and modifiers). One possibility for TGA to happen is that miRNA may have a complementary sequence with its seed sequence on the non-coding RNA. This hybridization induces the cleavage of the latter preventing recruitment of inhibitory chromatin effectors (figure 1.5 A). It results in de-repression of the promoter activity for the sense transcript (Morris et al., 2008; Modarresi et al., 2014). (B) The second model is that the miRNA seed sequence still recognizes the long non coding RNA and recruits protein complexes implicated in active chromatin changes (figure 1.5 B). There is then a shift of the chromatin state to have a permissive state at the sense promoter allowing gene transcription (Matsui et al., 2013).

As mentioned previously, miRNA are also able to induce transcription gene silencing (TGS). An Example

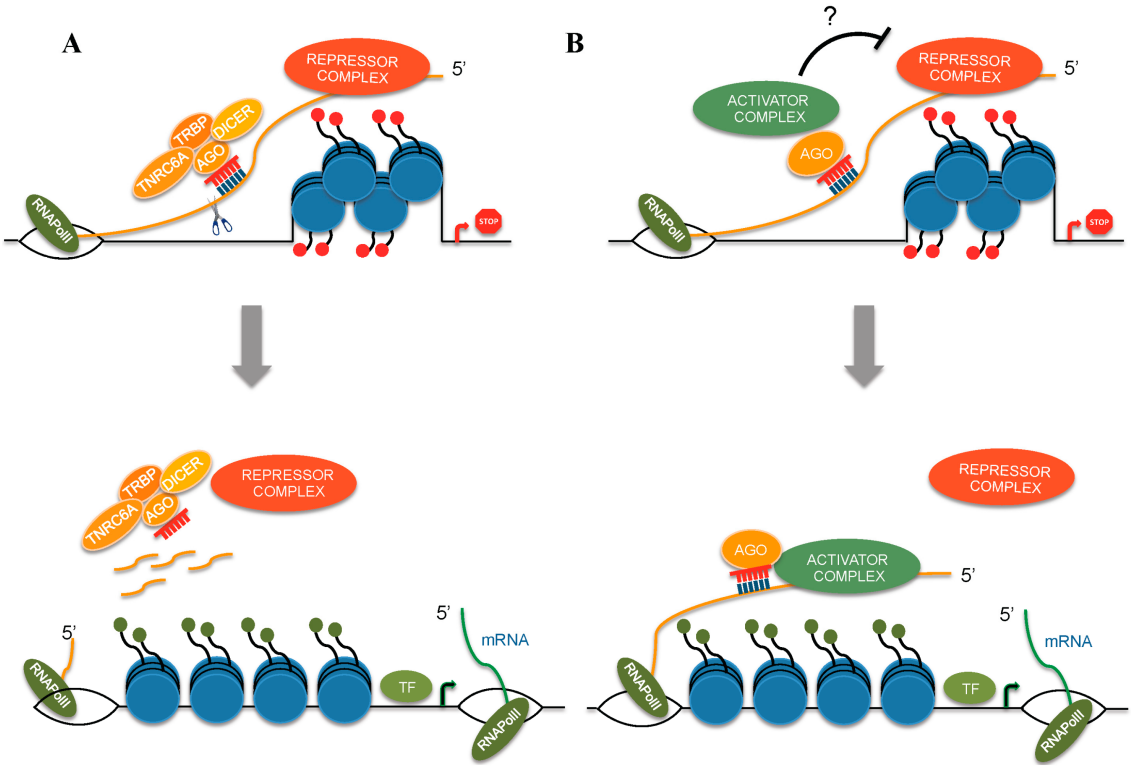

Figure 1.5: **Potential mechanisms of miRNA guided transcriptional gene activation**. Antisense orientated long non coding RNAs (lncRNAs, orange line) is able to bind on sense promoter. The binding of the lncRNA induces repression of gene expression through recruitment of repressive complexes (chromatin effectors and modifiers). A: miRNA may have a complementary sequence with its seed sequence on the lncRNAs. This hybridization induces the cleavage of the latter preventing recruitment of inhibitory effectors. It results in de-repression of the promoter activity for the sense transcript. B: miRNA seed sequence still recognizes the lncRNA and recruits protein complexes implicated in active chromatin changes. There is then a shift of the chromatin state to have a permissive state at the sense promoter allowing gene transcription. TNRC6A for trinucleotide repeat-containing 6A. TRBP for human immunodeficiency virus transactivating response RNA-binding protein. AGO for argonaute. TF for transcription factor. (Catalanotto et al., 2016)

is the miR-223 repressing *nfI-A* (Zardo et al., 2012). NFI-A is a transcription factor from the nuclear factor I family protein and is implicated in the fate of myeloid precursors. The overexpression of *nfI-A* drives erythropoiesis whereas repression of *nfI-A* drives granulopoiesis (Zardo et al., 2012). In cells undergoing granulopoiesis, miR-223 is overexpressed and it binds on *nfI-A* promoter. This hybridization triggers the recruitment of polycomb (PcG) repressor complex 1 proteins member such as YY1 protein. This protein induces chromatin compaction by addition of negative chromatin marks such as histone 3 lysine 27 trimethylation mark (H3K27me3). Two mechanisms are possible to induce TGS. The first mechanism is that the antisense non-coding transcript serves as docking platform and miRNA bind directly on it with its seed sequence. It allows the recruitment of RISC elements (Ago, Dicer), PcG elements (YY1, EZH2 and SUZ12) and chromatin modifiers (figure 1.6 A) (Catalanotto et al., 2016). Another mechanism is the formation of a triplex RNA*DNA:DNA (figure 1.6 B) (Toscano-garibay and Aquino-jarquin, 2014). MiRNA would be able to bind directly on the target promoter and recruit protein complexes involved in negative chromatin remodeling. A study worked on the repression of the major promoter of dihydrofolate reductase gene DHFR by non-coding RNA (Martianov et al., 2007). The latter induced promoter specific transcriptional repression through disruption of the pre-initiation complex.

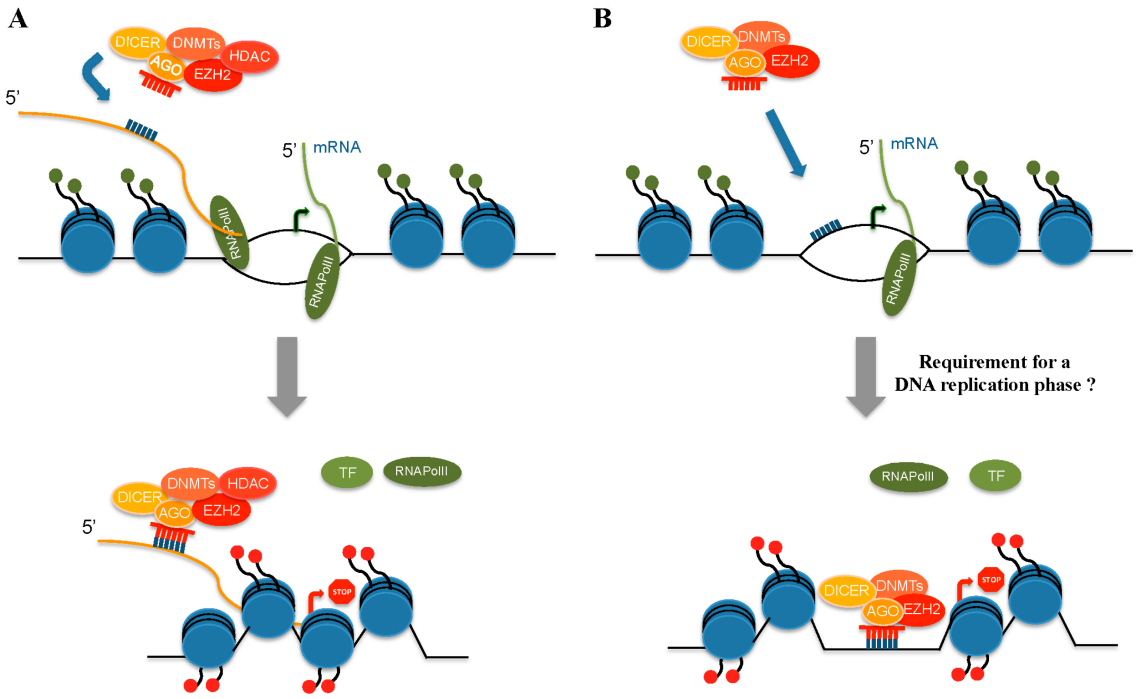

Figure 1.6: **Potential mechanisms of miRNA guided transcriptional gene silencing**. A: the antisense non-coding transcript serves as docking platform and miRNA bind directly on it with its seed sequence. It allow recruitment of polycomb (PcG) elements (EZH2) and chromatin modifier (histone deacetylase (HDAC)). B: Formation of a triplex RNA*DNA:DNA. MiRNA would be able to bind directly on the target promoter and recruit protein complexes involved in negative chromatin remodeling. DNMTs for DNA methyltransferases. HDAC for histone deacetylase. AGO for argonaute. EZH2 for enhancer of zeste homolog 2. TF for transcription factor. RNApolII, RNA polymerase II. (Catalanotto et al., 2016)

1.2 Epigenetic

1.2.1 General context

Epigenetic is the study of heritable DNA structure modification without alteration of sequence. This implies a regulation of gene expression during development and in embryonic cells (Mason et al., 2012). These modifications on genome are involved in normal development as well as in disease like cancer or neurological disorder (Timp and Feinberg, 2013). Epigenetic is implicated in several mechanisms during the development of an individual. It plays a role on cell differentiation, X-chromosome inactivation and genomic imprinting. These modifications include histone post-translational modifications (HPTMs), DNA methylation and the Long Non coding RNA recently described as triggers of the processes (Timp and Feinberg, 2013; Harries, 2012).
Throughout life these modifications may not persist. There are precise modifications at specific stages, in several species. Some studies describe the possible impact of the environment on DNA methylation and histone modifications alteration (Feil and Fraga, 2012).

For example in insects, they undergo important morphological modifications due to stress from environment. In the presence of predators or when the population density is too high, aphids can pass from wingless to winged individual (figure 1.7 A). This change occurs during the early stage of development. This arthropod, as Daphnia, possesses orthologues of vertebrate DNMTs but it is unknown if there is alteration of DNA methylation during this phenomenon (Feil and Fraga, 2012).

In plants there are also epigenetics changes. For example, *Linaria vulgaris* (yellow toadflax) presents a phenotypical plasticity. Indeed, their symmetry can be heritably radial or bilateral (figure 1.7 B), these

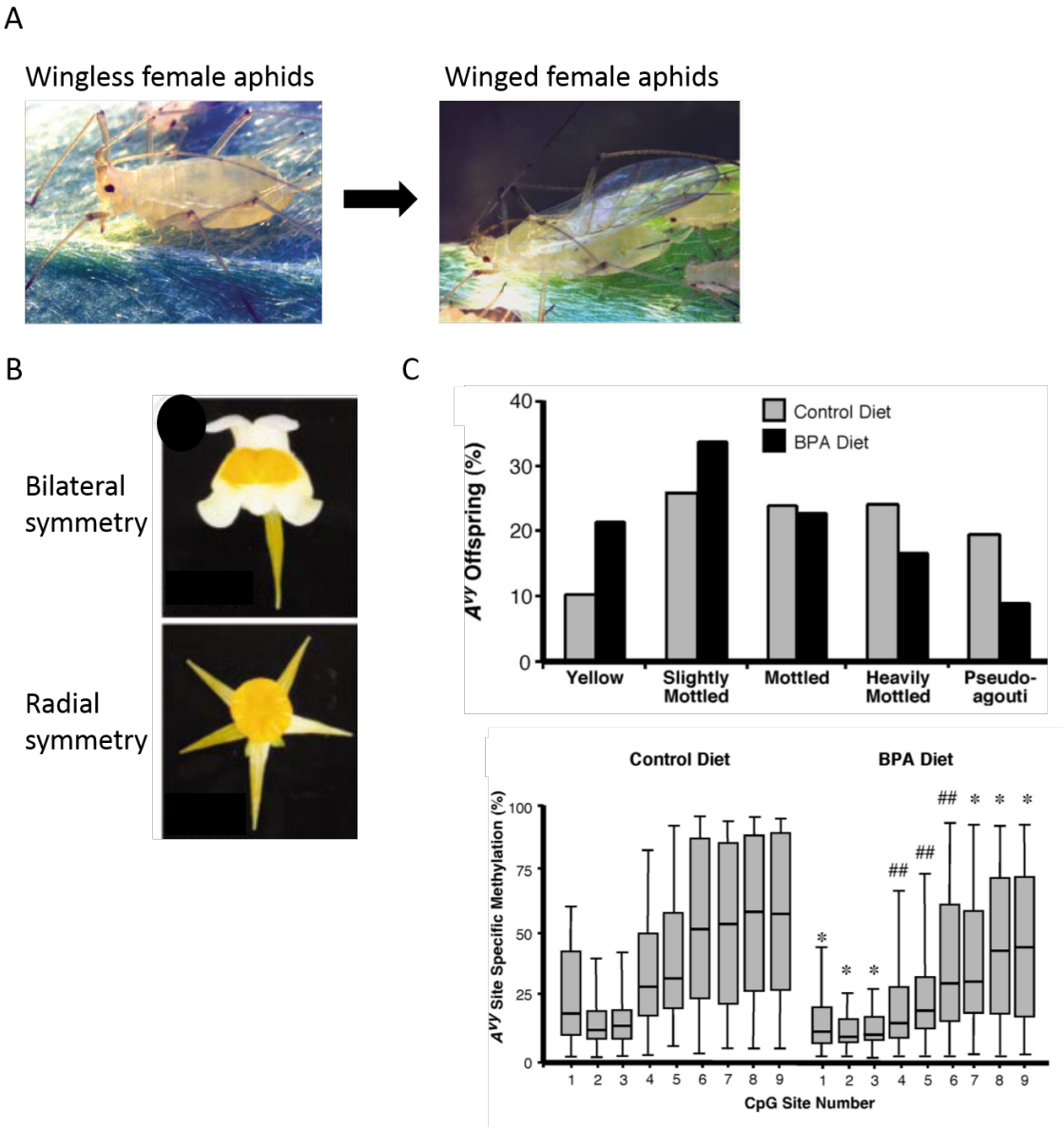

Figure 1.7: **Links between epigenetic modifications and environmental factor**. A: Female genetic polymorphism, two morphological morphs: wingless and winged individual. B. Face view of a *Linaria vulgaris* with bilateral (up) and radial (down) symmetry. C: Mother diet (bisphenol A, BPA) influences coat color (up) and reduced DNA methylation on Avy gene (down) of offspring born. (Braendle et al., 2006; Cubas et al., 1999; Dolinoy et al., 2007)

two symmetries are possible thanks to DNA methylation alteration at a gene (*cycloidea* gene) encoding a transcription factor that controls dorsal asymmetry (Cubas et al., 1999).

The diet and contact with toxic component can also lead to phenotypic effects in animals. Small modifications of DNA methylation were observed. Several studies are trying to determine whether these small modifications have an impact on developmental and metabolic events at the early life stage (Heijmans et al., 2008; Tobi et al., 2009). The epigenetic alteration mainly occurs during the early stage of development because this is during this phase that epigenetic pattern takes place. Then, the alteration phenomenon is amplified due to cell division and thus it affects a large cell number. In mammals different diet components (folate, vit B6, vit B12, methionine, choline,…) have been found to alter DNA methylation and histone epigenetic marks. The CpG methylation level of the agouti viable yellow (*Avy*) promoter, in mouse, showed a decrease in the next offspring when mother diet is supplemented with bisphenol A (BPA, a compound used in plastic) (figure 1.7 C). In the next generation it was observed a large number of yellow coat mice with diabetes and obesity problem (Dolinoy et al., 2007). These results show that epigenetic modifications during early developmental stages can have a deep impact on health.

1.2.2 Histone post-translational modifications (HPTMs)

Several HPTMs exist on the histone N-terminal tail and they affect the chromatin structure. Thereby, modifications influence gene transcription and other mechanisms as repair, replication and recombination (Bannister and Kouzarides, 2011). The best described modifications are histone acetylation, phosphorylation and methylation (Moore et al., 2013). Histone phosphorylation will not be described.
There are over one hundred of HPTMs described in literature. Depending on what we observed, one histone modification or a combination of these modifications, the effect on promoter activity is not the same. For example, the trimethylation of histone 3 lysine 4 (H3K4me3) and the acetylation of the histone 4 lysine 16 (H4K16Ac), separately, generate a permissive stage of the chromatin. But when these two modifications are present in combination that generates a restrictive stage of the chromatin (Rando, 2012). The combination of different histone modifications is called histone code. This mechanism is not fully understood and some scientists don't agree with this hypothesis.

Histone acetylation

Histone acetylation consists on the transfer of an acetyl group from acetyl-CoA to a lysine residue (figure 1.8). Two types of enzymes having opposite functions regulate this reaction: histone acetyl-transferase (HAT) and histone deacetylase (HDAC).

HAT give the acetyl group on the histone 4 at lysine 5 (H4K5) and lysine 12 (H4K12) and certain lysine residues on histone 3 (H3) (Table 1.1) (Bannister and Kouzarides, 2011). This reaction allows the development of the permissive state of chromatin called euchromatin. This state promotes the gene transcription by enhancing the DNA accessibility to transcription factors. Conversely, HDAC deacetylates lysine residues. HDAC is actually composed of a variety of enzymes and is functional by association with

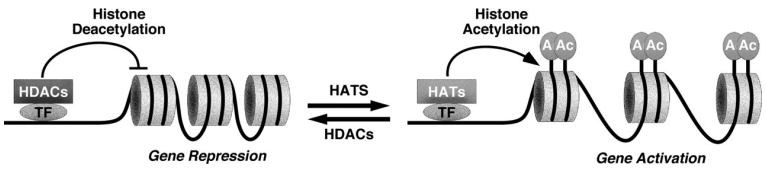

Figure 1.8: Histone acetylation with histone acetyl transferase (HAT) and histone deacetylase with deacetylation with histone deacetylase (HDAC). TF stands for transcription factor. Ac stands for acetyl group.

other HDAC family members. For instance, HDAC 1 is found together with HDAC 2 within a complex called Nucleosome remodeling and deacetylase (NuRD) (figure 1.9) (Yang and Seto, 2008). There are four classes of HDAC. They are sorted according to their sequence similarity and their function. Class I HDACs (HDAC 1, 2, 3 and 8) are located in the nucleus and possess the highest activity. They are ubiquitous and play crucial roles in normal cells (Adhya and Basu, 2010). Class II HDACs (HDAC 4, 5, 6, 7, 9 and 10) can shuttle from the nucleus to the cytoplasm and vice versa. This class possesses a tissue specific expression and has a lower deactylase activity than class I (Ruijter et al., 2003). These two first classes are Zn2+ - dependent classical HDAC and they are inhibited by trichostatin A (TSA). The third class is the sirtuins (class III HDAC). The class III members are considered as NAD+ dependent and insensitive to TSA (Adhya and Basu, 2010). The fourth class is atypical but possesses similarity in sequence with the other classes (HDAC 11) (Adhya and Basu, 2010).

Histone methylation

Methylation of specific lysine residues located on histones is a mechanism commonly used in plants, animals and fungi. This mechanism aims both at defensing genome integrity and at regulating gene expression (Smith et al., 2010).
Histone methylation occurs on lysine and arginine on the side chain of histones. This modification is more complex than acetylation. Indeed, lysine can be mono-, di- or tri-methylated and arginine can be mono-, symmetrically or asymmetrically dimethylated (figure 1.10) (Bannister and Kouzarides, 2011). The methyl group is provided by the S-Adenosyl methionine (SAM). Specific lysines are methylated by histone lysine methyltransferase (HKMT) at precise degrees. For instance in human, DIM5 (histone methyltransferase) tri-methylates H3K9 causing the restrictive stage of chromatin called heterochromatin whereas SET7/9 (SET-domain protein methyltransferase superfamily) only mono-methylates H3K4 causing the activated stage of chromatin, euchromatin (Table 1.1) (Xiao et al., 2003). For arginine, it exists two types of arginine methyltransferase, type-I and type-II. These two types are family members of the protein arginine N-methyltransferase 1 (PRMT). The most known members are PRMT 1, 4, 5 and 6 (Bedford and Clarke, 2009).

Histone demethylases are less known enzymes; the first lysine demethylase was identified in 2004 and is called lysine specific demethylase 1 (LSD-1). When LSD-1 is associated with the Co-REST repressor complex, it demethylates histone H3K4 while when it is associated with androgen receptor, it demethylates H3K9 (Bannister and Kouzarides, 2011). This allows LSD-1 having either an activator or a repressor function. Following the discovering of LSD-1, other classes of lysine demethylases were described like JMJD2. The latter is able to demethylate a tri-methylated lysine as H3K9me3 (Whetstine et al., 2006).

It should be noticed that there are less known histone modifications such as ADP ribosylation, ubiquiti-

Table 1.1: **effect of histone modifications on chromatin**

Modification type	Histone					
	H3K4	H3K9	H3K14	H3K27	H4K5	H4K12
Mono-me	+	+		+	+	+
Di-me	+	-		-		
Tri-me	+	-		-		
Acetylation	+	+	+			

Table notes: . + represents positive mark of histone modification (open chromatin (euchromatin)). – represents negative mark of histone modification (close chromatin (heterochromatin)). H3K4 stands for the lysine 4 of the histone 3. me stands for methyl group.

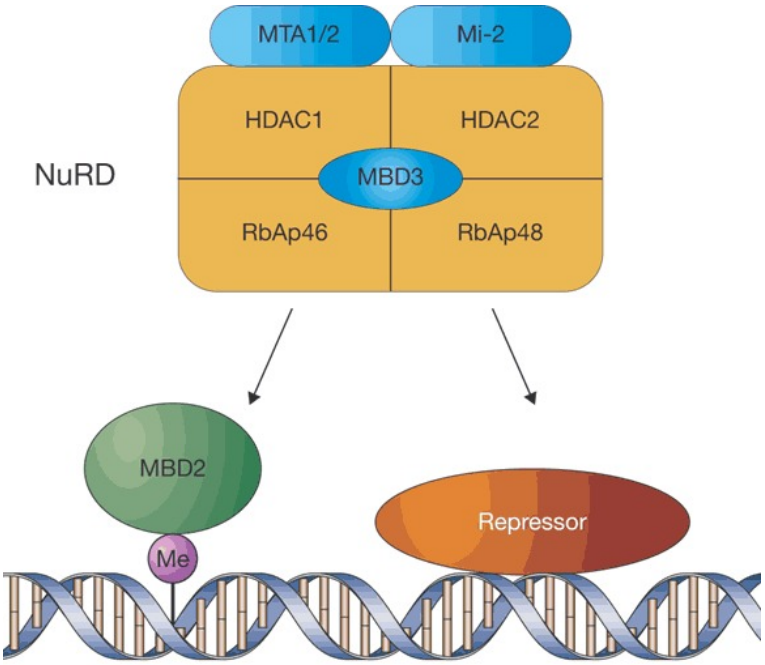

Figure 1.9: The NuRD complex is composed of the histone deacetylase HDAC1 and HDAC2, the histone-binding proteins RbAp46 and RbAp48, the nucleosome remodeling enzyme Mi-2, the metastasis-associated proteins MTA1 and MTA2 and the methyl binding domain MBD3. The NuRD complex interacts with sequence-specific DNA-binding proteins (repressors, e.g. MeCp2, an ubiquitin-like with PHD and Zn2+ domain (UHRF) proteins) and/or is recruited by MBD2 to methylated DNA resulting in transcriptional repression. (Crook et al., 2006)

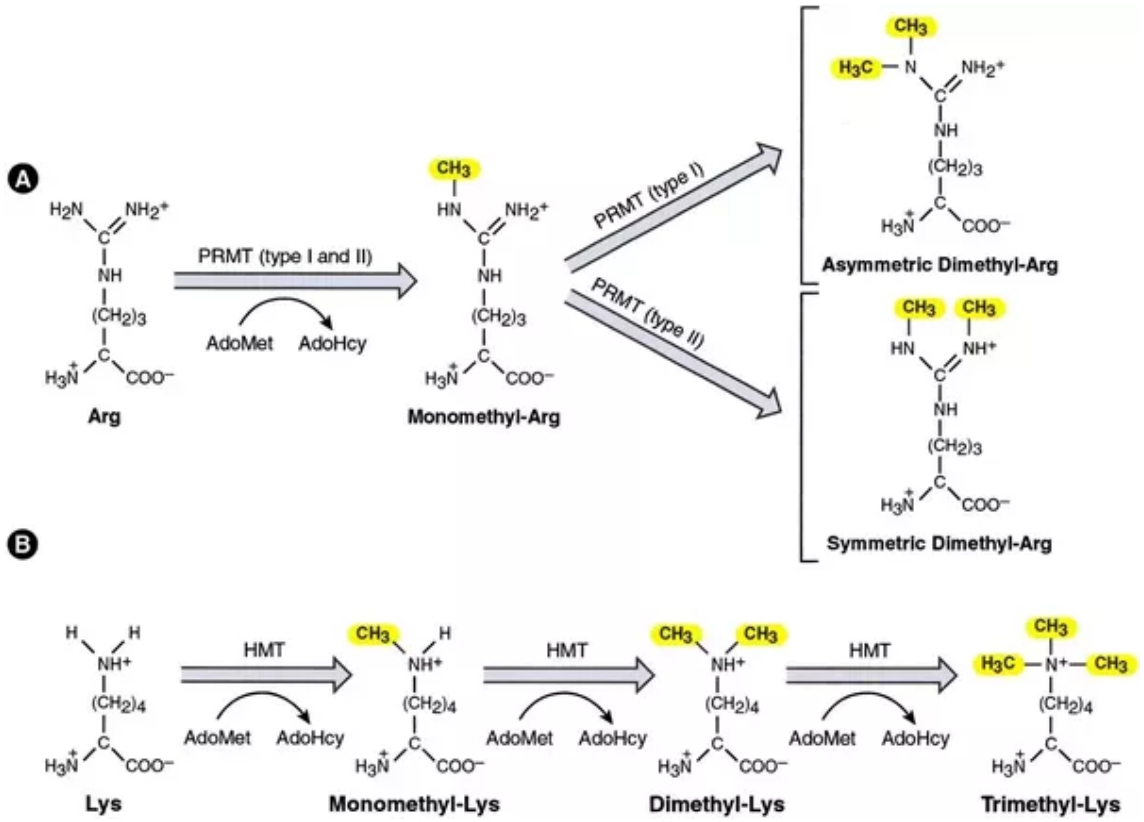

Figure 1.10: **The protein methyltransferase family**. A: Protein arginine methyltransferase family (PRMT). In this picture is represents the class I and II. These two classes are able to mono-methylated arginine. The type I enzyme catalyzes the asymmetric dimethylarginine generation and the type II enzyme generates the symmetric methylarginine. (B) Protein lysine methyltrasferase family (histone methyl transferase (HMT)). The different type of methylation pattern on histone lysine. It is unknown if this is the same enzyme that make all the different methyl-lysines. (Bedford and Richard, 2005; Zhang and Reinberg, 2001)

nation and symoylation but these will not be detailed in this paper.

1.2.3 DNA methylation

DNA methylation was discovered long time ago in the 1940's. Several studies, until 1980's, have shown that DNA methylation could play a crucial role in the gene expression and cell differentiation (Moore et al., 2013). Actors involved in the DNA methylation are DNA methyltransferases (DNMTs). Their source of methyl group is already introduced (SAM). There are two types of DNMTs. The first category adds a methyl group on a previously unmodified DNA (DNMT3a and DNMT3b, De novo DNMTs). The second type is active during DNA replication and allows the preservation of DNA methylation pattern on the newly synthetized DNA strand (DNMT1, perpetuating DNMT) (figure 1.11) (Moore et al., 2013).

Different roles of DNA methylation are identified such as regulation of gene expression according to the cell type, X chromosome inactivation and the genomic imprinting (Moore et al., 2013). DNMT3a and 3b possess a conserved DNA binding domain PWWP (proline-tryptophan-tryptophan-proline) (Ge et al., 2004). It is however unclear how DNMT3a and 3b recognize their specific binding site. There are two theories to explain the recognition of a specific DNA sequence. The first one is that RNA interference could help DNMT to reach the appropriate DNA sequence. The second one is that transcription factors could recruit DNMT to specific site (Moore et al., 2013). DNA methylation is recognized by different kinds of protein family: methyl-CpG binding domain (MBD) proteins and ubiquitin-like with PHD and zing finger domain (UHRF) proteins (Nan et al., 1993). The best known member of the MBD family is MeCP2 (Methyl CpG binding protein 2); it induces a chromatin restrictive stage. Indeed, it recruits chromatin repressors like histone deacetylase present in the nucleosome remodeling and deacetylase complex (NuRD) (figure 1.9) and histone methyltransferase (Chavez et al., 2010). In addition, MeCP2 can recruit DNMT1 to hemimethylated DNA during replication (Kimura and Shiota, 2003).

DNA methylation mainly occurs in CpG Islands, which designate DNA regions rich in dinucleotide cytosin/guanine (CpG), showing a CG content higher than 40 percent and spreading over a minimum of 200 base pairs. CpG Islands are primarily located in promoter region. Transcriptionally active promoters are associated with unmethylated CpG islands. Moreover DNA methylation might be associated with transcriptional repression or activation. This is due to the fact that transcription factors (activators or silencers) are either impaired or favored to bind their DNA response element when they are methylated (Chavez et al., 2010). A recent study showed that DNA methylation not only occurs on promoter and in

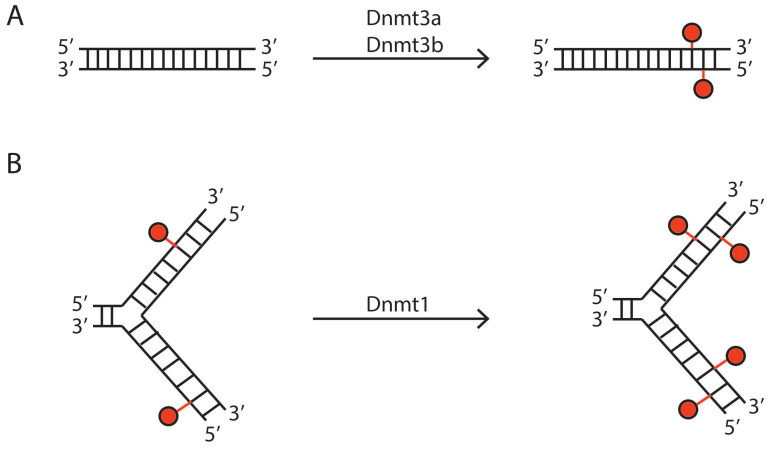

Figure 1.11: **The DNA methyl-transferase (DNMT) family**. A: DNMT3A and 3b add a methyl group on an unmodified DNA. B: DNMT1 acts during DNA replication and allows the preservation on DNA methylation pattern on the newly synthetized DNA strand (Moore et al., 2013).

classical CpG islands (Irizarry et al., 2009). This study showed the existence of a sequence that mapped up to hundreds Kb far from the transcriptional start site (TSS) called "CpG island shores" (Irizarry et al., 2009). These regions are actively involved in the regulation of gene expression.

1.3 Splicing mechanism

In eukaryotic cells, most of the primary messenger RNA transcripts (pre-mRNA) transcribed by RNA polymerase II undergo post-transcriptional modifications allowing their maturation and their regulation. One of these modifications is the splicing of the pre-mRNA to a mature mRNA which will be translated into a protein in the cytoplasm. This phenomenon happens on more than 90 % of eukaryotic genes and is carried out by the spliceosome. It is a ribonucleoprotein complex characterized by the presence of small nuclear RNAs U1, U2, U4/U6 and U5 organized in small nuclear ribonucleoprotein particles (snRNPs) (Baralle and Baralle, 2017). In a subset of eukaryotes, there is also a minor spliceosome, involved in the splicing of 1 % of human genes, formed by U11/U12 and U4atac/U6atac snRNPs, that are functionally comparable to the components of the major one (Turunen et al., 2013).

1.3.1 Splicing consensus sequences

Spliceosome complex assembly is directed by the consensus sequences that mark the exon/intron boundaries. The exon/intron junction at the 5' of the intron is called the donor site (5' splice site), the intron/exon junction at the 3' of the intron is called the acceptor site (3' splice site) and the internal sequence of the intron is called the branch point (figure 1.12). The branch point contains an adenine important for the first trans-esterification step of the splicing reaction and is localized in a pyrimidine rich region.

As mentioned previously, it exists two types of spliceosomes in eukaryotes. The major spliceosome is an U2-dependent spliceosome and the minor one is an U12-dependent spliceosome. In contrast to U2-type spliceosome found in all eukaryotes, the U12-type spliceosome is only present in vertebrates, some fungi, nematodes and plants (Turunen et al., 2013).

The U2-type intron is conserved with a donor site corresponding to a AG/GU sequence, an acceptor site corresponding to a YAG/R (Y=C/U;R=A/G) and an adenine at the branch point located in a CURACU sequence called the polypyrimidine tract (PPT) (figure 1.12A). The U12-type intron is characterized by a /RUAUCCUUU sequence at the donor site and a UUCCUURAY sequence at the branch site (figure 1.12B) (Turunen et al., 2013). As the U2-type intron, the most used termini by the U12-type spliceosome are /GU-AG/. Nevertheless, one third of the termini found was /AU-AC/ (figure 1.12B) (Dietrich et al., 2005). The U12-type introns were suggested to have a role in regulating the expression of specific sets of genes since they were found in genes related to information processing function such as DNA replication and repair, transcription, RNA processing and translation (Turunen et al., 2013). They were also found to be present at genes related to cytoskeletal organization, vesicular transport, and voltage-gated ion channel activity (Turunen et al., 2013).

1.3.2 Spliceosome assembly

The two spliceosomes coexist in the eukaryotic cells and although their snRNPs composition is different, the splicing mechanism is very similar (Turunen et al., 2008).

The spliceosome catalytic conformation or complex C is formed in several successive steps with the formation of intermediate complexes names complex E, A and B (Turunen et al., 2013). It is important to note

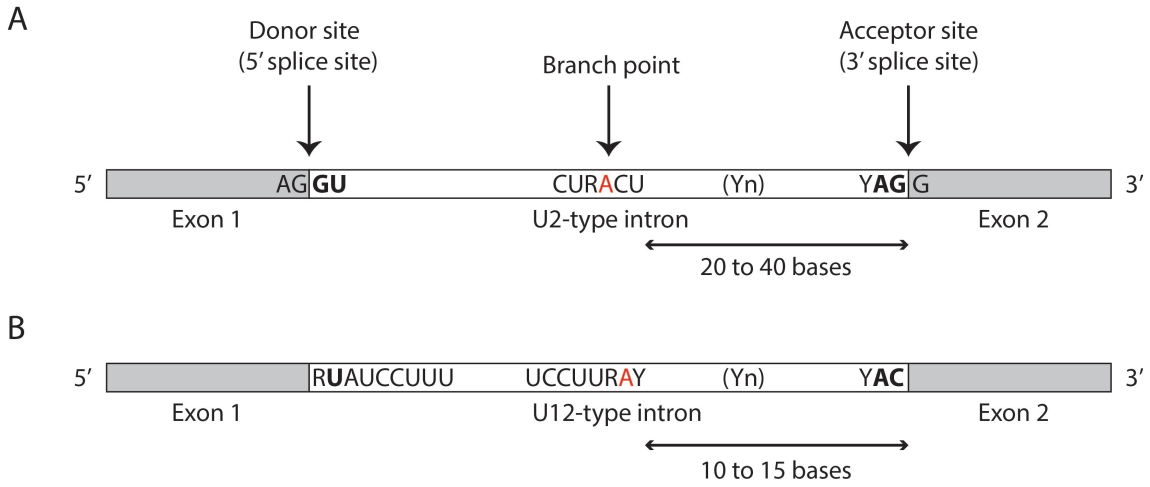

Figure 1.12: **Comparison of the U2- and U12-type regulatory consensus sequences**.A: U2-type intron representation. B: U12-type intron representation. The adenine from the branch point is written in red. Bold nucleotides represent conserved nucleotides.

that no complex E is formed during a U12-type splicing event (figure 1.13). The splicing carried out by U2-type spliceosome begins with the binding of the snRNP U1 at the donor site, splicing factor 1 (SF1) at the branch point and the U2 snRNP auxiliary factor (U2AF) dimer at region rich in pyrimidine (U2AF65) and the acceptor site (U2AF35) to form the complex E (figure 1.13A). At the second step, the SF1 factor is replaced by the snRNP U2 at the adenine from the branch point. This leads to the formation of the complex A then B by addition of the tri-snRNP U4/U5/U6 (figure 1.13B and C). Thereafter, the catalytic complex is formed with the release of U4 and U1 allowing the interaction of the three snRNP U2/U5/U6. U6 and U2 interaction leads to the rapprochement of the acceptor site and the branch point. This rapprochement between these two structures allows the first trans-esterification reaction (complex C) (figure 1.13D). The second trans-esterification reaction is carried out by U5 and leads to the exons ligation and introns excision. Finally, the spliceosome breaks up to release the snRNPs. They will be re-used for another splicing event.

Apart from the first step, U12-type spliceosome assembly is similar to the U2-type spliceosome. U12 and U11 form a stable complex binding at the acceptor site and the branch point leading to the formation of the complex A (Turunen et al., 2008). Then the snRNPs from the U12-type spliceosome interact with the pre-mRNA as the snRNPs of the U2-type spliceosome (figure 1.13) (Schneider et al., 2002).

1.3.3 Alternative splicing

More than 95 % of genes undergo alternative splicing (Keren et al., 2010). This phenomenon is implicated in the regulation of tissue-specific gene expression regulation by generating different protein isoforms playing roles in various cellular processes (Maniatis and Tasic, 2002). Around 50 % of the alternative splicing events produce variable quantities of the relevant isoforms in different tissues leading to a tissue-specific isoform expression (Wang et al., 2008a). These are responsible for development and tissue identity (Baralle and Baralle, 2017). It exists several types of alternative splicing patterns through which exons can be included or skipped (cassette exon), extended or shortened by using alternative 5' donor or 3' acceptor sites as well as alternative promoters or polyA signal, and introns can be removed or retained (figure 1.14) (Baralle and Baralle, 2017).

In order to define exons from introns and to regulate splicing event, auxiliary sequences are essential for both constitutive and alternative splicing. These sequences are classified according to their effect and position as exonic splicing enhancers and silencers (ESE and ESS, respectively) and intronic splicing enhancers

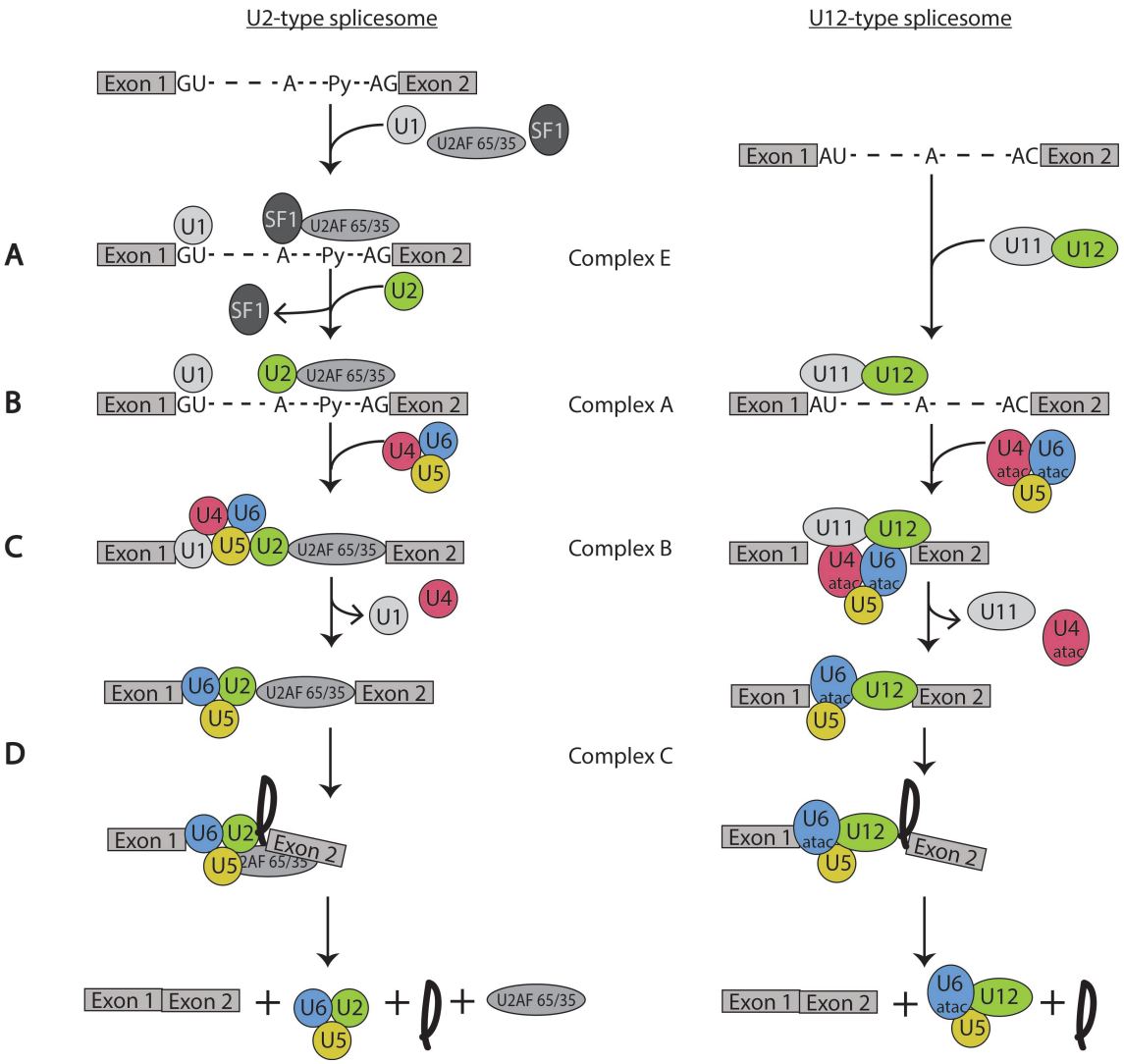

Figure 1.13: **Comparison of the different steps of U2- and U12-type spliceosome assembly**. U1, U2, U4/U6, U5, U11/U12, U4atac and U6atac represent small nuclear RNAs organized in small nuclear ribonucleoprotein particles (snRNPs). SF1 stands for splicing factor 1. U2AF stands for U2 snRNP auxiliary factor, it represents a dimer comprised of U2AF65 and U2AF35 (U2AF65/35)

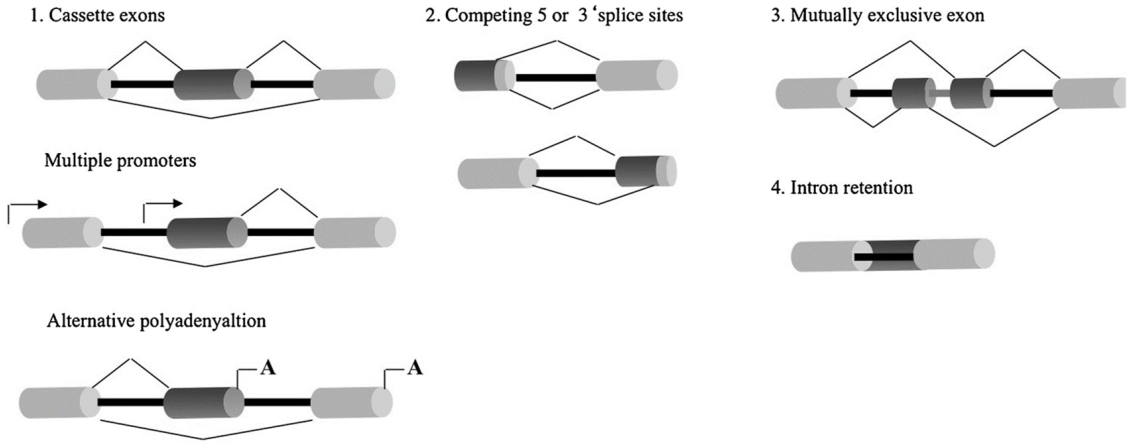

Figure 1.14: **Schematic representation of possible pre-mRNA splicing outcomes**. 1. Cassette exon: inclusion or exclusion can occur at varying proportions. Exon inclusion/exclusion can be altered by changes in transcription initiating site through the use of different promoters, forming alternative 5'-terminal exons joining to a common 3' exon. This mechanism can also define the use of alternative polyadenylation sites. 2. When more than one splice site is present within an exon, the exon length can vary due to the use of alternative 5' and 3' splice sites. Other two less common splicing events are 3. mutually exclusive splicing, where one exon or the other is present in the mature mRNA but not both and 4. intron retention (Baralle and Baralle, 2017).

and silencers (ISE and ISS, respectively) (Pagani and Baralle, 2004). Several common characteristics are that these auxiliary sequences are short (less than 10 nt), their sequences are variable, most of the time they are present in multiple copies and they are weakly active individually.

The vast majority of enhancer elements contain purine-rich sequences, which are binding sites for serine/arginine-rich (SR) proteins (Long and Caceres, 2009). SR proteins are characterized by the presence of one or two RNA recognition motifs (RRM) at the N-terminal and an arginine/serine (RS) domain at the C-terminal (Manley and Krainer, 2010). Generally speaking, these proteins interact through their RS domains with each other, with U1 snRNA, SR related proteins and with U2AF35, facilitating splicing by forming interactions across exons and introns (Baralle and Baralle, 2017). Furthermore, it has also been observed that enhancer activity can occur via recognition of SR proteins at ESE sites allowing the recruitment of the spliceosome (U2 and U12) at the adjacent intron. These proteins also act for the formation of complexes B and C by promoting the incorporation of the tri-snRNP U4/U5/U6 and the binding between U2 and U6.

The silencer elements are principally bound by heterogeneous nuclear ribonucleoproteins (hnRNPs), which bind pre-mRNA without necessarily forming stable association with other RNA-protein complexes. As well as SR proteins, hnRNPs possess one or several RRM domains and one auxiliary domain implicated in protein-protein interaction (Krecic and Swanson, 1999). At least 20 proteins are part of the family; they are designated from A1 (34 Kda) to U (120 Kda). The mechanisms through which exonic and intronic silencers interfere with splicing include inhibition of splice site recognition by sterically blocking the recruitment of snRNPs (Tange et al., 2001). Some hnRNP such as hnRNPA1 may repress the splicing by blocking the spliceosome assembly initial steps by the fixation of several molecules all along the RNA on specific or non-specific sequences (figure 1.15A). They may also inhibit the effect of some nearby regulatory sequences (figure 1.15B). Finally, the repression of the splicing may be due to the formation of a loop in the pre-mRNA preventing splicing machinery to recognize the exon site (Baralle and Baralle, 2017) (figure 1.15C).

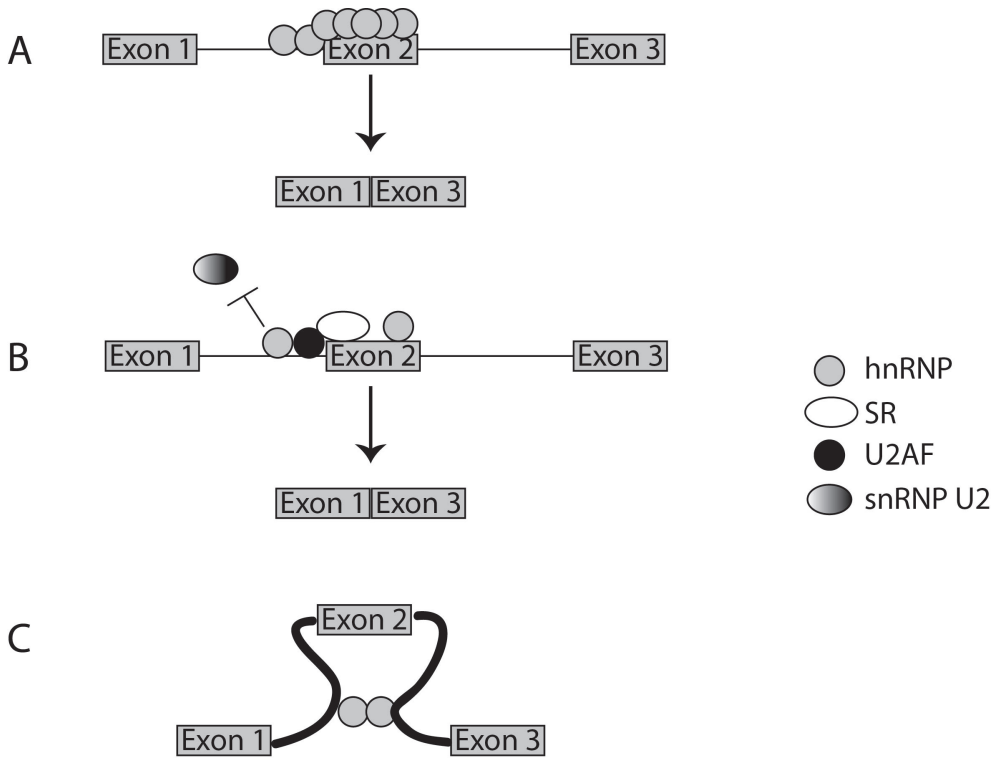

Figure 1.15: **Repressor role of hnRNP on splicing**. A: Inhibition of spliceosome assembly by hnRNP polymerization. B: Repression of U2 binding by steric hindrance. C: Inhibition of splicing by hnRNP dimerization and creation of loop inside the pre-mRNA. Exons and introns are represented by gray rectangle and black horizontal bars, respectively. hnRNP stands for heterogeneous nuclear ribonucleoproteins. SR stands for serine/arginine-rich proteins. U2AF stands for U2AF stands for U2 snRNP auxiliary factor. snRNP U2 stands for the small nuclear ribonucleoprotein particles.

1.4 The *Herpesviridae* family

1.4.1 Nomenclature and classification

The order *Herpesvirales* contains three families: the *Malacoherpesviridae* infecting bivalves, the *Alloherpesviridae* infecting fishes and amphibians and the *Herpesviridae* infecting mammals, birds and reptiles (Davison, 2010) (figure 1.16). The family *Herpesviridae* is the largest and is composed of three subfamilies, the *alpha-*, *beta-* and *gammaherpesvirinae*, classified according to their biological properties. This viral family contains at present more than 130 species sharing common features (Ackermann, 2004; Davison et al., 2009). They are spherical viral particles measuring from 150 to 300 nm. They possess a linear double stranded DNA ranging from 124 to 230 kilobase pairs (kbp) accompanied by some rare RNA molecules (Bechtel et al., 2005; Bresnahan and Shenk, 2000; Jochum et al., 2012). These nucleic acids are associated with an icosahedral capsid composed of 162 capsomers surrounded by proteins with regulatory functions which constitutes the tegument. Finally the virus is covered by an envelope made from cellular membranes and containing glycoproteins which spike on its surface (figure 1.17). Herpesviruses are also classified according to their genome arrangement. Six sequence arrangements are described and are designated by the letters A to F (figure 1.18).
This thesis focuses on an oncogenic herpesvirus from the *Alphaherpesvirinae* subfamily named *gallid herpesvirus-2* (GaHV-2). During this work, a very virulent strain of GaHV-2 was used (RB-1B strain), Genbank n°EF523390 and its genome is 178.246 bp of length. This herpesvirus presents a genome arrangement belonging to the E group (figure 1.19). Two unique sequences termed unique long (U_L) and unique short (U_S) are surrounded by inverted repeats namely terminal and internal repeat long (TR_L/IR_L) for U_L and internal and terminal repeat short (IR_S/TR_S) for U_S. Structural and conserved genes are mainly encoded in U_L and U_S regions whereas most genes involved in the latency and the transformation steps are located in the repeated regions, such as latency associated transcript (LAT), the viral oncoprotein meq and viral microRNAs (figure 1.19).

Besides these morphological properties, all family members of *Herpesviridae* family share several common biological properties (Ackermann, 2004). They possess a large array of enzymes implicated in nucleic acid metabolism (e.g., thymidine kinase), protein processing (e.g., protein kinase) and DNA synthesis (e.g., DNA polymerase, helicase) which occurs in the nucleus of infected cells. In addition, the production of infectious progeny virus generally ends by the destruction of the infected cells. Following lytic infection, herpesviruses systematically establish latency in their natural host in specific cell types.

The classification into three subfamilies was mainly established on the basis of the host range, but also on the spectrum of cells capable of supporting viral latency *in vivo* (figure 1.16) (Davison et al., 2009).

Alphaherpesvirinae subfamily includes four genera designated *Mardivirus*, *Iltovirus*, *Simplexvirus* and the *Varicellovirus*. *Mardivirus* and *Iltovirus* infect avian hosts while *Simplexvirus* and *Varicellovirus* infect mammalian hosts (Smith, 2012) (figure 1.16). Alphaherpesviruses are characterized by a broad host range, a short replication cycle, a rapid proliferation in cell culture and for some of them an efficient lysis in infected cells. Members of this subfamily mostly infect epithelial or neuronal cells before establishing latency mainly in non-dividing sensory neurons. GaHV-2 from the *Mardivirus* genus is an oncogenic herpesvirus causing the Marek's disease (MD). Unlike most of the members of this subfamily, GaHV-2 latently infect T CD4+ lymphocytes and is able to trigger tumor formation in avian species. Its biological properties are closer from the members of the *Gammaherpesvirinae* than from the *Alphaherpesvirinae* (Osterrieder et al., 2006). The main discriminative features of this alphaherpesvirus are that the replicative infection does not lead to cell lysis and that intra host viral transmission is exclusively mediated through cell-to-cell spreading.

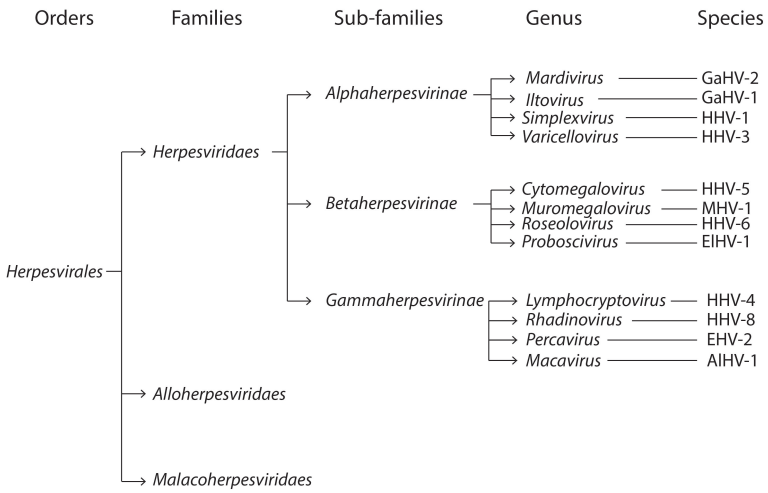

Figure 1.16: ***Herpesvirales* order classification**. Representative species are specified for each gender. GaHV for *gallid herpesvirus*. HHV for human herpesvirus. MHV for murine herpesvirus. ElHV for elephantid herpesvirus. HHV5 or human cytomegalovirus (HCMV)

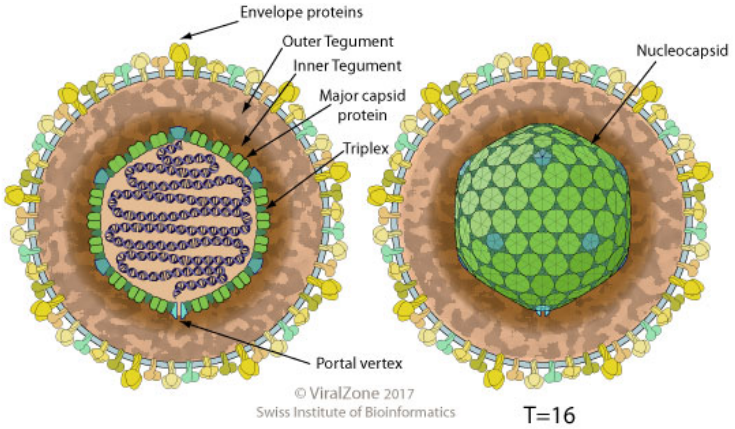

Figure 1.17: **Herpesvirus morphology**. Schematic representation of a viral particle. The nucleocapsid is the association of viral capsid proteins with viral nucleic acid. The capsid is composed of a major capsid protein named viral protein 5 (VP5) and a triplex composed of VP23 and VP19C proteins. Around the nucleocapsid is found an unorganized structure named tegument. This is composed of viral proteins. Finally, the virus is surrounded by an envelope containing glycorpoteins. T stands for triangulation number.

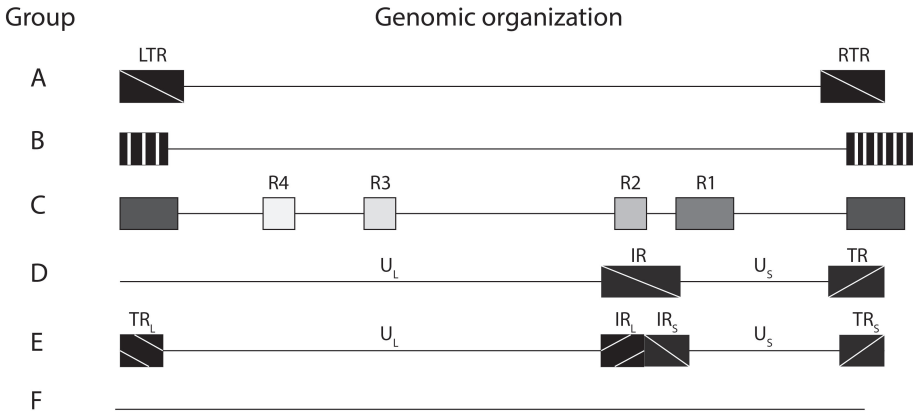

Figure 1.18: **Genomic herpesvirus organization from group A to F**. This schema represents the six genome classes in *Herpesviridae* family. The unique regions are represented by horizontal lines and boxes represent the repeated regions. In the A group LTR and RTR stand for Left Terminal Repeat and Right Terminal repeat. In the B group terminal sequences are repeated with a variable number of repeats. The C group is composed of internal domains from R1 to R4 and the D group shows internal and terminal repeated domain (IR and TR). The E group is composed of two unique regions unique long (U_L) and unique short (U_S) such as the D group. Each unique region is surrounded by inverted repeats: U_L is surrounded by the terminal repeat and the internal repeat long (TR_L and IR_L) and U_S is surrounded by internal repeat and terminal repeat short (IR_S and TR_S). No repetition was found in the F group.

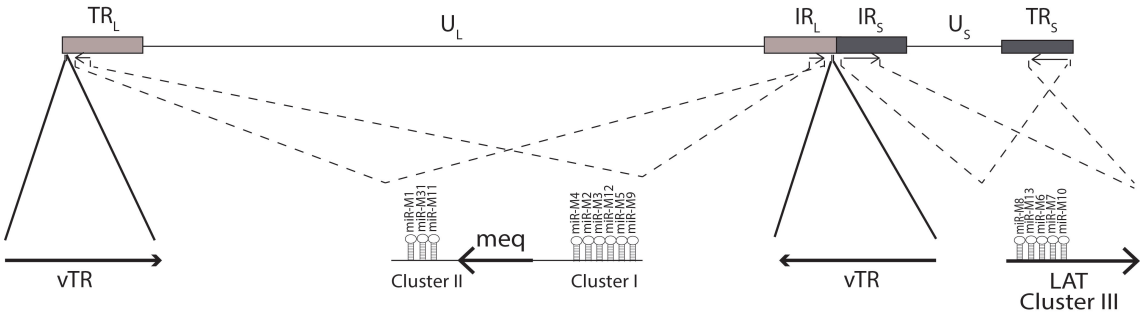

Figure 1.19: **Crucial factors implicated in tumorigenesis during GaHV-2 infection.** U_L and U_S represent the long and the short unique regions, respectively. They are surrounded by long and short terminal and internal inverted repeats: TR_L/IR_L around U_L and IR_S/TR_S around U_S. vTR stands for viral telomerase RNA. microRNAs cluster are named cluster I, II and III. LAT stands for Latency Associated Transcript.

Betaherpesvirinae subfamily is composed of four genera designated *Cytomegalovirus*, *Muromegalovirus*, *Roseolovirus* and *Proboscivirus* (figure 1.16). They are characterized by a narrower host range, a longer multiplication cycle and a less pronounced proliferation in cell. They establish latency essentially in secretory glands, lymphoreticular cells and kidney in mammalian hosts (Bernard N Fields, 2007).

Finally, *gammaherpesvirinae* subfamily contains also four genera: *Lymphocryptovirus*, *Rhadinovirus*, *Percavirus* standing for perissodactyl and carnivore and *Macavirus* standing for malignant catarrhal fever. They latently infect monocytes, dendritic cells and usually either B or T lymphocytes in mammalian hosts (figure 1.16) (Dupuy et al., 2012). Under specific circumstances, they are also able to transform latently infected cells and induce lymphoproliferative disease and/or other non-lymphoid cancers in their infected hosts.

1.4.2 Viral replicative cycle

Productive infection

The multiplication cycle is similar in all members of the group (figure 1.20). Viral life cycle begins with the entry into the cell with a first reversible attachment between viral glycoproteins (gC and gB) and cellular glycosaminoglycans at the cell surface (Herold et al., 1991). Thereafter a stable attachment is made possible with the viral glycoprotein gD interacting with cellular receptors such as tumor necrosis factor superfamily, cell adhesion molecules of the immunoglobulin superfamily and 3-O-sulphated heparan sulfate (Krummenacher et al., 2005; Donnell et al., 2006; Mettenleiter et al., 2009). This stronger interaction induces fusion of the viral envelope with the cellular membrane, leading to the release of proteins from the tegument into the cytoplasm. Two mechanisms of entry are described (Connolly et al., 2011). First, the entry might be performed by the fusion of the viral envelope with the cellular plasma membrane. Second, the entry is possible by endocytosis of the viral particle followed by fusion of the endosomal membrane with the viral envelope. Once inside the cytoplasm, the released nucleocapsid travels along microtubules to the nuclear pore where viral DNA is transferred into the nucleus (Mettenleiter et al., 2009). This phenomenon implies the dynein/dynactin protein motor (Sodeik et al., 1997). In the nucleus the viral genome circularizes. In this form, it triggers the initiation of viral genes transcription in three sequential phases by the cellular RNA polymerase II. The first phase of transcription is initiated by the regulatory tegument proteins and allows the transcription of genes called "immediate early" (IE) or α. One important tegument protein implicated in transcription is VP16 wich is part of a protein complex containing two cellular factors (cellular octamer DNA binding protein (Oct1) and HCF) (Wysocka and Herr, 2003). Once translated, these IE proteins mainly act as transactivators of transcription. They stimulate the transcription of the "early" genes (E) or β. These genes are requested for DNA replication. The last phase, named late "L" or γ, leads to the synthesis of structural proteins such as the envelope glycoproteins and the capsid proteins. L genes are transcribed after DNA replication. The synthesis of viral genome occurs through the mechanism of "rolling circle" generating units consisting of concatemeric structures separated by sequences that are specifically targeted for enzymatic cleavage and packaging (Nicoll et al., 2012). The cleavage results in the insertion of one copy of the viral genome during encapsidation.

Finally, virus assembly and egress take place in order to infect neighboring cells. Late proteins, requested to form the icosahedral capsid, are transported into the nucleus via the nuclear localization signal (NLS) and assemble by an autocatalytic process regulated by the viral protease (Heming et al., 2017). Nucleocapsid egress from the nucleus to the cytoplasm is still a matter of debate. Several mechanisms have been proposed : the model of nuclear pore egress, the "luminal" model and finally the "envelopment/deenvelopment/reenvelopment" model (figure 1.21) (Mettenleiter and Minson, 2006). The most likely one being the latest. According to this model, the mature capsid buds into the inner nuclear membrane (primary envelopment). Pre-enveloped viruses are then localized between the inner and the outer nuclear

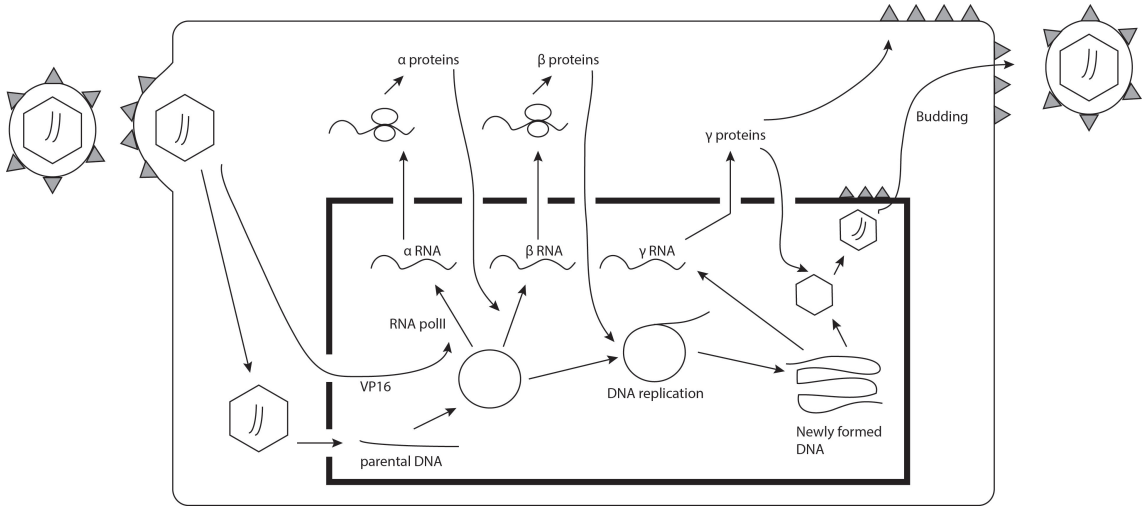

Figure 1.20: **Schematic representation of herpesvirus replicative cycle.** Grey triangles represent viral glycoproteins. VP stands for viral protein. α RNA stands for immediate early RNAs. β RNA stands for early RNAs. γ RNA stands for late RNAs. (created from (Roizman and Whitley, 2001)).

membrane. The primary envelope is acquired during budding through the inner nuclear membrane and is then lost by fusion of this membrane with the outer nuclear membrane (de-envelopment) leading to the release of the nude capsid in the cytoplasm (Mettenleiter et al., 2009). The naked capsid will re-envelop by budding into the Golgi compartment. The mature enveloped particle is eventually secreted by exocytosis from the infected cell (figure 1.21) (Mettenleiter, 2002; Mettenleiter and Minson, 2006).

Latent infection

Latency is a hallmark of the herpesvirus life cycle. It is described as the maintenance of the virus in the host cell in the absence of progeny virus production. During latency only a small subset of viral genes are expressed and the viral genome generally persists as episome in the nucleus of infected cells. In dividing cells, the viral genome replicates simultaneously with the cellular DNA and viral episomes are distributed equally between daughter cells (Vogel et al., 2010). For some herpesviruses such as GaHV-2 and HHV-6, viral genomes integrate into the host genome. All along latency herpesviruses are able to undergo reactivation that ultimately results in a new production of viral particles. Furthermore, some herpesviruses from *Alphaherpesvirinae* (*gallid Herpesvitrus-2* (GaHV-2)) and *Gammaherpesvirinae* (Human HerpesVirus-4 (HHV-4) and HHV-8) subfamilies are associated with tumorigenesis during latency in their natural hosts.
Viral and cellular factors associated to latency establishment are numerous and not yet all identified. Some might trigger oncogenesis as an unwanted effect during infection. These factors regulation and implication during GaHV-2 infection form the subject of the following chapter.

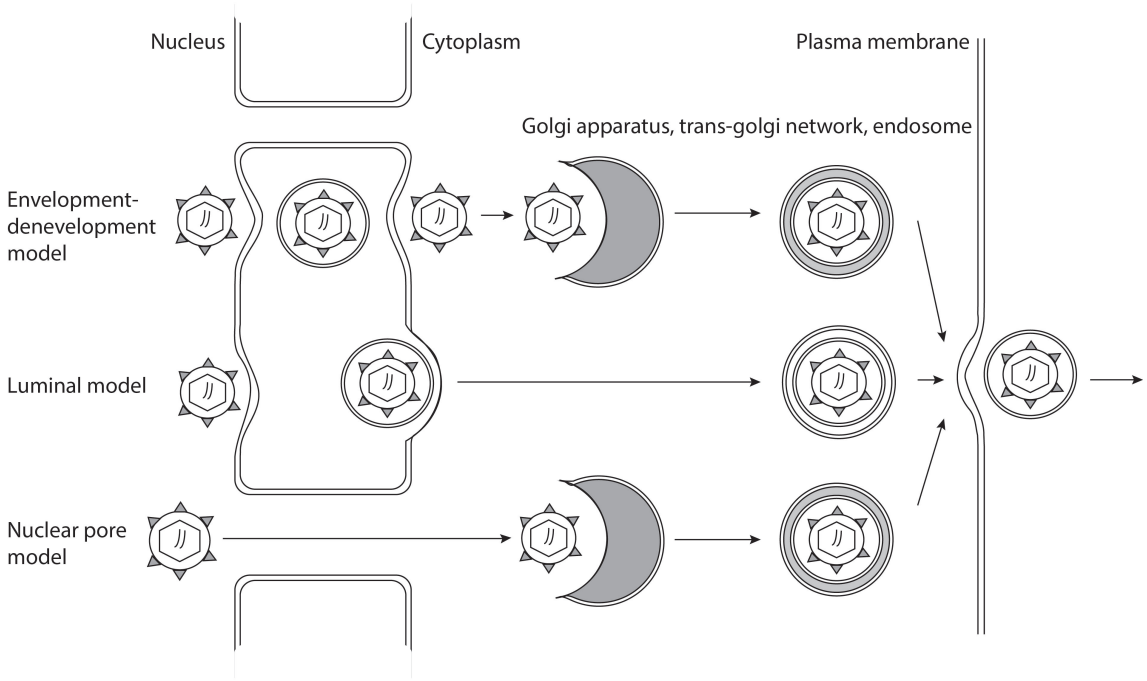

Figure 1.21: **Herpesvirus egress model.** The grey triangles represent viral glycoproteins anchor at the viral envelop. The hexagon represents the viral capsid. Grey vesicle represents the golgi apparatus and the trans-golgi network.

1.5 Marek's disease : genetic regulation of gallid herpesvirus 2 infection and latency (updated from Gennart et al., 2015)

Abstract
gallid herpesvirus-2 (GaHV-2) is an oncogenic alphaherpesvirus that causes Marek's disease (MD), a T cell lymphosarcoma (lymphoma) of domestic fowl (chickens). The GaHV-2 genome integrates by homologous recombination into the host genome and, by modulating expression of viral and cellular genes, induces transformation of latently infected cells. MD is a unique model of viral oncogenesis. Mechanisms implicated in the regulation of viral and cellular genes during GaHV-2 infection operate at transcriptional, posttranscriptional and post-translational levels, with involvement of viral and cellular transcription factors, along with epigenetic modifications, alternative splicing, microRNAs and post-translational modifications of viral proteins. Meq, the major oncogenic protein of GaHV-2, is a viral transcription factor that modulates expression of viral genes, for example by binding to the viral bidirectional promoter of the pp38-pp24/1.8 kb mRNA, and also modulates expression of cellular genes, such as *bcl-2* and matrix metalloproteinase 3 (*mmp-3*). GaHV-2 expresses viral telomerase RNA subunit (vTR), which forms a complex with the cellular telomerase reverse transcriptase (TERT), thus contributing to tumorigenesis, while vTR independent of telomerase activity is implicated in metastasis. Expression of a viral interleukin 8 homologue may contribute to lymphomagenesis. Inhibition of expression of the pro-apoptotic factors JARID2 and SMAD2 by viral microRNAs may promote the survival and proliferation of GaHV-2 latently infected cells, thus enhancing tumorigenesis, while inhibition of interleukin 8 by viral microRNAs may be involved in evasion of immune surveillance. Viral envelope glycoproteins derived from glycoprotein B (gp60 and gp49), as well as glycoprotein C, may also play a role in immune evasion.

1.5.1 Introduction

Marek's disease (MD) is a contagious lymphoproliferative disease of domestic fowl (chickens) first described in 1907 by Jozsef Marek. The disease is caused by an oncogenic alphaherpesvirus, *gallid herpesvirus type 2* (GaHV-2), and is characterized by formation of T cell lymphosarcomas (lymphomas) and paralysis (Burgess et al., 2004). MD is present worldwide and has a major economic impact on the poultry industry. Until the 1950s, the disease was associated with a polyneuritis syndrome, with a low rate of mortality (figure 1.22). Concomitant with increasing industrialisation in the 1960s, an acute form of the disease appeared, with a higher mortality rate (10–30 %) (Biggs and Nair, 2012). This acute form was characterized by visceral tumors in addition to nervous system lesions described initially. In the classical (paralytic) form, the disease has an incubation time of 3–9 weeks. The first clinical signs are locomotor dysfunction, followed by the onset of paralysis; the chicken usually dies of starvation (Mazzella et al., 1986). The acute (lymphomatous) form leads to the death of the chicken after 4 weeks and is characterized by multiple T cell tumors arising in visceral organs.

In the late 1960s, GaHV-2, also known as MD virus type 1 (MDV-1), was identified as the causative agent of MD (Churchill and Biggs, 1967). Currently, herpesviruses belonging to the gender *Mardivirus* are divided into three viral species, designated GaHV-2, GaHV-3 and meleagrid herpesvirus type 1 (MeVH-1) (Table 1.2) (Bulow and Biggs, 1975a,b). The GaHV-2 group contains all the oncogenic viruses and includes four pathotypes: moderate (m; strain CU-2), virulent (v; strain HPRS-16), very virulent (vv; strains RB-1B or Md5) and very virulent + (vv+; strain RK-1) (Witter, 1997).
In the late 1960s, two vaccines against MD were generated; the first was based on the GaHV-2 oncogenic

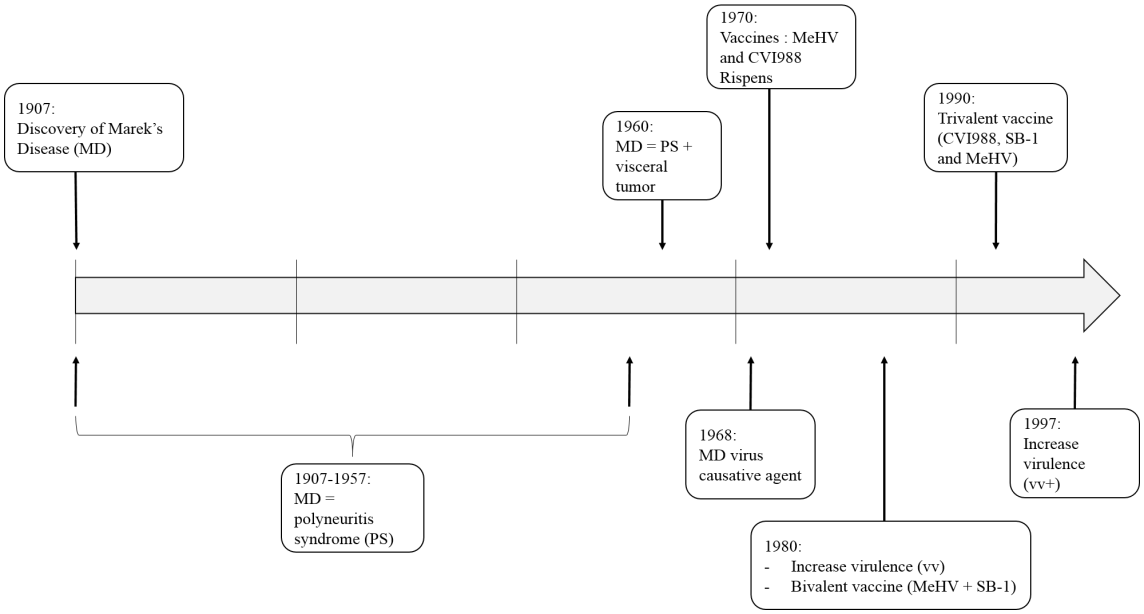

Figure 1.22: **Time line representing the history of increase virulence and usage of vaccine against *gallid herpesvirus-2* (GaHV-2).** Black bars represent every 20 years. MeHV for meleagrid herpesvirus.

strain HPRS-16, attenuated by more than 30 passages in chicken kidney cell cultures (Table 1.2) (Churchill and Chubb, 1969). A few years later, this vaccine was replaced by a vaccine based on MeHV-1 (strain FC-126) (Table 1.2). Since vaccine failures were still encountered when using HPRS-16 or FC-126, a new vaccine was developed based on a naturally attenuated GaHV-2 strain, CVI988-Rispens, which exhibits low pathogenicity (Table 1.2). In the 1980s, the virulence of GaHV-2 increased further with the emergence of a vv strain. The vaccine strategy was modified; a bivalent vaccine composed of FC-126 (MeHV-1) and SB-1 (GaHV-3) was introduced (Table 1.2) (Calnek et al., 1982; Witter and Lee, 1984). Ten years later, there was a further increase in the incidence of MD due to the emergence of hypervirulent (vv+) GaHV-2 (Table 1.2). In response, the three species of Mardivirus were combined into a trivalent vaccine composed of CVI988 (GaHV-2), SB-1 (GaHV-3) and FC-126 (MeVH-1). Currently, the disease is controlled with this updated vaccine strategy. Vaccination prevents the development of MD, but not virus transmission or infection, so there is a risk that increases in virulence may occur in the future (Read et al., 2015). New strategies, such as recombinant virus, e.g. GaHV-2 Δ Marek EcoQ (Meq), might be needed in the future to control infection (Lee et al., 2008, 2012).
The aim of this review is to examine the genetic regulation of viral and cellular genes during the infectious cycle of GaHV-2 and to explore how the virus modulates gene expression.

Table 1.2: **Description of the gender *Mardivirus***

Genotype	**Pathotype**	**Oncogenicity**	**Strains**
GaHV-2 (MDV-1)	Hypervirulent (vv+)	+++	RK-1, 584A, 648A
	Very virulent (vv)	++	RB-1B, Md5, Md-11
	Virulent (v)	+	GA, HPRS-16, JM
	Moderate (m)	No	Rispens, CU-2, HPRS17
GaHV-3 (MDV-2)	Moderate (m)	No	SB-1, HPRS-24, HN-1
MeHV-1 (MDV-3)	Moderate (m)	No	FC-126, WTHV, HPRS-26

Table notes: GaHV for *gallid herpesvirus*. MeHV, meleagrid herpesvirus.

1.5.2 Infectious cycle of gallid herpesvirus type 2 *in vivo*

The infectious cycle of GaHV-2 contains four phases: (1) the early productive phase; (2) the latent phase; (3) the late productive phase; and (4) the transformation phase (figure 1.23). Infection begins by inhalation of dust or dander containing infectious particles released from feather follicles. Within the lung, the virus is phagocytosed by macrophages and transported to secondary lymphoid tissue, such as the spleen, thymus and bursa of Fabricius (Barrow et al., 2003). In these organs, the early productive phase, with active replication of virus, takes place mainly in B lymphocytes (Shek et al., 1983). The virus then transferred via cell-to-cell spread to neighboring cells in which latency is established. In the latent phase, the virus persists in target cells, mainly CD4+ T lymphocytes, without replicating; genome expression is limited to specific genes required to maintain latency, no progeny virions are produced and the latent virus remains undetected by the immune system (Davison and Nair, 2004). The GaHV-2 genome integrates by homologous recombination into the host genome within specific telomeric regions in chromosomes of any size (macro- and micro-chromosomes) (Robinson et al., 2010). The GaHV-2 linear genome possesses telomeric repeats identical to host telomere sequences (TTAGGG)n (Kaufer et al., 2011). Viral integration appears to enhance cellular transformation and tumor formation. The late productive phase consists of reactivation of the virus in a subpopulation of latently infected cells (figure 1.23). During this phase, viral replication occurs within epithelial cells in feather follicles and is associated with horizontal transmission of the virus. Finally, the transformation phase appears within latently infected cells, which spread in peripheral nerves and visceral organs, causing T cell lymphoma and paralysis (Davison and Nair, 2004).

During the viral replication cycle, transcriptional modification and epigenetic changes (DNAmethylation, histone post-translational modifications and non-coding RNAs), along with post-transcriptional and post-translational modifications, regulate expression of cellular and viral genes (figure 1.24). These allow GaHV-2 to switch between the productive and latent phases, and to induce transformation of infected cells.

1.5.3 Transcriptional modification in GaHV-2 infected cells

Transcriptional regulation by the oncoprotein Meq

During GaHV-2 infection, some latently infected T cells undergo transformation and form generalized T lymphomas in chickens. Meq, a nuclear phosphoprotein of 339 amino acids (aa), is expressed in the lytic and latency phases and is considered to be the major oncogenic protein of GaHV-2. The gene encoding *meq* is localized within the repeat regions surrounding the long unique region of the GaHV-2 genome, known as the terminal and internal repeats of the unique long region (TR_L and IR_L, respectively) (Qian et al., 1995). The C-terminal region is a proline-rich region with a transactivation domain (figure 1.25 A) (Qian et al., 1995, 1996). Its N-terminal region possesses a basic-leucine zipper (bZIP) structure similar to that of the cellular transcription factors (proto-oncogenes) c-Jun/c-Fos (figure 1.25 A) (Qian et al., 1995, 1996). This bZIP domain allows Meq to form homodimers or to dimerize with other proteins possessing a bZIP domain, such as c-Jun, for which it has a strong affinity (Levy et al., 2003). Meq is also able to bind to cellular factors, such as c-AMP response element-binding protein (CREB), musculoaponeurotic fibrosarcoma oncogene homologue (MAF), sucrose non-fermentable (SNF) and activating transcription factors (ATFs) 1, 2 and 3 (Liu and Kung, 2000; Levy et al., 2003), p53, retinoblastoma protein (pRB), cyclin-dependent kinase 2 (CDK2), infected cell protein 4 (ICP4) (Liu and Kung, 2000) and COOH terminal-binding protein (CtBP) (Brown et al., 2005). The heterodimer Meq/Jun and the homodimer Meq/Meq bind to specific nucleotide sequences, designated the Meq responsive element I (MERE I, 5' -GAGTGATGACGTCATC- 3') and MERE II (5' -ACACACA- 3'), respectively (figure 1.25 B). MERE I sites are found in viral promoters, such as ICP4, a transactivator of the replicative phase, and glycoprotein B (gB), along with cellular promoters, including interleukin (IL)-2, B cell lymphoma 2 (Bcl-2), matrix metallo-proteinase-3 (MMP-3) and gga-miR-21 promoters (figure 1.25 B) (Qian et al., 1995; Levy et al., 2003, 2005; Ajithdoss et al., 2009; Stik et al., 2013). The binding of Meq/c-Jun to MERE I activates transcription of genes that have roles associated with cell transformation and viral replication. GaHV-2 transforms latently infected cells through

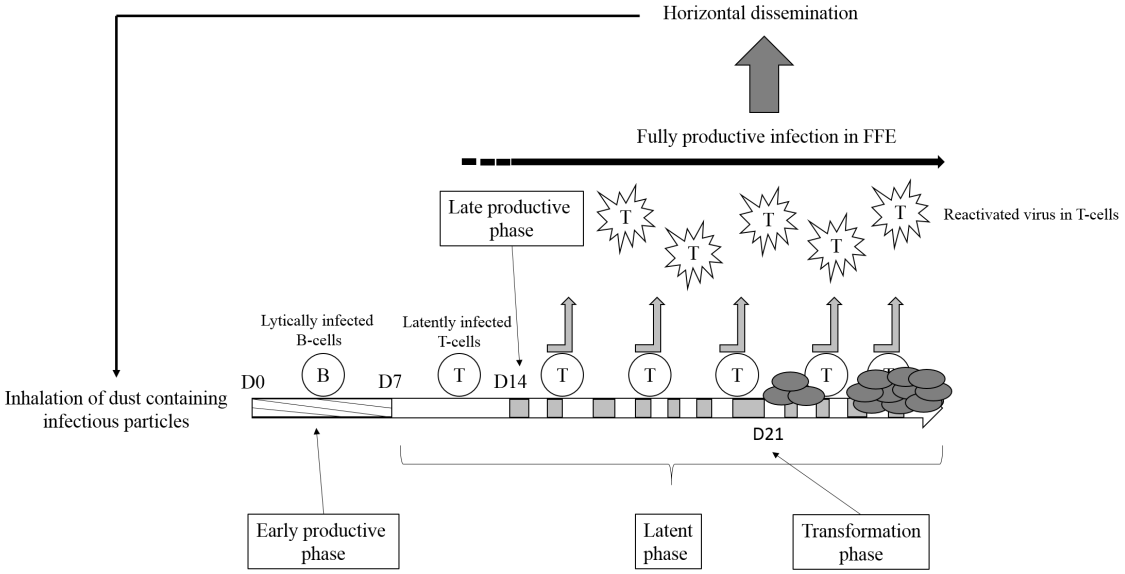

Figure 1.23: **The different infection phases of the oncogenic strain *gallid herpesvirus-2* (GaHV-2).** The number of days (D) in this diagram was derived from experimental observations (natural infection is slower). Day 0 represents the day of the inhalation of the viral particle in the lung followed by its migration to lymphoid organs. The darker light grey arrows represent the reactivation of the virus from latently infected T-cells. At around 14 days post-infection, infectious particles are detected in dust from feather follicles. At the late productive phase, viral replication in feather follicle epithelium (FFE) is fully productive; enveloped, infectious and cell-free viruses are released in the environment (horizontal dissemination). B surrounded with a round represent B lymphocytes. T surrounded with a round represent latently infected T lymphocytes. T surrounded with a star represent T lymphocytes infected with reactivated viruses. Dark grey rounds represent transformed latently infected T cells.

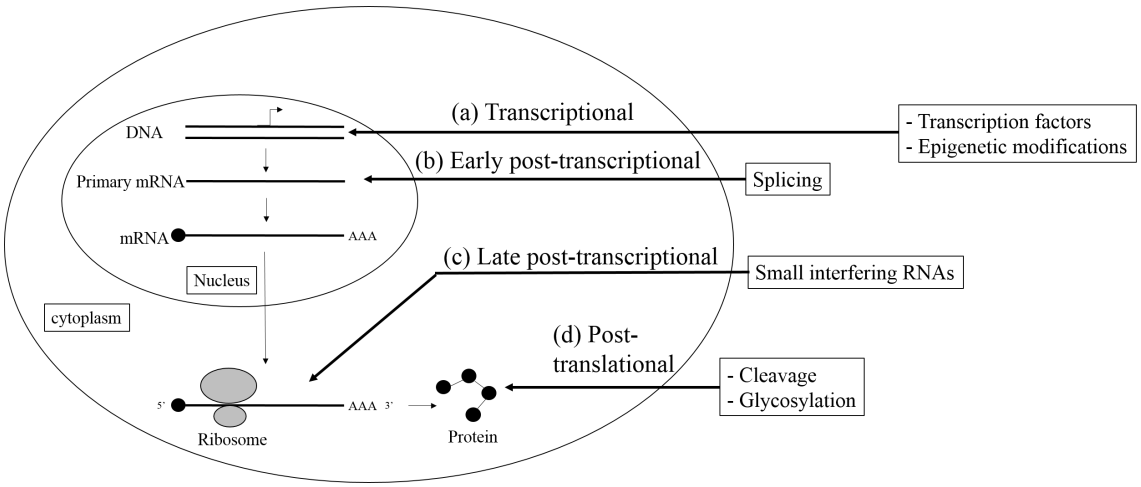

Figure 1.24: **Overview of genetic regulation of *gallid herpesvirus -2* (GaHV-2) infection.** (a) Transcriptional level. (b) Early post-transcriptional level. (c) Late post-transcriptional level. (d) Post-translational modification. Each modification level comprises one or multiple examples of factors implicated in the modification of gene expression (black frame).

activation of gene expression of *bcl-2*, which is anti-apoptotic and facilitates cell survival, and the enzyme MMP-3, which degrades the extracellular matrix, facilitating dispersion of transformed cells. The cellular micro-RNA (miRNA) gga-miR-21 is also implicated in transformation; it targets tumor suppressor genes, such as programmed death cell 4 (*pdcd4*), a protein implicated in apoptosis (Meng et al., 2007; Asangani et al., 2008). At another level, Meq regulates viral replication through activation of ICP4, implicated in viral replication, and gB, implicated in cell-to-cell spread of GaHV-2 in cultured cells (Schumacher et al., 2000; Levy et al., 2003).

Although gene expression is enhanced after binding of the Meq/c-Jun heterodimer to the MERE I site, the binding of the homodimer Meq/Meq to MERE II represses gene expression. A MERE II site has been localized in the viral bidirectional promoter controlling on one hand the *pp38-pp24* genes and on the other hand the 1,8 Kb mRNA (Qian et al., 1996; Liu and Kung, 2000; Levy et al., 2003). Since pp38 and pp24 are late lytic proteins, Meq prevents viral DNA replication and productive infection by targeting this promoter (figure 1.26). The heterodimer pp38/pp24 also targets its own promoter (figure 1.26) (Jiabo et al., 2008); pp38 is located at the IR_L/U_L junction, with its 5' end in U_L, while pp24 is located at the TR_L junction with its 3' and in U_L. The pp38 and pp24 proteins have their N-terminal extremity in common (Shigekane et al., 1999). The binding of the heterodimer pp38/pp24 induces upregulation of the bidirectional promoter (Shigekane et al., 1999; Chen et al., 2009). In this condition, a 14 kDa protein is encoded by the 1.8 kb mRNA. This protein promotes the transition from G1 to S phases of the cell cycle and enhances viral DNA replication (Hayashi et al., 1999). In addition, pp38 is involved in lytic infection of B lymphocytes in the spleen and maintains the viability of infected T lymphocytes by limiting apoptosis (Gimeno et al., 2005). Increased activity of the bidirectional promoter is associated with immortalization and proliferation of latently infected cells.

Transcriptional regulation of the GaHV-2 viral promoter by cellular transcription factors

Cellular transcription factors, such as p53 and c-Myc, bind to and modulate the expression of viral genes, such as the latency associated transcripts (LATs) and viral telomerase RNA subunit (vTR) (Shkreli et al., 2007; Stik et al., 2010). LATs are long, non-coding, RNAs (10 kb) localized in the internal and terminal repeats of the unique short region (IR_S/TR_S) of the GaHV-2 genome. These non-coding RNAs are mainly expressed during viral latency and lymphomagenesis, suggesting that LATs have a role in the maintenance of latency and/or cell transformation. A cluster of microRNAs (cluster III, designated the MDV-1-miRM8-M10 cluster) has been identified in the first intron of the LATs gene (miR-M8, miR-M13, miR-M6, miR-M7 and miR-M10) (Burnside et al., 2006; Strassheim et al., 2012). These miRNAs are overexpressed in GaHV-2 transformed cells and in latently infected cells (Stik et al., 2010). This overexpression is due to the binding of p53, a tumor suppressor, to the LATs promoter, which consists of a series of 60 bp repeats. Each of the repeats contains a conserved functional p53 binding site. The number of 60 bp repeats varies according to strain of virus and may be associated with the level of virulence (figure 1.27) (Spatz and Silva, 2007; Stik et al., 2010).

The cellular proto-oncogene c-Myc induces expression of the vTR promoter (Shkreli et al., 2007). The telomerase complex is composed of two subunits: telomerase reverse transcriptase (TERT), which has telomerase activity, and telomerase RNA (TR), which is recognized by TERT and is used as a template to copy the repeat motif at the end of the chromosome (Chbab et al., 2010). GaHV-2 is the only virus known to encode a vTR (Fragnet et al., 2003). This RNA component is functional when it associates with chicken TERT and is expressed at a higher level than its avian homologue, chicken TR (Fragnet et al., 2005; Shkreli et al., 2007). The vTR/chTERT complex contributes to the establishment and maintenance of GaHV-2 induced tumorigenesis (Trapp et al., 2006), while vTR independent of telomerase activity is implicated in metastasis (Kaufer et al., 2010). The vTR gene is regulated indirectly by Meq; the Meq/c-Jun heterodimer binds to and induces expression of the c-myc promoter, then c-Myc transactivates vTR (figure 1.25) (Levy

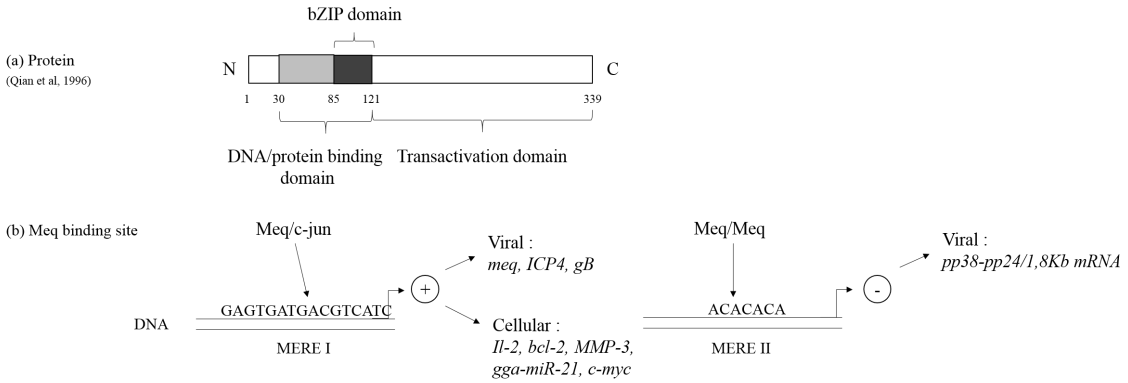

Figure 1.25: **The oncoprotein Meq is a transcriptional factor encoded by *gallid herpesvirus-2* (GaHV-2)**. A: The bZIP domain has homology with the basic leucine zipper domain (bZIP) of the cellular transcription factors c-Jun and c-Fos, which mediates dimerization. The transactivation domain is a proline-rich domain triggering either activation or repression of the promoter activity. B: Meq can form heterodimers (Meq/c-Jun) or homodimers (Meq/Meq), which bind to Meq response element I (MERE I) and MERE II, respectively. Binding of Meq/c-Jun to viral and cellular promoters promotes gene transcription, while the binding of Meq/Meq represses gene expression. ICP4 for infected cell protein 4. gB for glycoprotein B. Il-2 for interleukin 2. Bcl-2 for B cell lymphoma 2. MMP-3 for matrix metalloproteinase 3. Gga-miR-21 for chicken microRNA-21. pp38 for phosphoprotein.

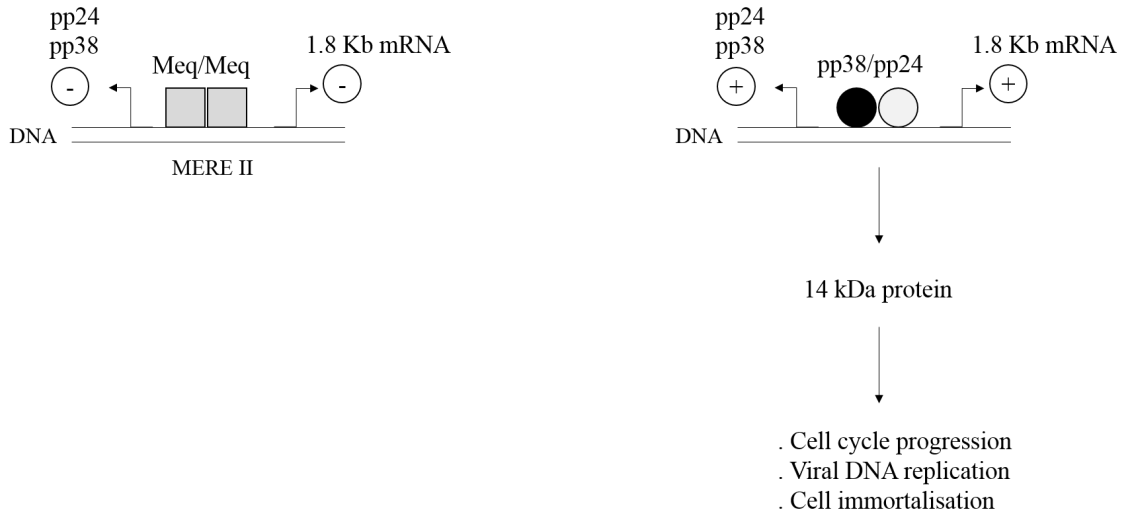

Figure 1.26: **Dual functioning of the bidirectional promoter pp38-pp24/1.8 kb mRNA by binding of viral transcription factors**. The grey squares represent the oncoprotein Meq. The black circle represents the late lytic protein pp38 and the light grey circle represents the late lytic protein pp24. + for positive regulation on the promoter activity allowing gene expression. – for negative regulation on the promoter activity generating repression of gene expression.

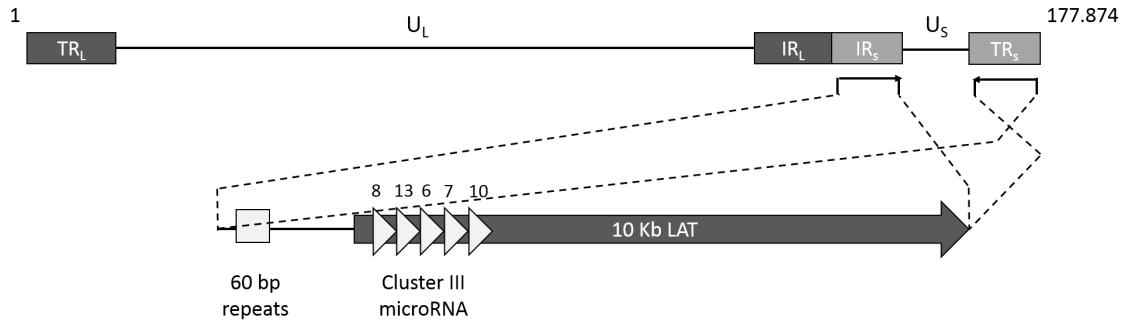

Figure 1.27: **Alignment of genomic regions containing 60 base pair (bp) repeats in the latency associated transcript (LAT) promoter in different *gallid herpesvirus type 2* (GaHV-2) strains**. White boxes represent repeat motifs of 60 nucleotides. The black box upstream represents the sequence localized between telomeres and the 60 nucleotide repeat motif. The different strains are ranged by pathotype: avirulent (a), moderate (m), virulent (v), very virulent (vv) and hypervirulent (vv+) (adapted from (Spatz and Silva, 2007)).

et al., 2005; Shkreli et al., 2007).

Transcriptional regulation by epigenetic modification

Epigenetic is the study of heritable DNA structure modification without alteration of sequence. This implies a regulation of gene expression during development and in embryonic cells (Mason et al., 2012). These modifications on genomes are involved in normal development as well as in disease like cancer or neurological disorder (Moore et al., 2013). Epigenetic marks include histone post-translational modifications (HPTMs), DNA methylation (DNAme) and the long non coding RNA recently described as triggers of the processes (Harries, 2012). Epigenetic modifications are implicated in the modulation of viral gene expression during GaHV-2 infection and contribute to the switch from latency to the lytic phase (figure 1.24). The GaHV-2 genome is methylated during latency, suggesting that methylation restricts gene expression during the latent phase (Kanamori et al., 1987). In a study of repeat regions of the GaHV-2 genome, a region encoding the lytic phase protein pp38 was more methylated than a neighboring region encoding latent phase transcripts (LATs and Meq) (Brown et al., 2012). In the same study when GaHV-2 promoters were analyzed during latency, the region containing the pp38 (lytic) promoter and the origin of replication (ori) where shown to possess restrictive marks of epigenetic modification, such as histone 3 lysine 27 trimethylation (H3K27me3) and H3K9me3, while the region containing the microRNA-M9/M4 cluster, meq and LATs (latent) promoters possessed permissive marks, such as H3K4me3 and H3K9 acetylation (H3K9Ac) Table 1.3 (Brown et al., 2012). Recently, two studies demonstrated a differential DNA methylation pattern at promoters of two immediate early genes, ICP4 and ICP27 (Strassheim et al., 2016; Rasschaert et al., 2018). Each of these viral genes possesses two alternative promoters: a proximal and a distal one. The research was undertaken in three cell types representing the productive, the latent and the reactivation phase. For ICP4, the distal promoter is associated with its expression during the productive and the latent phases, whereas the proximal promoter is associated with its expression during the reactivation phase. Both promoters are regulated by DNA methylation during the viral life cycle and are hypermethylated during latency (Rasschaert et al., 2018). Description of the ICP27 transcripts showed that this gene transcribed either by its own promoter (pICP27) or by the glycoprotein K promoter (pgK). The pgK can generate a spliced ICP27 transcript giving an N-terminal-deleted ICP27 isoform (ICP27delatN). The pICP27 is essentially active during the productive phase and is associated with a low level of DNA methylation (Strassheim et al., 2016). The alternative promoter, pgK, preferentially generates the gK transcript during the productive phase and the ICP27deltaN during the latent phase. DNA methylation analysis showed that pgK was systematically demethylated (Strassheim et al., 2016). Altogether these data indicate that DNAme and HPTMs may play a role in the restriction of specific genes during the GaHV-2 replication cycle, but further investigation is needed.

Epigenetic changes play a role in regulating DNA replication and virus reactivation in other herpesviruses (Table 1.3). Only HPTMs have been found during infection with the alphaherpesviruses human herpesvirus-1 (HHV-1, herpes simplex virus type 1) and HHV-3 (varicella-zoster virus) (Liang et al., 2009) while both DNA methylation (DNAme) and histone post-translational modifications (HPTMs) have been observed during HHV-4 and HHV-8 (Epstein–Barr virus and Kaposi's sarcoma associated virus; gammaherpesvirus) infections (Table 1.3) (Murata and Tsurumi, 2013, 2014; Kuss-duerkop et al., 2018). HHV-8 induces different cancers such as kaposi's sarcoma, primary effusion lymphomas (PEL) and multicentric castleman disease. Oncogenesis processes were observed in endothelial cells and in B cells with activation of angiogenesis by viral miRNAs and oncoproteins such as Latency associated nuclear antigen (LANA) (Purushothaman et al., 2016). LANA is expressed in HHV-8 positive cancer and is known to induce angiogenesis and activates host cell cycle by degrading p53 and by stabilizing c-myc (Wei et al., 2016). LANA was observed to interact with DNMTs and recruit DNMT3A at host chromatin (Shamay et al., 2006) inducing hypermethylation at specific host promoters such as transforming growth factor β (TGF-β) type II receptor (TβRII). This hypermethylation inhibits TβRII expression and lead to KSHV-induced PEL development (Bartolo et al., 2008). The TGF-β pathway is implicated in development, cell proliferation, differentiation, apoptosis and homeostasis (Mishra et al., 2007; Massagué et al., 2000). Deregulation of this pathway may lead to cancer development and progression (Colak and Dijke, 2017). Moreover, another viral protein of HHV8 (viral interleukin-6 (vIL-6)) enhances DNMT1 expression triggering global hypermethylation in endothelial cells leading to cell proliferation and migration (Wu et al., 2014). Epigenetic changes were also observed on latent and lytic genes during viral life cycle of HHV-8 (Table 1.3) (Günther and Grundhoff, 2010; Purushothaman et al., 2016). During latency, negative marks such as hypermethylation and H3K27me3 were observed at promoters of lytic genes (Purushothaman et al., 2016). An example, is the repression by hypermethylation of the immediate early gene promoter RTA during latency. The viral protein LANA was shown to recruit EZH2, a methyltransferase which triggers H3K27me3, on lytic promoter during latency (Purushothaman et al., 2016).

HHV-4 induces different B cell lymphomas (Burkitt's and Hodgkin's lymphomas) but also carcinomas of nasopharynx (NPC) and stomach (Li et al., 2016). Several miRNAs and proteins were shown to enhance B cells and epithelial cells transformation such as latent membrane proteins (LMP) 1 and 2 which activate oncogenic pathways (Raab-traub, 2012). EBV possesses three latency stages and according to these stages different DNMTs are overexpressed. In EBV associated gastric cancer (EBVaGC) latency I, LMP2A transactivates DNMT1 and DNMT3B (Hino et al., 2009). In EBV associated NPC (latency II), LMP1 enhances transcription of DNMT1, DNMT3A and DNMT3B (Tsai et al., 2006). In hodgkin's lymphoma cell lines (latency II) as well as in lymphoblastoid cell lines derived from EBV positive germinal center B cells (latency III), DNMT3A is overexpressed but the viral protein implicated in this overexpression was not identified (Leonard et al., 2011). These overexpression of DNMTs lead to hypermethylation of specific host promoter such as IFN regulatory factor 5 (IRF5) in Burkitt's lymphoma and EBVaGC (Yamashita et al., 2010). A repression of IRF5 was also observed in breast cancer tissues. The repression of IRF5 due to EBV mediated methylation gives a role in host immune invasion during viral persistence and oncogenesis. A summary of epigenetic changes of these two gammaherpesviruses (HHV4 and 8) is related in Table 1.3.

The alteration of epigenetic mechanisms induced by DNA viruses are still an understudied field. Proteins implicated in these aberrant epigenetic patterns are not fully known. The results presented above suggest that DNA viruses trigger repression of host gene expression by epigenetic marks in order to facilitate persistent viral infection and to promote virus-induced cancer progression. Viruses are able to hijack different epigenetic mechanisms (DNA methylation and HPTMs) at specific host promoters. Many questions remain about how viruses interfere with host epigenetic machinery.

Table 1.3: **Epigenetic overview of alpha and gamma-herpesviruses**

		Latency phase	**Lytic phase**	**Example**	**reference**
Alphaherpesviruses					
HHV-1	Lytic promoter	H3K9me3 (-)	H3K4me3 (+)	ICP8, ICP4, ICP27	(Liang et al., 2009)
		H3K27me3	H3K9Ac (+)	ICP0	(Nicoll et al., 2012)
		H3K9me2 (-)	H3K14Ac (+)		
	Latent promoter	H3K9Ac (+)		LATs	(Nicoll et al., 2012)
		H3K14Ac (+)			
HHV-3	Lytic promoter	H3K9me3 (-)	H3K4me3 (+)	ICP0	(Liang et al., 2009)
GaHV-2	Lytic promoter	H3K9me3 (-)		pp38, orilytic	(Brown et al., 2012)
		H3K27me3 (-)			
		DNAme			
	Latent promoter	H3K4me3 (+)		Cluster microRNA	(Brown et al., 2012)
		H3K9Ac (+)		Meq, LATs	
Gammaherpesviruses					
HHV-4	Lytic promoter	H3K27me3 (-)	H3K4Ac (+)	BZLF1 (Zta)	(Murata and Tsurumi, 2013)
		H3K9me3 (-)	H3K9Ac (+)		(Murata and Tsurumi, 2014)
			H3K4me3		
HHV-8	Lytic promoter	H3K27me3 (-)	H3K4me3 (-)	ORF50 (Rta)	(Günther and Grundhoff, 2010)
		DNAme	H3K9Ac (+)		
			H3K14Ac (+)		
	Latent promoter	H3K4me3 (+)		Cluster microRNA	(Günther and Grundhoff, 2010)
		H3K9Ac (+)			
		H3K14Ac (+)			

Table notes: HHV for human herpesvirus. GaHV for *gallid herpesvirus*. ICP for infected cell protein. LATs, latency associated transcripts. pp38 for phosphoprotein 38. BZLF1 for Epstein–Barr virus (EBV) immediate-early protein. ORF for open reading frame.

1.5.4 Early post-transcriptional modification: splicing

In GaHV-2, an immediate early protein, ICP27, prevents the splicing of a viral (viral interleukin 8, vIL-8) and a cellular (chicken telomerase reverse transcriptase, chTERT) mRNA by interaction with serine/arginine dipeptide rich (SR) proteins, which promote splicing by regulating splice site selection and spliceosome assembly (Table 1.4) (Amor et al., 2011). Repression of chTERT splicing might have an effect during the lytic and reactivation stages, when chTERT activity is low (Amor et al., 2011). Deletion of vIL-8 impairs tumor formation in GaHV-2 infected chickens (Engel et al., 2012). vIL-8 is able to bind and attract B cells (the main target during the lytic phase) and T cells (the main target during latency and transformation) (Engel et al., 2012; Haertle et al., 2017). Inhibition of vIL-8 may slows down the GaHV-2 replicative cycle. Different transcript isoforms have been observed during the latent and lytic phases of GaHV-2. During latency, the cluster miR-M9-M4 forms a long spliced transcript containing the sequences encoding Meq and vIL-8 (Coupeau et al., 2012). During the lytic phase, miR-M9-M4, Meq and vIL-8 are expressed as three independent and unspliced transcripts (Coupeau et al., 2012). vIL-8 is also encoded by other splice variants; two splice variants containing exon II and III of vIL-8 with either RLORF5a or RLORF4 were observed 4 days post-infection (Jarosinski and Schat, 2007).
Numerous herpesviruses modulate the splicing of viral and/or cellular pre-mRNA, positively or negatively, allowing the viruses to switch from latent to lytic phases and to escape from the immune system (Table 1.4). These herpesviruses are mainly alphaherpesviruses (HHV-1 and HHV-2) and gammaherpesviruses (HHV-4 and HHV-8) (Hardwicket and Sandri-goldin, 1994; Hardy and Sandri-Goldin, 1994; Ruvolo et al., 2004; Majerciak et al., 2008; Sedlackova et al., 2008; Nojima et al., 2009; Amor et al., 2011; Tang et al., 2013).

Table 1.4: **Splicing event modulation occurring during herpesvirus infection**

Herpesvirus Species*	**Viral factors influencing the splicing (+ or -)****	**mRNA target and splicing**	**Function**	**Reference**
Alphaherpesviruses				
HHV-1	ICP27 (-)	**Cellular:** β-actin and β-globin		(Hardwicket and Sandri-goldin, 1994)
		Viral: ICP0, ICP22, ICP47, UL45	Establishment and/or maintenance of latency	(Hardy and Sandri-Goldin, 1994)
		and gC (cell associated from (US) and soluble (S))	Protection of virus and host cell from host complement	(Sedlackova et al., 2008)
HHV-2	ICP27 (-)	**Cellular:**PML (PML III (US) and PML V (S))	Persistence of the virus in the cells	(Nojima et al., 2009)
		Viral:ICP34.5 (ICP34.5β (US) and ICP34.5α (S))	Role in neuro-virulence and cell survival	(Tang et al., 2013)
GaHV-2	ICP27 (-)	**Cellular:**chTERT	Impact during the lytic and reactivation stage	(Shkreli et al., 2007)
		vIL8		(Amor et al., 2011)
Gammaherpesviruses				
HHV-4	SM (-)	**Cellular:**hGH		(Ruvolo et al., 2004)
HHV-8	ORF57 (+)	**Viral:**ORF50	DNA replication	(Majerciak et al., 2008)
		K8α (S) and K8β (US)	Switch latent to lytic phase	

Table notes: HHV for human herpesvirus. GaHV for *gallid herpesvirus*. PML for promyelocytic leukaemia. hGH for human growth hormone. S for spliced isoform. US for unspliced isoform. + for splicing is promoted. − for splicing is inhibited.

1.5.5 Late post-transcriptional modification: microRNAs

Twenty-five mature miRNAs from 13 pre-miRNAs have been identified in GaHV-2 (Burnside et al., 2006, 2008; Yao et al., 2009; Muylkens et al., 2010; Stik et al., 2014). These 25 mature miRNAs are expressed from three clusters located in repeat regions (IR_L/TR_L and IR_S /TR_S). Cluster I (MDV-1-miR-M9-M4) is located upstream of the oncogene meq, while cluster II (MDV-1-miR-M11-M1) is located downstream of meq. Cluster I is composed of six pre-miRNAs (miR-M9, miR-M5, miR-M12, miR-M3, miR-M2 and miR-M4), while cluster II is composed of three pre-miRs (miR-M11, miR-M31 and miR-M1) (Burnside et al., 2008). Cluster III (MDV-1-miR-M8-M10), located in short repeat regions (IR_S/TR_S), is situated in the first intron of the LATs gene. Most of GaHV-2 miRs are overexpressed during virus induced oncogenesis and in transformed T cell lines (Stik et al., 2014).

Functional GaHV-2 microRNAs in the regulation of the cell cycle

The MDV-1-miR-M4-5p (cluster I) exhibits the highest expression during GaHV-2 lymphomagenesis (Muylkens et al., 2010). This miRNA is implicated in GaHV-2 lymphomagenesis (Zhao et al., 2009; Muylkens et al., 2010; Parnas et al., 2014; Yao and Nair, 2014). MDV-1-miR-M4-5p has the same seed region as miR-155, an oncogenic cellular miRNA (gga-miR-155) (Zhao et al., 2009; Muylkens et al., 2010). Expression of miR-155 has been associated with several human cancers (Yao and Nair, 2014). In GaHV-2 transformed cell lines and GaHV-2 induced lymphoma, gga-miR-155 is downregulated, while MDV-1-miR-M4-5p is upregulated (Muylkens et al., 2010); MDV-1-miR-M4-5p may substitute for gga-miR-155 during latent infection with GaHV-2. A common cellular target of these orthologous miRNAs is the histone methyltransferase jumonji AT rich interactive domain 2 (*jarid2*), which promotes apoptosis; inhibition of *jarid2* by MDV-1-miR-M4-5p may promote the survival and proliferation of GaHV-2 latently infected cells, thus enhancing tumorigenesis (figure 1.28) (Parnas et al., 2014).

Three other miRNAs from cluster I (MDV-1-miR-M3-5p, MDV-1-miR-M2-3p and MDV-1-miR-M9-5p) target cellular mRNA during GaHV-2 infection. MDV-1-miR-M3-5p targets *smad2*, which promotes apoptosis (Xu et al., 2011); inhibition of *smad2* may promote the survival of GaHV-2 latently infected cells and the formation of tumors (figure 1.28). MDV-1-miR-M2-3p and miR-M9-5p target the *IL-18* mRNA (Parnas et al., 2014); *IL-18* is a pro-inflammatory cytokine stimulating interferon γ production by T cells and inhibition of this cytokine may allow the virus to escape from immune surveillance (figure 1.28).

Functional GaHV-2 microRNAs in the regulation of the viral replication cycle

Pre-MDV-1-miR-M4 generates two mature miRNAs (MDV-1- miR-M4-5p and MDV-1-miR-M4-3p), which target viral proteins, inhibiting expression of *ul28* and *ul32*, respectively (Muylkens et al., 2010) (figure 1.28); UL28 and UL32 are implicated in viral DNA cleavage and packaging, so their inhibition may help to maintain latency. MDV-1-miR-M7-5p (cluster III) targets the coding region of two viral immediate early (IE) mRNAs, *icp27* and *icp4* (Strassheim et al., 2012). Along with ICP22, ICP27 and ICP4 are the first proteins expressed during lytic infection; they trigger expression of early (E) and late viral genes (Strassheim et al., 2012). Overexpression of this miRNA may promote establishment of the latent phase.

Altered regulation of cellular microRNAs

Expression of some cellular miRNAs is modulated during GaHV-2 infection and may play a role in tumorigenesis (Table 1.5) (Yao et al., 2009; Stik et al., 2013; Tian et al., 2012). These three studies assessed the over- or under-expression of several cellular miRNAs in *in vivo* and *in vitro* samples representing the tumorigenesis phase of the viral life cycle. In these three studies, miR-126 was found repressed. This repression will

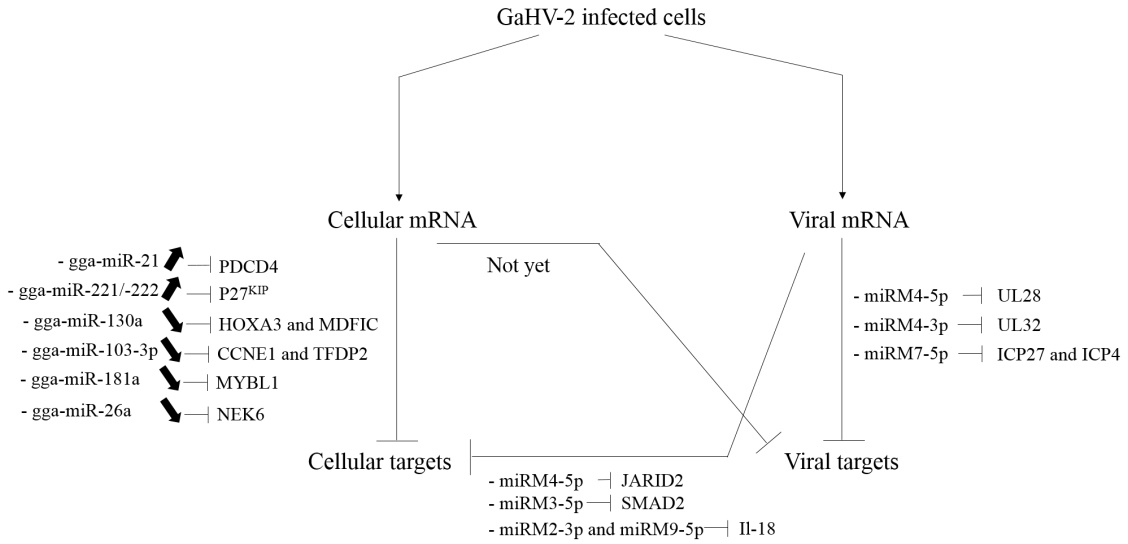

Figure 1.28: **Regulation of cellular and viral targets by RNA interference during *gallid herpesvirus type 2* (GaHV-2) infection**. $--> $ Increased miR expression. $--|$ Repression of expression.

be characterized in the context of this PhD program and the role of this cellular miRNA during GaHV-2-induced lymphomagenesis will be addressed. The miRNAs gga-miR-21 and gga-miR-221/miR-222 are expressed at high levels during GaHV-2 latency and tumorigenesis (Lambeth et al., 2009; Stik et al., 2013). These miRNAs inhibit expression of a regulator of the cell cycle, p27Kip1 (Lambeth et al., 2009), a cyclin dependent kinase inhibitor which controls the transition from G0 to G1 phases of the cell cycle; inhibition of P27Kip1 promotes cell proliferation and growth (figure 1.28). Inhibition of PDCD4 by gga-miR-21 may promote survival and proliferation of GaHV-2 infected cells (Stik et al., 2013).

The miRNAs gga-miR-130a, gga-miR-103-3p, gga-miR-181a, gga-miR-26a and miR-126 are repressed during GaHV-2 tumorigenesis (Han et al., 2016a,b; Lian et al., 2015; Li et al., 2014b; Stik et al., 2013). All these miRNAs have a potential role as tumor suppressor with an impact on cell proliferation and migration. The repression of gga-miR-130a allows the expression of the protein homeobox A3 (HOXA3) and MyoD family inhibitor domain containing (MDFIC) implicated in cell proliferation and migration (Han et al., 2016b). HOXA3 is a DNA binding transcription factor which is part of a cluster of homeobox genes. This transcription factor was shown to regulate gene expression, morphogenesis, differentiation, cell proliferation and migration (Chisaka and Kameda, 2005; Mace et al., 2005). MDFIC is a member of the MyoD family protein characterized by a specific cysteine-rich C-terminal domain. It participates to cellular processes by modulating the Wnt and c-jun N-terminal kinase pathways (Kusano and Raab-traub, 2002). Gga-miR-103-3p was shown to target cyclin E1 (CCNE1) and the transcription factor E2F dimerization partner 2 (DP-2) (TFDP2). These proteins are implicated in cell cycle by allowing transition G1/S phase during the cell cycle (Ekholm and Reed, 2000; Mazumder et al., 2004) and activation of cell cycle regulated genes, respectively. Despite the fact that these two proteins are implicated in cell cycle regulation, repression of gga-miR-103-3p promotes cell migration (Han et al., 2016a). The two last miRNAs, gga-miR-181a and gga-miR-26a, were shown to be implicated in cell proliferation by targeting MYBL1 and NIMA related kinase 6 (NEK6), respectively (Lian et al., 2015; Li et al., 2014b). MYBL1 is part of the Myb proto-oncogene family and is a transcription factor which transactivates promoter by recognition of a specific sequence (YAACG/TG). It has a role in cell proliferation, in B cell differentiation and cell lymphoma transformation (Lian et al., 2015). NEK6 is a mitotic kinase belonging to the NEK family. It contributes at establishing the microtubule-based mitotic spindle and controls cell proliferation and survival (Li et al., 2014b).

In human, other herpesviruses from gammaherpesvirus subfamily (HHV-4 and HHV-8), able to induce tumorigenesis, were shown to also modulate miRNA expression during infection (Fiorucci et al., 2015).

Table 1.5: **Cellular microRNAs found over- or under-expressed during GaHV-2-induced tumorigenesis**

	Stik *et al*,2013	Tian *et al*,2012	Yao *et al*,2009
Cellular miRNAs repressed			
miR-126	0.4	1.8	0.5
miR-26a	0	1.4	1
miR-155	0	ND	0.3
miR-181a	0.5	0.5	ND
miR-223	0.25	1.6	0.2
Cellular miRNAs overexpressed			
miR-146a	ND	0.4	ND
miR-146b	0	ND	3
miR-146c	5	ND	ND
miR-21	2	0.6	ND
miR-221	3.5	1.7	4

Table notes: ND stands for not determined. Numbers represent the factor of overexpression or repression evaluated in the three different studies.

As mentioned previously, HHV-4 triggers the formation of different cancers. In EBV-positive Burkitt's lymphoma miR-28 is repressed contributing to lymphomagenesis (Fiorucci et al., 2015). In NPC, miR-204 was shown repressed while miR-155 was shown overexpressed. The repression of miR-204 is associated to the most aggressive and poor prognostic phenotype of NPC (Ma et al., 2014). Moreover, this miRNA was found to directly target cell division cycle 42 (*cdc42*) contributing to the inhibition of cell invasion and metastasis (Ma et al., 2014). The overexpression of miR-155 stimulates the ability of NPC to control cell proliferation, cell migration and invasion (Zhu et al., 2014).
On the other hand, HHV-8 induces several cancers. Little is known about the impact of cellular microRNAs on these cancers. Several miRNAs were observed to be repressed (miR-125b-1-3p and miR-1183) while others were observed to be overexpressed (miR-126-3p, miR-199a-3p and miR-16-5p) in Kaposi's sarcoma samples compare to adjacent healthy tissues (Wu et al., 2014). The deregulation of these miRNAs could have a crucial role in the progression of Kaposi's sarcoma.

The analysis of functional mRNA target of miRNAs helps to understand mechanisms implicated in virus infection, replication and tumorigenesis. The modulation of viral and cellular miRNA expression helps the virus to escape from immune system, maintain latency, transformation of latently infected cells and propagated tumoral cells into organisms.

1.5.6 Post-translational modifications

Little is known about post-translational modifications during GaHV-2 infection. The capsid precursor (pro-capsid) is composed of four proteins surrounding an internal scaffold (Laurent et al., 2007). This scaffold is composed of two proteins, UL26 and UL26A, which are processed from a common precursor with protease activity at two cleavage sites. The precursor first undergoes autocleavage at the R site, releasing the N-terminal protease domain, which cleaves at the M site, releasing a 25 amino acid segment promoting the attachment of the scaffold to the capsid.
The glycoprotein gB represents the major component of the GaHV-2 viral envelope and is composed of three glycoproteins (Yoshida et al., 1994); gp100 is formed by the homodimer pr44/pr44 (pr88) and is glycosylated (figure 1.29 A, B) , while gp60 and gp49 are formed after cleavage of gp100 (figure 1.29 C) and may play a role in immune evasion (Sithole et al., 1988). Glycoprotein C (gC) is the major antigenic protein of GaHV-2 (Isfort et al., 1986); it is secreted from infected cells and may play a role in immune evasion.

A 47 kDa precursor protein, pr47, is cleaved to another precursor, pr44 (44 kDa), which undergoes N-glycosylation, leading to the formation of two mature proteins, gp57 (57 kDa) and gp61 (61 kDa), differing in the addition of a sugar residue (figure 1.29 D, E) (Isfort et al., 1986). These post-translational modifications support correct scaffolding of the capsid and may influence the immune response to GaHV-2.

1.5.7 Conclusion

Cancers are characterized by six hallmarks (figure 1.30); during GaHV-2 infection, numerous viral proteins and transcripts contribute to lymphomagenesis by acting on at least four of these hallmarks. Regulation of viral and cellular gene expression during GaHV-2 infection is a complex process that is not fully understood; several major questions remain unanswered: (1) what are the factors controlling the switch from latency to the lytic phase; (2) what is the contribution of latency to transformation; and (3) what are the stimuli for and events involved in reactivation? Further studies are necessary to investigate all these processes.

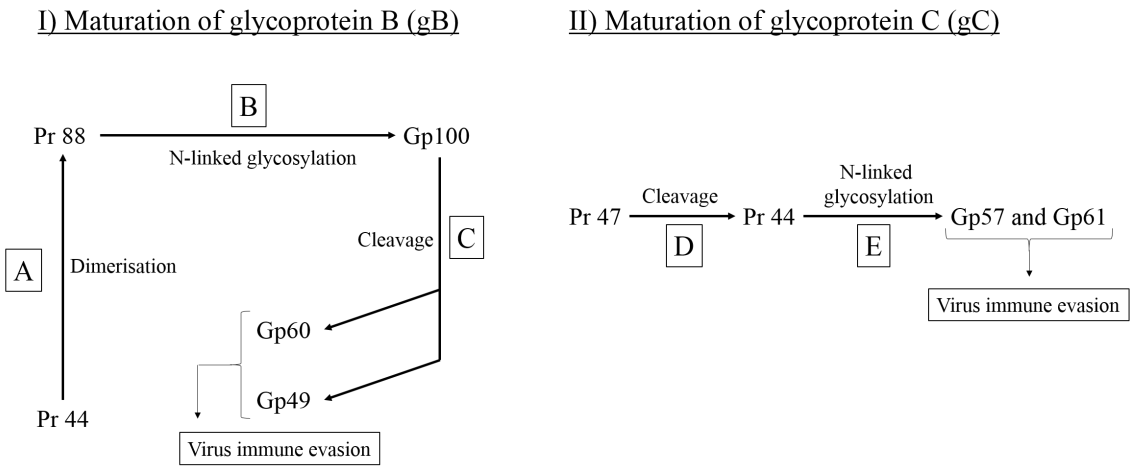

Figure 1.29: **Maturation of *gallid herpesvirus type 2* (GaHV-2) glycoproteins**. (I) GaHV-2 glycoprotein B complex contains two functional glycoproteins (gp60 and gp49). These are formed from the precursor pr44, which homodimerizes into another precursor, pr88 (A). This precursor undergoes N-linked glycosylation (B) to give the glycoprotein gp100, which is cleaved into the two mature glycoproteins gp60 and gp49 (C). (II) GaHV-2 glycoprotein C can have molecular weights of 57 and 61 kDa (gp57 and gp61, respectively), which can be explained by the addition or removal of a sugar group on this glycoprotein. The maturation begins with the cleavage of the precursor pr47, pr44 (D), which is then N-linked-glycosylated to produce gp57 and gp61 (E) (Isfort et al., 1986).

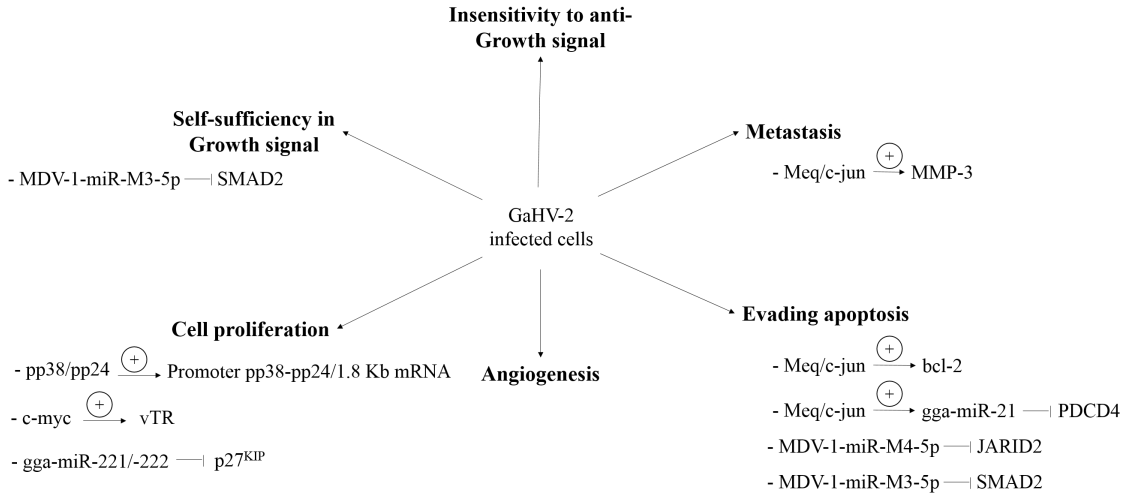

Figure 1.30: **The six hallmarks of cancer and mechanisms used by *gallid herpesvirus-2* (GaHV-2) to induce cancer**. + for positive transcriptional regulation. Repression of gene expression. B cell lymphoma 2 (Bcl-2) is an anti-apoptotic protein. Programmed cell death protein 4 (PDCD4), mothers against decapentaplegic homologue 2 (SMDA2) and Bcl-2l13, a member of the Bcl2 family, are pro-apoptotic proteins. Matrix metalloprotease-3 (MMP-3) is implicated in the degradation of the extracellular matrix. vTR is the viral RNA telomerase subunit. P27KIP1 is a cyclin dependent kinase (CDK) inhibitor and controls passage from the G0 to G1 phase.

1.6 microRNA-126

1.6.1 Host gene

In human cells, the host gene of miR-126 is the epidermal growth factor like-7 (*egfl-7*). This gene possesses 10 exons and the pre-miRNA-126 is localized in the seventh intron into a cpG island (Saito et al., 2009). EGFL-7 was shown to be mainly expressed in endothelial cells (Nikoli et al., 2013; Nikolic et al., 2010; Schmidt et al., 2007). It was also observed to be expressed in primordial germ cells (Campagnolo et al., 2008) and neurons (Nikolic et al., 2010) indicating that EGFL-7 has several biological functions and not only in vascular system.

At transcriptional level, it was found that *egfl-7* possesses three alternative isoforms transcribed from three separate promoters (figure 1.31) (Saito et al., 2009). A long, a short and an alternative transcripts. The long transcript covers the *egfl-7* full length coding region, the short transcript begins in the seventh exon (just upstream the pre-miR-126 location) and the alternative transcript begins from an alternative exon localized in the CpG island of intron 2 and cover the coding region of the gene (Saito et al., 2009).

EGFL-7 protein is secreted in the extracellular matrix (ECM) at the surface of endothelial cells via the interaction with a component of the ECM named $\alpha_V \beta_3$ integrin (Nikoli et al., 2013). This protein possesses five different domains (Nikolic et al., 2010). From N-terminal to C-terminal, the first domain consists on a signal peptide, followed by an emilin like domain (EMI). This domain is a cysteine rich repetitive element often detected in ECM proteins. Close to EMI domain, two EGF-like domains are present giving the name of the protein. The last element is the coiled coil domain binding Ca2+ (Nikolic et al., 2010).

Box1: definition
Vasculogenesis: refers to the differentiation of endothelial precursor cells, or angioblasts, into endothelial cells and the *de novo* formation of a primitive vascular network.
Angiogenesis: refers to the growth of new capillaries from preexisting blood vessels either via sprouting or intussusception (new blood vessel is created by splitting of an existing blood vessel in two).

EGFL-7 is implicated mainly in the vasculogenesis and to a less extent to the angiogenesis process (definition of vasculogenesis and angiogenesis in box 1) (Nikolic et al., 2010; Kuhnert et al., 2008). A deletion of EGFL-7 in a model of zebrafish embryo led to the incapacity of endothelial cells to form a tube with a lumen (Parker et al., 2004; Strilic et al., 2009). During vasculogenesis, EGFL-7 is assumed to create an environment which facilitates the local motility of endothelial cells during tube formation. Nevertheless, this hypothesis is controversial. A study showed a positive impact of EGFL-7 on endothelial cells migration in mouse (Campagnolo et al., 2005), while others did not observe migration of endothelial cells (HUVEC)

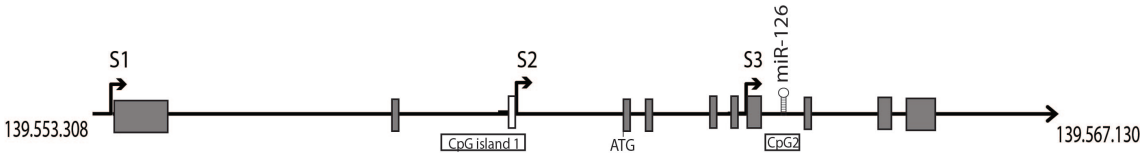

Figure 1.31: **Human epidermal growth factor like-7 (*egfl-7*) alternative transcript**. Black bars represent intron. Grey boxes represent exons. The white box represents an alternative exon. S1, S2 and S3 are the three alternative transcriptional start sites of the human *egfl-7* gene. The ATG represents the start codon. (adapted from (Saito et al., 2009)).

with EGFL-7 alone or in combination with some ECM proteins (Soncin et al., 2003; Parker et al., 2004; Schmidt et al., 2007). During angiogenesis, it was found that EGFL-7 supports weak adhesion between endothelial cells (Parker et al., 2004) and creates an environment where cells attach and detach easily from each other until they acquire their correct position (Nikolic et al., 2010). In mice, if EGFL-7 is deleted there is formation of an oversized sprout leading to a loss of endothelial cells migration and finally to a delayed vascularization in the animal. Finally, EGFL-7 was shown to be a negative regulator of vascular elastogenesis (Lelièvre et al., 2008). EGFL-7 inhibits the enzyme lysyl oxidase 2 (LOXL2) implicated in the deposition of elastin. Hence, EGFL-7 may be implicated in the shaping of the ECM affecting indirectly the migration of endothelial cells.

EGFL-7 expression is important during the development of the vascular system of the embryo. Hence, EGFL-7 is overexpressed at the embryonic and neonatal stages. Then, EGFL-7 is repressed in mature blood vessels. EGFL-7 may be expressed again in adult either during a wound requiring the renewal of blood vessels or during tumorigenesis. However, EGFL-7 is still expressed in highly proliferative tissues (Schmidt et al., 2007).

1.6.2 Regulation of EGFL-7 and miR-126 expression

Like EGFL-7, miR-126 was shown to be highly expressed in endothelial cells (Harris et al., 2010). MiR-126 expression was characterized in two studies from human model (Saito et al., 2009; Watanabe et al., 2012). The alternative transcript (with TSS located in a CpG island) of *egfl-7* was shown to be the main transcript producing miR-126 (Saito et al., 2009). According to this study, miR-126 expression was not driven by DNA methylation but by HPTMs in its promoter since a low percentage of methylation was observed in prostate primary tumor. In another context, Watanabe demonstrated the impact of DNA methylation in CpG islands on miR-126 expression (Watanabe et al., 2012). When the CpG island in the second intron was methylated, a decrease of miR-126 was detected while no impact of DNA methylation was observed in the CpG island surrounding the pre-miR-126. The hypermethylation in the second intron might be implicated in the inhibition of miR-126 expression from of the alternative transcript (Watanabe et al., 2012). Later, several studies in different human cancers such colorectal cancer, esophageal squamous cell carcinoma and glioma showed an impact of DNA methylation on miR-126 expression (Zhang et al., 2013; Liu et al., 2015b; Cui et al., 2016). Despite the result obtained by Saito, DNA methylation is most likely involved in the regulation of miR-126 expression.

The expression of miR-126 was also shown to be regulated by specific transcription factors (TF). Two response elements allowing the binding of enhancers (E26 transformation specific sequence-1 and -2 (Ets-1 and Ets-2)) were identified in human (Harris et al., 2010). Even after repression of these two TFs, a basal level of miR-126 expression remains. These results suggest that either Ets-1 and Ets-2 act in combination with other proteins or they perform a fine tuning of miR-126 expression. In another study, Krueppel-like factor 2 (KLF-2) was also identified as a transactivator of miR-126 expression in zebrafish (Nicoli et al., 2010). Monteys and collaborators demonstrated that miR-126 possesses an intronic promoter allowing partially the transcription of the short transcript (Monteys et al., 2010). In order to demonstrate that this region is a potential promoter, a plasmid containing the pre-miR-126 region alone or with the potential promoter sequence was transfected into cells that do not express miR-126. The plasmid with the pre-miR-126 sequence alone showed a low expression while the plasmid with the potential promoter sequence, presented a significant increase of miR-126 (Monteys et al., 2010). Recently, the transcription factor GATA2 was found to regulate positively miR-126 expression (Hartmann et al., 2016). *In silico* analysis was performed and putative binding sites of GATA2 were found in the alternative transcript and short transcript promoters of EGFL-7/miR-126. Nevertheless, GATA2 was found to only have an impact on miR-126 expression on its alternative transcript promoter and not on the promoter of the short transcript

just upstream pre-miR-126 sequence (Hartmann et al., 2016).

1.6.3 Functions

MiR-126 in vascular integrity

In a physiological context miR-126 is mainly expressed in endothelial cells (Fish et al., 2008). MiR-126 is expressed in highly vascularized tissues such as the heart, lungs and kidneys (Qin et al., 2012). As mentioned earlier, the main function of miR-126 is to participate in angiogenesis. Nevertheless, miR-126 takes also part in inflammation and carcinogenesis (Harris et al., 2010). Two studies showed the importance of miR-126 in vascular integrity and angiogenesis (Fish et al., 2008; Wang et al., 2008b). In zebrafish, a role of miR-126 in vascular integrity was found (Fish et al., 2008). They show that when miR-126 is deleted it leads to cranial hemorrhage. The blood vessels integrity, the endothelial tube organization were compromised. Moreover, collapsed lumen were also observed (Fish et al., 2008). The same conclusions were obtained by Wang and collaborators in mouse (Wang et al., 2008b). Knockout mice embryo (miR-126-/-) died during development or perinatally of severe edema, multifocal hemorrhages, blood vessel ruptures. These embryos dysplayed a lack of integrity in the blood vessels (Wang et al., 2008b).

Comparison of miR-126/EGFL-7 implication in angiogenesis

As mentioned earlier, EGFL-7 protein also has a function during angiogenesis and mainly during embryology. Kuhnert and collaborators found that miR-126 was more important during angiogenesis than EGFL-7 (Kuhnert et al., 2008). This team created two groups of transgenic mice. One group harbored a deletion of EGFL-7 gene but maintained the expression of miR-126 (EGFL-7-/-) and the other group harbored a deletion of miR-126 genes without affecting EGFL-7 expression (miR-126-/-). Mice EGFL-7-/- presented a high rate of viability compared to mice miR126-/-. Deletion of miR-126 led to edema formation due to a lack a vascular integrity. Since blood vessels are not fully mature, hemorrhages were observed in subcutaneous region and in the jugular vein of the embryo. In 50 % of cases, miR-126 deletion leads to death of the embryo while mice EGFL-7-/- presented almost a normal phenotype. Altogether these data indicate a more important role of miR-126 in angiogenesis in comparison to EGFL-7 in embryonic stages (Kuhnert et al., 2008).

MiR-126 targets

Targets in the context of angiogenesis The functions of miR-126 can be explained by the study of its targets. During angiogenesis, miR-126 is a regulator of the vascular endothelial growth factor (VEGF) induced-signaling pathway. When a tissue does not need formation of new blood vessels, the VEGF induced-signaling pathway is inhibited by sprouty-related protein with EHV-1 domain 1 (SPRED1) and the phosphatidylinositide 3-kinase regulatory subunit 2 (PI3KR2) also named p85β (figure 1.32) (Fish et al., 2008). When angiogenesis is required, miR-126 is expressed and targets SPRED1 and p85β preventing inhibition of VEGF signaling pathway (Fish et al., 2008). The repression of the regulatory subunit p85β is associated with the activation of the catalytic subunit named phosphatidylinositide 3-kinase (PI3K) (Sessa et al., 2012). The targeting of SPRED1 is also observed in perivascular cells (PVC) also named pericytes (Pitzler et al., 2016). PVC cover blood vessels and are implicated in their stabilization. When PVC and endothelial cells are co-cultured, an overexpression of miR-126 was observed leading to the inhibition of SPRED1 followed by the activation of the Erk1/2 signaling pathway (Fish et al., 2008) (figure 1.32). The upregulation of miR-126 was shown to enhance intercellular interactions between PVC and the endothelial cells (Pitzler et al., 2016). SPRED1 was also validated as a target of miR-126 in cultured primary chicken

hepatocytes (Wang et al., 2013). In this study, it was shown that SPRED1 was directly targeted by miR-126. In silico analysis demonstrated that the target site is conserved across species. The chicken predicted miR-126 binding site sequence is more than 90 % identical to the mouse, human, rat or rabbit. SPRED-1 is known to regulate the Ras-MAPK signaling in the liver. Some studies on Hepatocellular carcinoma (HCC) indicated the dysregulation of SPRED1 leading to an inhibition of HCC cells proliferation by decreasing MAPK activity (Han et al., 2012; Yoshida et al., 2006). Early in angiogenesis process, both VEGF and the angiopoietin 1 (Ang-1) are required to initiate capillary budding followed by stabilization of the neovessel for maturation by Ang-1 alone (Sessa et al., 2012). If VEGF is initiated in early embryonic stage alone, it would lead to an increased permeability of the vessels associated with edema. By targeting p85β, miR-126 allows the PI3K pathways to be initiated by the binding of Ang-1 to its receptor (figure 1.32) (Sessa et al., 2012).

Targets in the hematopoietic and immune contexts MiR-126 expression is also found in hematopoietic cells with a different function. It was demonstrated in zebrafish that miR-126 is an important factor implicated in the development of erythrocytes cells. Hematopoietic stem cells differentiate through the inhibition of the factor c-Myb. Two miRNAs are implicated in this inhibition : miR-126 and miR-150. A fine-tuning of both miRNAs at different developmental stages enhance the production of thrombocytes or erythrocytes. The stem cells will become erythrocytes when miR-126 is more expressed than miR-150. If the concentration of miR-126 does not increase, stem cells will differentiate into a megakaryocyte (Grabher et al., 2011).

MiR-126 was also found to have a role in the function of T CD4+ cells. T CD4+ cells play a role both in innate and adaptative immune responses. In the innate immunity, it maintains the balance of anti-inflammatory and proinflammatory responses. It was shown that miR-126 deficiency promotes the activation and the proliferation of T CD4+ *in vitro* through the targeting of insulin receptor substrate-1 (IRS-1) (Chu et al., 2017). *In vivo*, in a model of dextan sulphate sodium (DSS)-induced autoimmune colitis in mouse, a repression of miR-126 enhances this pathology with a higher number of T CD4+ cells in splenocytes and an elevated activation phenotype (Chu et al., 2017). IRS-1 is implicated in several pathways (Akt, Erk and NF-kappaB pathways) associated with cell proliferation and cell migration triggering TCD4+ proliferation (figure 1.33).

1.6.4 Tumor suppressor and pro-tumoral roles

MiR-126 was shown to be repressed in numerous human cancers (gastrointestinal cancers, cancers of endocrine glands and genital tracts and other cancers such as lung cancer and osteosarcoma) leading to the conclusion that miR-126 would have a role as tumor suppressor (Ebrahimi et al., 2014). A first example, is the repression of miR-126 in lung carcinoma (Crawford et al., 2008). In non-small cell lung cancer and in lung cancer cell lines, V-crk sarcoma virus CT10 oncogene homolog (Crk) was shown to be directly targeted by miR-126. Crk is known to have a role in signaling pathways implicated in cell adhesion, proliferation and migration (Feng et al., 2010). An overexpression of miR-126 led to a decrease level of Crk and to a repression of cell adhesion, migration and invasion (Crawford et al., 2008; Miller et al., 2003). In this cancer, miR-126 level is correlated with the survival rate of patients. Crk was also shown to be targeted by miR-126 in gastric carcinoma (Feng et al., 2010). An overexpression of miR-126 in this other cancer type leads to the same observation than in lung cancers. In both situations, miR-126 overexpression induced a decrease of cell proliferation by arrest of the cell cycle at G0/G1 stage and a decrease of cell migration and invasion *in vitro* and *in vivo* (Feng et al., 2010). In addition of targeting Crk in gastric cancer, miR-126 was shown to synergistically target PIK3R2 and polo-like kinase 2 (PLK2), an oncogene and a tumor suppressor genes respectively (Liu et al., 2014). Indeed, the targeting of a tumor suppressor gene by miR-126 in gastric cancer was also observed in 2011 by Ostubo et al (Otsubo et al., 2011). These authors showed that miR-126 was able to inhibit sex determining region Y box 2 (SOX2), giving to miR-126 a role as oncogene.

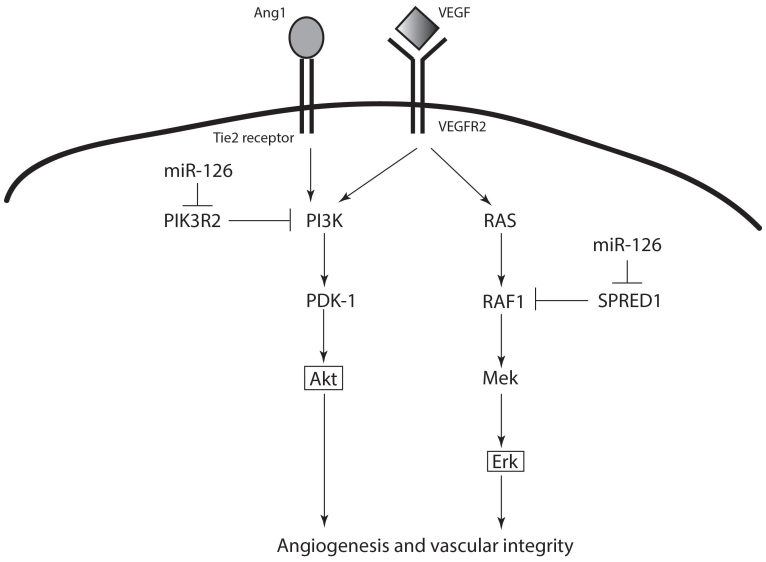

Figure 1.32: **Schematic representation of signaling pathways regulated by miR-126**. Akt and Erk signaling pathways. Ang1 for Angiopoietin 1. VEGF for vascular endothelial growth factor. VEGFR2 for VEGF receptor 2. Tie2 receptor for angiopoietin receptor. PIK3R2 for phosphatidylinositide 3-kinase regulatory subunit 2. PI3K for phosphatidylinositide 3-kinase. PDK-1 for phosphoinositide-depedent kinase-1. Akt also known as protein kinase B (PKB). RAS, RAF, Mek and Erk are part of the MAPkinase pathway. Erk for extracellular signal-regulated kinases. SPRED1 for sprouty-related protein with EHV-1 domain.

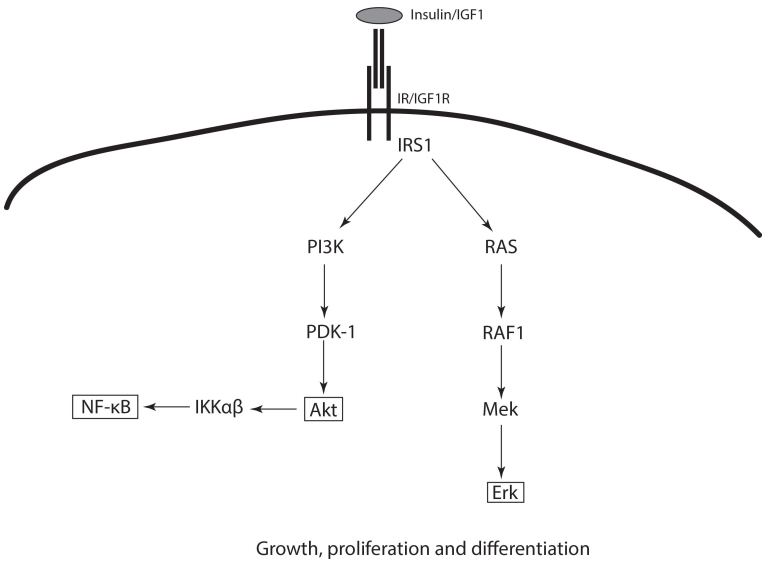

Figure 1.33: **Schematic representation of IRS1 pathway**. IGF1 for isulin-like growth factor-1. IR for isulin receptor. IGF1R for IGF1 receptor. IKK$\alpha\beta$ for Iκkinase$\alpha\beta$. NF-κB for nuclear factor kappa-light-chain-enhancer of activated B cells. PI3K for phosphatidylinositide 3-kinase. PDK-1 for phosphoinositide-depedent kinase-1. Akt also known as protein kinase B (PKB). RAS, RAF, Mek and Erk are part of the MAPkinase pathway. Erk for extracellular signal-regulated kinases. SPRED1 for sprouty-related protein with EHV-1 domain 1.

Sox2 has a role in growth inhibition via cell cycle arrest and apoptosis. The overexpression of miR-126 in some cultured and primary gastric cancer cells showed a role of miR-126 in gastric carcinogenesis (Otsubo et al., 2011).

In B- and T-lineage acute lymphoblastic leukemia (ALL), the expression level of miR-126 varied according to molecular lesions observed (Fulci et al., 2009). In leukemia with the rearrangement break cluster region/Abelson murine leukemia viral oncogene homolog 1 (BCR/ABL), an overexpression of miR-126 was observed. While cells having the molecular lesion E2A/PBX1 and mixed-lineage leukemia (MLL/AF4), showed a repressed expression of miR-126 (Fulci et al., 2009).

In acute myeloid leukemia (AML), miR-126 plays a role in cell survival *in vitro* (Li2011, article de base). They showed in two cell lines derived from acute monocytic leukemia (THP-1) and eosinophilic myelomonocytic leukemia (ME-1) an overexpression of miR-126 leading to a decrease of apoptosis. On the other hand, an inhibition of miR-126 decreases cell survival by increasing apoptosis of cancerous cells. These data shows an oncogenic role of miR-126 in AML although its tumor suppressor functions are better studied (Li2011).

All these studies suggest that miR-126 has a more complicated behavior than only a tumor suppressor gene and that miRNA function is not dependent of one target gene but rather from a competition or balance among these targets genes for a specific type of cancer (Otsubo et al., 2011; Liu et al., 2014).

1.7 Objectives

MicroRNAs (miRNAs) are small single stranded around 21-24 nucleotides long non coding RNAs processed from precursor RNAs showing a hairpin structure. MiRNAs are active in very diverse cellular processes such as differentiation, proliferation and apoptosis. Their function has been underlined in organ development and in maintaining the proper functioning of differentiated tissues. Deregulation of the normal miRNA expression play a critical role in a wide range of human and animal diseases, particularly in tumors (Pichler and Calin, 2015).

During latency, herpesvirus infection results in the establishment of a dormant state in which a highly restricted set of viral genes are expressed. This is associated with extensive methylation of CpG motifs in non-expressed viral genes. Together with these alterations of the viral genome, several host genes undergo epigenetic modifications during the latent infection. In some of the human and animal herpesvirus infections, these epigenetic dysregulations of cellular genes are involved in the development of cancer. This PhD program was carried out in an animal model of virus induced lymphoma causing the MD in chicken. This lymphoproliferative disease is the ultimate consequence of chicken infection with virulent strains of GaHV-2. This *Alphaherpesvirinae* actually shares several properties with *Gammaherpesvirinae* (such as human herpesvirus-4 and -8) which are associated with the development of tumors under specific conditions in latently infected lymphoid cells. GaHV-2 was shown to modulate the expression of several cellular miRNAs in chicken. Altered expressions of host-encoded miRNAs were analyzed *in vitro* and *in vivo* samples in several studies (Stik et al., 2013; Tian et al., 2012; Yao et al., 2009). Although only few of the cellular miRNA deregulations triggered by GaHV-2 were analyzed in depth, these studies suggested that altered expressions of host miRNAs are involved in the molecular pathways of GaHV-2 oncogenicity.

During this PhD program we focused on a host miRNA, miR-126 since several pieces of evidence suggested it might be downregulated during GaHV-2 tumorigenesis (Stik et al., 2013; Tian et al., 2012; Yao et al., 2009). Originally described as a miRNA mediating proper angiogenesis and vascular integrity, miR-126 has been

reported to impair cancer progression through signaling pathways that control tumor cell proliferation, migration, invasion and survival. MiR-126 was shown to be downregulated in several human cancers such as in oral, stomach, colon, lung, breast, cervix, bladder and prostate carcinoma (Ebrahimi et al., 2014). MiR-126 is an intronic miRNA integrated in the intron 7 of a cellular gene, the epidermal growth factor like domain 7 (*egfl-7*). In human, the latter gene is controlled by three promoters generating three transcripts, a long, an alternative and a short transcript (Saito et al., 2009). It was shown that epigenetic modifications have an impact on the alternative transcript expression by remodeling the alternative promoter (Saito et al., 2009; Monteys et al., 2010).

In this context, the core issue of this PhD program is whether miR-126 downregulation is a key event involved in GaHV-2 oncogenicity. To address this general issue, the following questions will be answered successively:

- When and to which level is miR-126 actually downexpressed during the course of GaHV-2 infection?
- What are the transcriptional features (initiation of the transcription, splicing isoforms) of the gene hosting miR-126 in chicken?
- Is miR-126 misexpression related to specific epigenetic patterns in CpG islands of chicken miR-126 host gene?
- Does miR-126 inhibition participate to the cancerous process induced by GaHV-2?
- Is it possible to counteract some of the malignant features of GaHV-2 by restoring miR-126 expression at different steps of the GaHV-2 infection?

A series of experiments will be designed to address these questions and the data obtained will be reported in three sections. In the first study, a thorough quantification method of miR-126 expression will be set up. This tool is essential to compare in the most accurate way miR-126 expression level in any situation corresponding to the different stages of GaHV-2 infection. This method relies on the use of several reference genes to normalize the expression of the gene of interest allowing to obtain reliable results.

In the second study, we will investigate the miR-126 expression level at the key steps of GaHV-2 infection (*in vitro* and *in vivo*) together with associating this expression level with DNA methylation pattern found in the host gene. The miR-126 expression level will be evaluated by using the reliable quantification method set up in the first study. In order to determine whether low miR-126 expression levels are associated with specific epigenetic signatures and with peculiar transcription profiles, DNA methylation patterns and transcriptional isoforms will be established at miR-126 genomic locus. Another objective of this study is to test the biological consequences of miR-126 silencing during MD lymphoma development. Since miR-126 repression likely plays a pivotal role in altering gene expression patterns during cell transformation, we will investigate the impact of miR-126 restoration. A strategy will be developed to overexpress miR-126 and control miRNAs in transformed CD4+ T cells propagated from MD lymphoma. To this end, we will use a conditional expression system (Tet-on inducible expression plasmids after selection and integration) that was recently used to modify gene expression in the MDV transformed cells propagated *in vitro* (Rasschaert et al., 2016). After induction of miRNA expression, cancerous cells will be characterized in order to assess cell survival and proliferation.

The objective of the third study is to interfere with miR-126 silencing during the natural course of GaHV-2 infection. To this end, a recombinant GaHV-2 will be produced in order to obtain infectious viruses that constitutively express the miRNA lost during lymphoma development. The goal of this strategy is to characterize anti-tumoral potential of miR-126 and to see whether GaHV-2 oncogenic potential might be impaired when miR-126 expression is maintained along the infection. First steps of this project will be

carried out in the present PhD program to prepare the material that will be further tested in an *in vivo* trial. Recombinant viruses expressing either wild-type or mutated versions of miR-126 will be generated from a very virulent GaHV-2 strain (RB-1B) cloned as an infectious Bacterial Artificial Chromosome (BAC). Infectious viruses will be amplified and characterized *in vitro* to evaluate if the recombinant viruses replicate to similar levels as the original strain and if this strategy is adapted to overexpress miR-126 together with viral amplification.

Chapter 2

Discussion and perspectives

Since several human viruses are associated with cancer development, animal models are needed to explore the mechanisms and processes linking viral infection and oncogenesis. This study focuses on gene regulation and functional roles of a cellular miRNA (miR-126) in an animal model of virus-induced lymphoma causing the MD in chickens. During latency, herpesvirus infection results in the establishment of a dormant state in which a highly restricted set of viral genes are expressed; this is associated with extensive methylation of CpG motifs in non-expressed viral genes. Together with these alterations of the viral genome, several host genes undergo epigenetic modifications during the latent infection. In some of the human and animal herpesvirus infections, these epigenetic dysregulations of cellular genes are involved in the development of cancer.

MD is the ultimate consequence of chicken infection with virulent strains of GaHV-2 and is a unique natural animal model for herpesvirus-induced lymphomagenesis. Several properties of MD lymphoma illustrate the relevance of the animal model. (i) MD lymphoma cells overexpress the CD30 antigen, a member of tumor necrosis factor receptor II family. CD30 overexpression (CD30hi) is a conserved signature found in neoplastic transformation in human and chicken lymphomas of different etiologies (Burgess et al., 2004). (ii) GaHV-2 expresses a long non coding RNA, the vTR (viral Telomerase RNA), a functional component of the telomerase complex that promotes MDV induced lymphomagenesis (Kaufer et al., 2011; Chbab et al., 2010). As a reminder, telomerase reactivation is associated with 85 % of the human malignancies. (iii) The modulation of gene expression observed in MD induced lymphoma shows the high adaptation level of the virus to the cell transcriptional network either through the cooperation with cellular TF or through the expression of viral TF to modulate viral and cellular gene expression. Functional examples of this crosstalk between cellular and viral transcriptional components were recently reviewed by Gennart and collaborators (Gennart et al., 2015). (iv) During oncogenesis, GaHV-2 regulates viral and host gene expression by inhibiting gene translation through a large set of viral miRNAs. In addition, GaHV-2 modulates the expression of several host miRNAs as described for the upregulated chicken miRNA, miR-21 and the cluster miR-221/ -222 and for the down-regulated chicken miRNAs miR-26a (Stik et al., 2013; Lambeth et al., 2009; Li et al., 2014b). In this context of host miRNAs dysregulated during GaHV-2 infection, this piece of work focused on miR-126 to better understand the mechanisms that control the expression of this host miRNA and to address the consequences of its downregulation during MD.

The salient findings reported in this PhD thesis are :

- The setting up of thorough quantification method permitting the most accurate assessment of miR-126 expression in different biological contexts.
- The demonstration of the repression of miR-126 during the course of GaHV-2 infection and more precisely during the lymphomagenesis phase.
- The association of miR-126 repression with hypermethylation in CpG islands of chicken miR-126 host gene (*egfl-7*).
- A preliminary functional analysis showing that miR-126 inhibition might participate to the cancerous process induced by GaHV-2 with a role in cell proliferation.
- The development of an original strategy to overexpress miR-126 during GaHV-2 infection by generating a recombinant GaHV-2 possessing a functional miR-126 expression cassette.

Previously, several studies demonstrated the repression of miR-126 in the context of MD (Stik et al., 2013; Tian et al., 2012; Yao et al., 2009). These studies limited their quantification analysis in *in vitro* and *in vivo* samples representing the lymphomagenesis phase of GaHV-2. During the PhD program, miR-126 expression pattern was investigated in all the key steps of GaHV-2 infection. Moreover, these quantifications were performed with the use of a thorough quantification method using several reference genes to ensure

reliable results.

In MD, little is known about how GaHV-2 modulates and regulates cellular miRNAs during the course of the disease. The use of epigenetic to deregulated cellular genes, the identification of alternative transcripts potentially implicated in gene expression regulation and functional analysis results obtained in this study brought new insights on miR-126 regulation during GaHV-2 induced lymphomagenesis.

Set up of a thorough quantification method

The study of viruses brought detailed understanding of the system biology inherent in virus-host interactions (Bernard N Fields, 2007). Cellular gene expression modulation during viral infection is widely studied in order to assess how viruses may persist in their hosts, how do they escape from the immune system and, for some viruses, how do they induce oncogenesis. In order to answer these questions animal experimental models are used. Most of the time, gene quantification is normalized with only one Reference Gene (RG) without preliminary test leading to misinterpretation of data. In this study it was demonstrated that according to the RG used data interpretation may lead to different conclusions. The use of several RGs for gene normalization is then crucial to obtain reliable results with a biological meaning especially if subtle changes in gene expression are expected.

To properly assess miR-126 expression levels, quantitative reverse-transcription PCR (qRT-PCR) was developped. This technique is evidencing advantages over other methods such as sensitivity, dynamic range and capacity for multiplexing (Huggett et al., 2005). However, the data analysis from qRT-PCR is challenging. The accuracy of the results is largely dependent on proper data normalization. As numerous variables inherent to a qRT-PCR experiment need to be controlled in order to differenciate experimentally induced variations from true biological changes. To limit these technical variations, several internal controls must be used as reference genes (RGs). In this study, six different usually used RGs (*gapdh*, β*-actin*, *sdha*, *18Sr-RNA*, *hmbs* and *U6*) were assessed in the different samples to normalize miR-126. These candidates belong to different functional classes, reducing the possibility of confounding co-regulation. Nevertheless, in literature, it is recommended to use other small non-coding RNAs for normalization of a microRNA since their biogenesis is similar (Mestdagh et al., 2009; Cassol et al., 2016; Li et al., 2014a). These include both small non-coding RNA such as U6 that is extensively used and small nucleolar RNAs. In human, several miRNAs were considered to be stably expressed (let-7a, miR-26a, miR-191 and miR-103) and widely used for normalization in tumor samples (Chang et al., 2010). The selection of appropriate reference genes to normalize miRNA expression was performed also in other models such as bovine and plant (Li et al., 2014b; Cassol et al., 2016). Six non-coding RNA expression were assessed in different bovine solid tissues (lung, muscle, fat, heart, kidney, spleen, uterus and small intestine) (Li et al., 2014b). In all these tissues, *U6*, miR-191 and let-7f were demonstrated to be the most stably expressed. As well as for coding-protein genes used for normalization or mRNA, preliminary experimental assay needs to be performed to select the most stably expressed miRNAs in the different samples representative of the experimental conditions used. It is important to note that miRNAs pose a significant challenge for normalization, since they represent as little as 0.01 % of the total mass of RNA in sample, and this percentage can vary significantly across different samples (Liang et al., 2007). Moreover, they are tightly regulated RNAs in numerous biological processes making difficult to find stably expressed miRNAs in the different experimental conditions studied. Non-coding RNAs were not systematically used as reference genes for miRNAs. A recent paper quantified miR-159 in leaves, roots and stem in cucumber after infection with the cucumber green mottle mosaic virus (CGMMV) and the authors normalized the miRNA with two protein-coding genes (Liang et al., 2018).

Repression of miR-126 expression during GaHV-2 induced lymphomagenesis

In the context of MD, studies were more dedicated to viral miRNAs characterization rather than host miRNAs (Burnside et al., 2006, 2008; Yao et al., 2009; Muylkens et al., 2010; Stik et al., 2014; Parnas et al., 2014; Xu et al., 2011; Yao and Nair, 2014). Expression and functional roles of these viral miRNAs were assessed all along GaHV-2 viral life cycle (Muylkens et al., 2010; Parnas et al., 2014; Xu et al., 2011; Yao and Nair, 2014). Several viral miRNAs were demonstrated to play important roles in tumor development with an implication in apoptosis, cell survival and immune escape.

Several studies suggested that mis-expression of host miRNAs is one of the corner stones in the herpesvirus induced oncogenesis (Fiorucci et al., 2015; Catrina et al., 2014; Lian et al., 2012). This work focuses on host-pathogen relation in the context of MD by studying a host miRNA (miR-126) repressed during GaHV-2 induced lymphomagenesis. Previous studies assessed host miRNAs expression pattern with high throughput sequencing methods (Lian et al., 2012; Tian et al., 2012; Stik et al., 2013; Yao et al., 2009). Some host miRNAs were found upregulated while other were found downregulated. Functional analysis were performed for only few of them (Stik et al., 2013; Han et al., 2016a,b; Lian et al., 2015). Nevertheless, this brought insights on host miRNAs implication during GaHV-2 induced lymphomagenesis with roles in cell survival, cell proliferation and migration. Since miR-126 was shown to be repressed during oncogenesis, we decided to assess if its inhibition participates to the cancerous process induced by GaHV-2. A strategy was developed to overexpress miR-126 and control miRNAs in transformed CD4+ T cell propagated from MD lymphoma. Preliminary experiment performed in the context of this PhD program showed a potential impact of miR-126 on cell proliferation. Other functional analysis are needed to have a comprehensive overview on how repression of miR-126 is implicated in tumor formation. Another strategy that may be used to address this question is the silencing of miR-126 expression in a cellular context where it is highly expressed.

Studies about host miRNAs during GaHV-2 infection are a thematic in expansion. While the focus was on viral genes, a few years ago other factors have been taken into account to understand this complex host-pathogen relation. The expression pattern of cellular mRNAs were assessed from samples of infected chickens (Dang et al., 2017; Chen et al., 2011; Haq et al., 2010). Different signaling pathways were shown to be modulated all along GaHV-2 infection such as the JAK/STAT signaling pathway implicated in cell differenciation, proliferation, development, apoptosis and inflammation (Dang et al., 2017). A study demonstrated that GaHV-2 transformed cells obtained from the liver present a different metabolism compared to uninfected cells (Chen et al., 2011).

Another factor that might be implicated in miR-126 repression is the major viral oncoprotein Meq. This protein possesses a bZIP domain allowing formation of homodimers or to dimerize with other cellular and viral proteins possessing a bZIP domain, such as c-Jun (Levy et al., 2003). This heterodimer targets the Meq responsive element I (MERE I) found in the cellular miR-21 promoter (Stik et al., 2013). This heterodimer is known as transactivating gene expression. The assumption is that the heterodimer might bind on miR-126 promoter and might induce its expression when DNA is not methylated. In the opposite case, no binding would be possible in the presence of a high level of methylation, preventing miR-126 expression during GaHV-2 induced tumorigenesis. Nevertheless, this assumption is unlikely since no MERE I was found within miR-126 host gene.
Another assumption would be the implication of two cellular transcription factors, c-Myc and myc associated factor X (MAX), in miR-126 expression regulation. C-Myc and MAX are able to form a heterodimer and are known to transactivate gene expression. C-Myc has been linked to immune dysfunction, cancer development and neoplastic transformation (Trop-Steinberg and Azar, 2018). *In silico* analysis was performed on miR-126 host gene and responsive elements (RE) for these two transcription factors were detected in the most discriminative region of the CpG island. Shifts in the DNA methylation patterns

detected in RE corresponding to these transcription factors might trigger differential binding of these transcription factors.

Downregulation of miRNA-126 and epigenetic regulation

In most tumors, global DNA methylation is low with local spots of high DNA methylation at particular gene promoters (Timp and Feinberg, 2013). This phenomenon is thought to be an adaptative advantage for the cancer cells due to silencing of tumor suppressor genes. *In vivo* miR-126 expression level was shown to be nearly extinguished in tumor CD4+ T cells infected with GaHV-2. Characterization of this repression revealed a hypermethylation within miR-126 host gene (*egfl-7*) at the CpG island 2 (CpG2) when comparing samples from infected to uninfected birds *in vivo*. The study of DNA methylation was focused on CpG2 since it was found in human to be implicated in miR-126 gene regulation (Saito et al., 2009). DNA methylation and histone post-translational modifications (HPTMs) were demonstrated to be implicated in miR-126 expression in prostate cancer cell lines (Saito et al., 2009). Several other studies in human cancers brought the importance of DNA methylation to induce miR-126 repression (Liu et al., 2015b; Cui et al., 2016; Saito et al., 2009; Watanabe et al., 2012). In human esophageal squamous cell carcinoma (ESCC) the repression of miR-126 is associated with the overexpression of DNA methyltransferase 1 (DNMT1). Quantification of the different DNMTs (*de novo* (DNMT3A and 3B) and maintance (DNMT1) enzymes) would be interesting to understand the upstream pathway involved in the hypermethylation.

Epigenetic modulations are evidenced in the context of cancer disease. During this work, the focus was restricted to one CpG island giving an incomplete view on how miR-126 gene expression is regulated. The role of DNA methylation in CpG island is part of the cancer epigenetics signature but the existence of CpG island shores (CGI shores) and nucleosome positioning were unknown until recently.

In colon cancer, most discriminative differences in methylation patterns between tissues occur outside CpG islands in sequences up to 2 Kb distant called CpG island shores (CGI shores) (Irizarry et al., 2009). These regions are implicated in physiological processes such as cell differenciation and reprogramming but also in disease such as cancer (Doi et al., 2009). Two potential roles of CGI shores are proposed: (i) they are sites of alternative transcription and enhancer regions. Hypomethylation of the CGI shores activates alternative TSS. (ii) CGI shores are boundaries surrounding CpG islands protecting them from DNA methylation. Erosion of these sharply defined boundaries results in altered gene expression. Cancers ignore the demarcated boundary between high and low methylation that is defined by CGIs (Timp and Feinberg, 2013). If boundaries between high and low methylation shifts outwards, the CGI shores become hypomethylated. Conversely, if boundaries between high and low methylation shifts inwards, toward CpG islands, CGI shores is found hypermethylated.

Another level of epigenetic regulation is the chromatin organization in euchromatin and heterochromatin that is controlled by nucleosome positioning and post-translational modifications of histone tails (Wang et al., 2007). Transcriptional activity is possible when there is nucleosome depletion (Kaplan et al., 2009), positive histone marks and the presence of histone variants such as H3.3 and H2A.Z (Zofall et al., 2009). In addition, bivalents regions exist with a combination of an active mark (H3K4me3) and a negative mark (H3K27me3). These regions are associated to hypermethylated CpG islands in cancer (Ohm et al., 2007).

In this study, we focused on small epigenetic structure (CpG island). New high-throughput techniques brought the existence of large epigenetic structures (Timp and Feinberg, 2013). The genome is partionated into large euchromatin and heterochromatin domains named large organized chromatin lysine modification (LOCKs) and lamina associated domains (LADs). These two domains increase in size during development and 80 % of them overlap (Timp and Feinberg, 2013; Hu et al., 2012; Peric-hupkes et al., 2010). (i) LOCKs are domains enriched with heterochromatin post-translational modification such as H3K9me2.

These domains expand during differentiation while a loss is observed in cancer (Wen et al., 2009). During oncogenesis process, epithelial-mesenchymal transition (EMT) occurs altering LOCKs domain composition (Timp and Feinberg, 2013). The chromatin is reprogrammed with a decrease of histone negative mark (H3K9me2) and an increase in histone positive marks (H3K4me3 and H3K36me3). (ii) LADs are DNA sequences associated with proteins from nuclear lamina (Zullo et al., 2008). Gene repression is associated with these regions. These two different large epigenetic structures allow to obtain large-scale epigenetic alteration in cancer.

All of these pieces of information about epigenetics underlines the fact that our study is very limited and would need a more global approach. DNA methylation should be studied in association with HPTMs. Two studies demonstrated that transcriptional gene silencing seems to be driven by histone modifications before DNA methylation changes (Bachman et al., 2003). It would be interesting to assess histone marks on the CpG island studied as well as a larger locus to identify potential implication of CpG shores on miR-126 expression.

Another point to discuss in this study is the very high percentage of DNA methylation observed in T CD4+ transformed cell lines propagated *in vitro* (86 %), especially when the situation is compared with *in vivo* samples (from 7 to 22 %). This statement was also made in a previous studies where marked hypermethylation of CGIs was observed in stable cell lines (mouse embryo cells) and immortalized cell lines (immortal fibroblast cell lines) (Timp and Feinberg, 2013). The pattern of DNA methylation in immortalized cell lines does not represent what is found during an *in vivo* challenge. Cell lines are therefore not the most appropriate sample to learn about epigenetic regulation. The immortalization process seems to modulate epigenetic machinery leading to aberrant DNA methylation pattern in the cells.

As mentioned in the introduction, several evidences indicated that miRNAs have nuclear functions. It was shown that mature miRNAs may shuttle from the cytoplasm to the nucleus (Catalanotto et al., 2016). Besides their cytoplasmic function as translation repressor, miRNA may associate with RISC components in the nucleus to regulate transcription. Even if only few examples have been studied in depth, strong evidences support that miRNAs contribute either to transcriptionnal repression (Zardo et al., 2012) or transcriptional activation (Place et al., 2008; Turner et al., 2014). Different mechanisms were described but altogether these studies indicated that miRNAs are able to recruit on specific DNA locus the chromatin remodelling machinery and the components of the DNA CpG methylation (Zardo et al., 2012). Thus miRNA may be considered as original triggers of epigenetic modifications that together result in transcriptional gene silencing (TGS) or transcriptional gene activation (TGA). It would be interesting to assess if repression of miR-126 is linked to miRNAs-induced silencing. The binding on a specific locus of miR-126 host gene might trigger epigenetic modification resulting in miR-126 repression during tumorigenesis.

Perspectives

In order to test the biological effects induced by miR-126 silencing during MD lymphoma development, a strategy was developed in order to restore miR-126 expression in transformed CD4+ T cells propagated from MD lymphoma. We used a conditional expression system (Tet-on inducible expression plasmids after selection and integration). After induction of miRNA expression, cancerous cells were characterized in order to assess cell survival and proliferation. The preliminary functional analysis showed that miR-126 inhibition might participate to the cancerous process induced by GaHV-2 with a role in cell proliferation. In order to complete the functional analysis, other phenotypes should be investigated such as the role of miR-126 in apoptosis, the influence of miR-126 in cell proliferation measured through colony formation assay after a long term miR-126 overexpression. Moreover, it would be interesting to characterize mechanistic consequences of miR-126 restoration by analyzing the expression level of several candidate

targets through high throughput RNA seq and proteomic approaches. A first analysis would be to decipher miR-126 interaction with two candidate target genes, namely CrK (Chicken Tumor Virus number 10 regulator of kinase), a previously characterized miR-126 target and TRPC6 (Transient recetpro potential canonical 6). Crk is known to have a role in signaling pathways implicated in cell adhesion, proliferation and migration (Feng et al., 2010). Recent data suggest that TRPC6-mediated elevation of intracellular Ca2+ stimulates cell proliferation and that inhibition of TRPC6 attenuates cell proliferation and invasion.

To complement the *in vitro* functional analysis, a part of this work concentrated on the creation of a recombinant GaHV-2 that constitutively expresses the miRNA lost during lymphoma development. The aim is to interfere with miR-126 silencing during the natural course of GaHV-2 infection. It is an original system to clearly define the role of miR-126 in MD progression.
An interesting perspective would be to create recombinant viruses with other cellular pre-miRNAs (separately or in cluster) already known repressed during the Marek's Disease such as miR-223 and miR-26a (Tian et al., 2012; Li et al., 2014b). miR-223 has a physiological role in the fate of myeloid precursor. Its deregulation was shown to be implicated in tumorigenesis in human (Li et al., 2011; Taïbi et al., 2014). miR-26a plays significant roles in growth, development and cell differentiation of different tissues (Gao and Liu, 2011). This miRNA was found downregulated in several human cancers such as bladder cancer, breast cancer and rhabdomyosarcoma (Maillot et al., 2009; Ciarapica et al., 2009).

Scientific production associated with this PhD program

- Review paper as first author:

  1) Gennart I., Coupeau D., Pejakovic S., Laurent S., Rasschaert D., Muylkens B. (2015). Marek's disease: genetic regulation of gallid herpesvirus 2 infection and latency. *The Veterinary Journal*, 205:339-348.

- Original paper as first author : submitted to PloS ONE (in revision):

  2) Gennart I., Regnier M., Rauw F., Wiggers L., Coupeau D., Lambrecht B., Muylkens B. Influence of anesthesia on gene expression analysis in experimental model of virus-induced lymphoma. *PloS ONE* (in revision)

- Original papers as second author:

  In collaboration with the University of Tours, DNA methylation patterns were established for ICP4 and ICP27 promoters, controlling the expression of these two Immediate Early genes of GaHV-2. This work was done during the second year of the PhD program in collaboration with the TLVI (Transcription Lymphome Viro-Induit) research group. These results were included in two original papers that are published in the "Journal of General Virology".

  3) Strassheim S., Gennart I., Muylkens B., Andre M., Rasschaert D., Laurent S. (2016). Oncogenic Marek's disease herpesvirus encodes an isoform of the conserved regulatory immediate early protein ICP27 generated by alternative promoter usage. *Journal of General Virology*, 97:2399-2410.

  Herpesvirus gene expression is temporally regulated, with immediate early (IE), early (E) and late (L) genes. ICP27, which is involved in post-transcriptional regulation, is the only IE gene product conserved in all herpesviruses. We show here that the ICP27 transcript of the oncogenic Marek's disease virus shares the same polyadenylation signal as the bicistronic glycoprotein K–ICP27 transcript and is regulated by alternative promoter usage, with transcription from its own promoter (pICP27) or that of gK (pgK). The pgK can generate a spliced ICP27 transcript yielding an N-terminal-deleted ICP27 isoform (ICP27DN) that, like ICP27, co-localizes with the SR protein in infected cells, but with a diffuse nuclear distribution. The pICP27 includes functional responsive elements (REs) for SP1, AP1 and CREB, is essentially active during the lytic phase and leads to exclusive expression of the native form of ICP27. The alternative promoter, pgK, including active REs for GATA, P53 and CREB, preferentially generates the gK transcript during the lytic phase and the spliced ICP27 transcript (ICP27DN) during the latent phase. An analysis of the DNA methylation marks of each promoter showed that pgK was systematically demethylated, whereas pICP27 was methylated during latency and demethylated during the lytic stage. Thus, MDV ICP27 gene expression is dependent on alternative promoters, the usage of which is regulated by DNA methylation, which differs between viral stages.

4) Rasschaert P., Gennart I., Boumart I., Dambrine G., Muylkens B., Rasschaert D., Laurent S. (2018). Specific transcriptional and post-transcriptional regulation of the major immediate early *ICP4* gene of GaHV-2 during the lytic, latent and reactivation phases. *Journal of General Virology,* 1-14.

Transcriptional and post-transcriptional mechanisms are involved in the switch between the lytic, latent and reactivation phases of the viral cycle in herpesviruses. During the productive phases, herpesvirus gene expression is characterized by a temporally regulated cascade of immediate early (IE), early (E) and late (L) genes. In alphaherpesviruses, the major product of the IE ICP4 gene is a transcriptional regulator that initiates the cascade of gene expression that is essential for viral replication. In this study, we redefine the infected cell protein 4 (ICP4) gene of the oncogenic Marek's disease virus (MDV or gallid herpesvirus 2) as a 9438 nt gene ended with four alternative poly(A) signals and controlled by two alternative promoters containing essentially ubiquitous functional response elements (GC, TATA and CCAAT boxes). The distal promoter is associated with ICP4 gene expression during the lytic and the latent phases, whereas the proximal promoter is associated with the expression of this gene during the reactivation phase. Both promoters are regulated by DNA methylation during the viral cycle and are hypermethylated during latency. Transcript analyses showed ICP4 to consist of three exons and two introns, the alternative splicing of which is associated with five predicted nested ICP4ORFs. We show that the ICP4 gene is highly and specifically regulated by transcriptional and post-transcriptional mechanisms during the three phases of the GaHV-2 viral cycle, with a clear difference in expression between the lytic phase and reactivation from latency in our model.

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