From the Department of Medicine, Solna
Karolinska Institutet, Stockholm, Sweden

ELUCIDATING THE ROLE OF DNA DAMAGE AND HUMAN CYTOMEGALOVIRUS IN MEDULLOBLASTOMA AND GLIOBLASTOMA

Jiri Bartek

Karolinska Institutet

Stockholm 2020

Published by Karolinska Institutet.
Printed by Arkitektkopia AB, 2020

ISBN 978-91-7831-722-6

Elucidating the role of DNA damage and Human Cytomegalovirus in Medulloblastoma and Glioblastoma

THESIS FOR DOCTORAL DEGREE (Ph.D.)

The thesis will be defended at Karolinska Universitetssjukhuset, Norrbacka S2:01 (Rehabsalen), Eugeniavägen 39, Solna

Thursday, April 2, 2020 at 8.30

By

Jiri Bartek

Principal Supervisor:

Cecilia Söderberg-Naucler, Professor
Karolinska Institutet
Department of Medicine, Solna
Division of Microbial Pathogenesis

Co-supervisors:

Afsar Rahbar, PhD
Karolinska Institutet
Department of Medicine, Solna
Division of Microbial Pathogenesis

Giuseppe Stragliotto, MD, PhD
Karolinska Institutet
Department of Medicine, Solna
Division of Microbial Pathogenesis

Mikael Svensson, Professor
Karolinska Institutet
Department of Clinical Neuroscience
Division of Neurosurgery

Opponent:

Einar Vik-Mo, PhD
Oslo University
Department of Clinical Medicine
Division of Neuroscience

Examination Board:

Susan Pfeifer, Professor
Uppsala University
Department of Women and Child Health
Division of Molecular Genetics

Leif Salford, Professor
Lund University
Department of Clinical Sciences
Division of Neurosurgery

Oscar Fernandez-Capetillo, Professor
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Genome Biology

To my family

ABSTRACT

The most common primary malignant brain tumor in children is Medulloblastoma, while Glioblastoma is the most common in adults. Treatment for both include some combination of surgery, radiation therapy, and chemotherapy. The evolution of most primary malignant brain tumors is unknown, although varying degree of genomic instability caused by defects in the DNA Damage Response (DDR) is suspected. Lately, even human cytomegalovirus (HCMV) has emerged as a suspected pathogen possibly implicated in malignant tumor evolution. Nevertheless, the causes of the chromosomal instability and its potential links with HCMV infection and/or resistance to genotoxic therapies (i.e. radiation and chemotherapy) remain largely unknown. Thus, the main aim of this PhD thesis is to investigate the role of HCMV in the context of DDR in human Medulloblastoma and Glioblastoma.

In the **1st** study, we turned our attention towards Glioblastoma (GBM). We examined the ability of HCMV to induce a more aggressive cancer stem cell (CSC)-like phenotype in primary GBM cell lines. HCMV infection induced a stem cell phenotype in primary GBM cell lines as determined by changes in the cellular gene expression profile and by the conferred ability of cells to grow as neurospheres *in vitro*, and this phenotype was prevented by treatment with the anti-viral drug ganciclovir. As CSCs are known to be resistant to chemotherapy, our results imply that HCMV may enhance the malignancy grade of the tumor, and possibly contribute to therapy resistance.

In the **2nd study**, we found pronounced endogenous DNA damage signaling and constitutive activation of DNA damage checkpoint kinase cascades across our medulloblastoma cohort. The bulk of the specimens also showed expression of HCMV immediate early and late proteins, in comparative analyses using three immunohistochemical protocols. Cell culture experiments validated the chronic endogenous replication stress in medulloblastoma cell lines and showed sharply differential, intriguing responses of normal cells and medulloblastoma cells to HCMV infection. Our results strongly indicate that in human medulloblastomas, the DDR checkpoint barrier is widely activated, at least in part due to replication stress. Furthermore, we propose that ~~unorthodox~~ the highly prevalent HCMV may impact the medulloblastoma host cell replication stress and DNA repair mechanisms.

In the **3rd study**, we examined cancer stem cell markers (CD133, CD15, VEGFR2) and HCMV protein expression in human medulloblastoma specimens and medulloblastoma cell lines, at the same time considering also the replication stress and DNA damage response, as cancer stem cells are often more resistant to standard-of-care radiation and chemotherapy treatments. Our immunohistochemistry analysis on clinical material identified widespread expression of the VEGFR2 receptor and CD15, yet more limited expression of CD133 compared to GBM. In addition, assessments

of expression of HCMV early and late proteins have been carried out in parallel, along with cell culture experiments with HCMV infection and replication stress responses in medulloblastoma cell lines. Remarkably, we found that unlike the 'non-stem cell' medulloblastoma cell lines, the cell line that showed robust stemness phenotype featured a very distinct response to DNA replication stress and HCMV infection, both emerging hallmarks of brain cancers.

In the **4th study**, we show that HCMV infection induced replication stress (RS) and triggered host DNA damage response (DDR) in permissive and non-permissive human cells. Further, we show that undergoing standard-of-care genotoxic radio-chemotherapy in patients with HCMV-positive glioblastomas correlated with elevated HCMV markers after tumor recurrence. We propose a model to explain oncomodulatory effects of HCMV, through RS induction, DDR subversion, cell death inhibition and host-cell's genome destabilization. Our findings provide fresh insights into HCMV pathobiology and inspiration for future strategies to combine radio-chemotherapy with anti-viral drugs for cancer treatment.

LIST OF SCIENTIFIC PAPERS

I. Fornara O, **Bartek J Jr**, Rahbar A, Odeberg J, Khan Z, Peredo I, Hamerlik P, Bartek J, Stragliotto G, Landázuri N, Söderberg-Nauclér C

Cytomegalovirus infection induces a stem cell phenotype in human primary glioblastoma cells: prognostic significance and biological impact.

Cell Death Differ. 2016 Feb;23(2):261-9.

II. **Bartek J Jr**, Fornara O, Merchut-Maya JM, Maya-Mendoza A, Rahbar A, Stragliotto G, Broholm H, Svensson M, Sehested A, Söderberg Naucler C, Bartek J, Bartkova J

Replication stress, DNA damage signalling, and cytomegalovirus infection in human medulloblastomas.

Mol Oncology. 2017 Aug;11(8):945-964.

III. **Bartek J Jr**, Merchut-Maya JM, Maya-Mendoza A, Fornara O, Rahbar A, Beltoft Brøchner C, Sehested A, Söderberg-Nauclér C, Bartek J, Bartkova J

Cancer cell stemness, responses to experimental genotoxic treatments, cytomegalovirus protein expression and DNA replication stress in pediatric medulloblastomas.

Cell Cycle (accepted for publication Feb. 2020)

IV. Merchut-Maya JM*, Maya-Mendoza A*, **Bartek J Jr**, Bartkova J, Lee M, Beltoft Brøchner C, Broholm H, Söderberg-Nauclér C, Bartek J

HCMV triggers replication stress and subverts host-cell's DNA damage response fueling genomic instability.

Submitted/under revision.

*shared first authorship.

Related publications

I. Estekizadeh A, Landázuri N, **Bartek J Jr**, Beltoft Brøchner C, Davoudi B, Broholm H, Karimi M, Ekström TJ, Rahbar A.

Increased cytomegalovirus replication by 5-Azacytidine and viral-induced cytoplasmic expression of DNMT−1 in medulloblastoma and endothelial cells.

Int J Oncol. 2018 Apr;52(4):1317-1327.

II. Kostopoulou ON, Mohammad AA, **Bartek J Jr**, Winter J, Jung M, Stragliotto G, Söderberg-Nauclér C, Landázuri N.

Glucocorticoids promote a glioma stem cell-like phenotype and resistance to chemotherapy in human glioblastoma primary cells: Biological and prognostic significance.

Int J Cancer. 2018 Mar 15;142(6):1266-1276.

III. Rasmussen RD, Gajjar MK, Tuckova L, Jensen KE, Maya-Mendoza A, Holst CB, Møllgaard K, Rasmussen JS, Brennum J, **Bartek J Jr**, Syrucek M, Sedlakova E, Andersen KK, Frederiksen MH, Bartek J, Hamerlik P.

BRCA1-regulated RRM2 expression protects glioblastoma cells from endogenous replication stress and promotes tumorigenicity.

Nat Commun. 2016 Nov 15;7:13398.

IV. **Bartek J Jr**, Ng K, Bartek J, Fischer W, Carter B, Chen CC.

Key concepts in glioblastoma therapy.

J Neurol Neurosurg Psychiatry. 2012 Jul;83(7):753-60.

V. Hamerlik P, Lathia JD, Rasmussen R, Wu Q, Bartkova J, Lee M, Moudry P, **Bartek J Jr**, Fischer W, Lukas J, Rich JN, Bartek J.

Autocrine VEGF-VEGFR2-Neuropilin-1 signaling promotes glioma stem-like cell viability and tumor growth.

J Exp Med. 2012 Mar 12;209(3):507-20.

CONTENTS

1 Introduction 1
1.1 Glioblastoma (GBM) 1
1.1.1 Epidemiology 1
1.1.2 Pathogenesis and Classification 2
1.1.3 Glioblastoma stem-like cells 5
1.1.4 Treatment 6
1.2 Medulloblastoma (MB) 7
1.2.1 Epidemiology 7
1.2.2 Pathogenesis and Classification 7
1.2.3 Medulloblastoma stem-like cells 10
1.2.4 Treatment 11
1.3 Cell cycle and checkpoint control 12
1.3.1 Genomic instability, DNA damage response (DDR) and tumorigenesis 13
1.3.2 The cellular DDR machinery 15
1.3.3 DNA Replication stress (RS): an emerging hallmark of cancer 16
1.3.4 Viruses and the DNA damage response 17
1.4 Human Cytomegalovirus (HCMV) 18
1.4.1 Replication and latency of HCMV 19
1.4.2 HCMV epidemiology, transmission and clinical course 21
1.4.3 Diagnosis of HCMV 22
1.4.4 Treatment of HCMV 23
1.5 HCMV and (brain) cancer 24
1.5.1 HCMV and DDR 30
1.5.2 HCMV as treatment target in brain tumor patients? 32
2 Aims of the thesis 33
3 Results and discussion 34
3.1 1st study: Cytomegalovirus infection induces a stem cell phenotype in human primary glioblastoma cells: prognostic significance and biological impact. 35
3.2 2nd study: Replication stress, DNA damage signaling, and cytomegalovirus infection in human medulloblastomas 36
3.3 3rd study: Cancer cell stemness, responses to experimental genotoxic treatments, cytomegalovirus protein expression and DNA replication stress in pediatric medulloblastomas 38
3.4 4th study: HCMV triggers replication stress and subverts host-cell's DNA damage response fueling genomic instability 40
4 Conclusion 43
5 Acknowledgments 46
6 References 47

LIST OF ABBREVIATIONS

5-ALA	5-Aminolevulinic acid
5-AZA	5-Azacytidine
8-OXO	8-Oxoguanine
APC	Anaphase-promoting complex
APOBEC	Apolipoprotein B mRNA editing enzyme catalytic
ATCC	American type culture collection
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia Rad-3 related
ATRT	Atypical teratoid/rhabdoid tumors
BER	Base excision repair
BMI1	Polycomb complex protein
BRCA	Breast cancer susceptibility genes
CD133	Prominin 1
CD15	Cluster of differentiation 15
CDC	Cell division cycle
CDK	Cyclin dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CIN	Chromosomal instability
CNS	Central nervous system
COX	Cyclooxygenase
CSC	Cancer stem cell
CSF	Cerebrospinal fluid
CTNNB1	Catenin beta-1
DC	Dendrit cell
DDR	DNA damage response
DNAPKcs	DNA-dependent protein kinase, catalytic subunit
DNMT-1	DNA (cytosine-5)-methyltransferase 1
DSB	Double strand breaks
E2F	E2 promoter binding factor
EBV	Epstein Barr virus
EGFR	Epithelial growth factor receptor

EGL	External granular layer
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic Reticulum
FACS	Flow cytometry
gB	Glycoprotein B
GBM	Glioblastoma
GCP	Granular cell progenitors
GCV	Ganciclovir
GENSAT	The Gene Expression Nervous System Atlas
GFAP	Glial fibrillary acidic protein
GLI1/2	Glioma-associated oncogene homolog 1/2
GSC	Glioma stem cells
GTR	Gross total resection
Gy	Gray
gH2AX	Phosphorylated histone 2AX
HCMV	Human Cytomegalovirus
HHV	Human Herpesvirus
HIF-1	Hypoxia-inducible factor-1
HIV	Human immunodeficiency virus
HNPCC	Hereditary nonpolyposis colorectal cancer
HPV	Human papillomavirus
hTERT	Telomerase reverse transcriptase
HTLV	Human T-cell leukemia retrovirus
HUVEC	Human umbilical endothelial cells
IDH	Isocitrat dehydrogenase type
IE/E/L	Immediate early/early/late
IFN	Interferon
IGL	Internal granular layer
IHC	Immunohistochemistry
IL	Interleukin
ISH	In-situ hybridization
LGG	Low Grade Glioma
LOH	Loss of heterogeneity
MAPK	Mitogen-activated protein kinase

MB	Medulloblastoma
MBSC	Medulloblastoma stem cells
MGMT	Methylguanine DNA methyltransferase
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MRN complex	Mre11-Rad50-Nbs1 complex
mRNA	Messenger RNA
MYCN	MYC proto oncogene
NADPH	Nicotinamide adenine dinucleotide phosphate
NER	Nucleotide excision repair
NF1	Neurofibromatosis type 1
NHEJ	Non-homologous end joining
NO	Nitric oxide
NOS	Not otherwise specified
NSAID	Non-steroid anti-inflammatory drugs
NSC	Neural stem cells
OCT4	Octamer-binding transcription factor 4
ORF	Open reading frame
OS	Overall survival
PCR	Polymerase chain reaction
PDGFRA	Platelet-derived growth factor receptor A
PFS	Progression free survival
PGE	Prostaglandin E
PIKK	Phosphatidylinositol-3-kinase like family
PTCH1	Protein patched homolog 1
PTEN	Phosphatase and tensin homologue
RAS	Rat sarcoma pathway
RB	Retinoblastoma
ROS	Reactive oxygen species
RPA	Replication protein A
RS	Replication stress
SCF	Skp, Cullin, F-box containing complex
SHH	Sonic hedgehog
SMC	Smooth muscle cells

SMO	Smoothened gene
SOX2	Sex determining region Y-Box
SSB	Single strand breaks
STAT3	Signal transducer and activator of transcription 3
STR	Subtotal resection
SUFU	Suppressor of fused protein
TCA	Tricarboxylic acid
TGF	Transforming growth factor
TMZ	Temozolomide
TNF	Tumor necrosis factor
TP53	Tumor protein 53
UL	Unique long
US	Unique short
VEGFR	Vascular endothelial growth factor
VGCV	Valganciclovir
WHO	World Health Organisation
WNT	Wingless-related integration site
VRC	Viral replication center

1 INTRODUCTION

1.1 Glioblastoma (GBM)

GBM is the most common primary malignant brain tumor in adults. Despite intensive research for several decades, GBM biology is still not fully understood, with the disease characterized by aggressive tumor growth, poor response to therapy and overall dismal prognosis.

1.1.1 Epidemiology

Being the most common malignant primary brain tumor, GBM accounts for 54% of all gliomas and 16% of all primary brain tumors [1]. The incidence of GBM is approx. 3 per 100.000 persons in the Western countries, with an increased frequency in males compared to females (1.6:1), and in Caucasians compared to Africans and Afro-Americans (2:1), and with lowest incidence in Asians and American Indians. The median age at diagnosis is 64 years, implying high absolute number of cases among elderly (peaking at 75-84 years), which, without an effective treatment, will keep increasing with growing and aging populations. The age at diagnosis tends to be higher for primary GBM (mean age of 55 and median age of 64) than for secondary GBM (mean age of 40 years) [1,2]. GBM is uncommon in children, representing only 3% of all central nervous system (CNS) and brain tumors among patients aged 0-19 years [2].

The supratentorial region is the preferred location for GBM – the highest incidence being in the frontal lobe, followed by the temporal and parietal lobes, and finally the occipital lobe [3]. GBM is rarely seen in the cerebellum and the spinal cord [3]. Risk factors for GBM include prior radiotherapy, immune factors and immune genes, decreased susceptibility to allergy and some single nucleotide polymorphisms detected by genomics [4-6]. Also, there is increased incidence of GBM in patients with hereditary tumor syndromes such as Turcot- [7] and Li-Fraumeni syndrome [8]. Inconsistent and inconclusive results have been recently published regarding the link between mobile phone usage and risk of glioma [9,10]. Besides the above-mentioned factors, GBM occurs sporadically without other known predispositions [11]. On the other side, protective factors include the use of anti-inflammatory drugs (i.e. NSAID) [12] and even genotypes that increase the risk of asthma and/or having allergies or atopic disease (i.e. eczema, asthma, psoriasis) [6,13].

The prognosis for GBM patients is poor, mainly decided by the level of treatment, but even by predictive clinical before treatment initiation such as age, comorbidity and functional performance status [14,15]. If left untreated, the median patient survival is 3 months, while maximal safe surgical resection, followed by radiation- and chemotherapy (the so called Stupp regimen) results in up to 15 months median

overall survival [16,17], with 2-year survival rates at 27%, while still less than 5% of patients survive 5 years following diagnosis [18]. Overall, survival rates for patients with GBM have shown no notable improvement in population statistics in the last three decades.

1.1.2 Pathogenesis and Classification

Pathogenesis

In cancer, the cell of origin refers to the normal cell that acquires the initial cancer promoting genetic hit or hits, which then leads to cancer- and/or tumorigenesis [19]. The cellular origin of gliomas remains a topic of controversy in cancer research. Nevertheless, recent advances in science have brought us new insights into the subject. Neural stem cells (NSC) give rise to progenitor cells that display a varying degree of potentiality, all of whom have experimentally been shown to induce various GBM subtype formation, depending on which driver line and driver mutations have been used [20-23]. As such, currently it is believed that different progenitor cells can function as the cell of origin. On the other hand, the role of post-mitotic, fully differentiated cells in the GBM formation is controversial, with astrocytes not easily forming glioma when targeted in experiments [21,22], while reports exist on differentiated neurons transforming into tumors [24], possibly caused by dedifferentiation of normal cells (i.e. astrocytes and neurons), reprogrammed into stem cells and then transforming into GBM [24]. Whether specific cells of origin are susceptible to certain mutations is not agreed upon, but data from experimental animal models suggest wide susceptibility to mutations in both stem- and progenitor cells [19]. Specific cell types may exhibit preferential vulnerability to certain mutations, and some of these combinations might lead to specific GBM types/subtypes [21]. Overall, these mechanisms and suggested interactions might also explain the genetic heterogeneity of GBM.

Classification

GBM is classified as grade 4 astrocytoma according to the World Health Organization (WHO) grading system of central nervous system malignancies [25,26]. The WHO grading system has so far been a histopathological grading system, taking into consideration numerous histopathological features, of which it is mainly necrosis and microvascular proliferation, in combination with rapid- (high mitotic index) and infiltrative growth that characterizes GBM and separates GBM from Low Grade Glioma (LGG) [26]. Recently (2016), the WHO grading system has been revised to incorporate molecular parameters and make use of layered diagnostic reports as stated in the ISN-Haarlem guidelines [27]. The ISN-Haarlem guidelines consist of a four-layered reporting system including the histological diagnosis, the histological grade and molecular information that when interpreted results in an integrated diagnosis. As such, the new classification subdivides GBM into IDH-wildtype (including Giant cell GBM, Gliosarcoma and Epitheloid glioblastoma), IDH-mutant

and NOS (Not Otherwise Specified) [26]. If no consensus can be reached between the histopathological and the molecular diagnosis, the molecular diagnosis will overrule the histopathological diagnosis and dictate the diagnosis. Also, since combined histopathological and molecular information might not be available for all tumors, the 2016 classification utilizes the NOS (Not otherwise specified) to name those diagnostic categories that are not precisely defined (**Figure 1**).

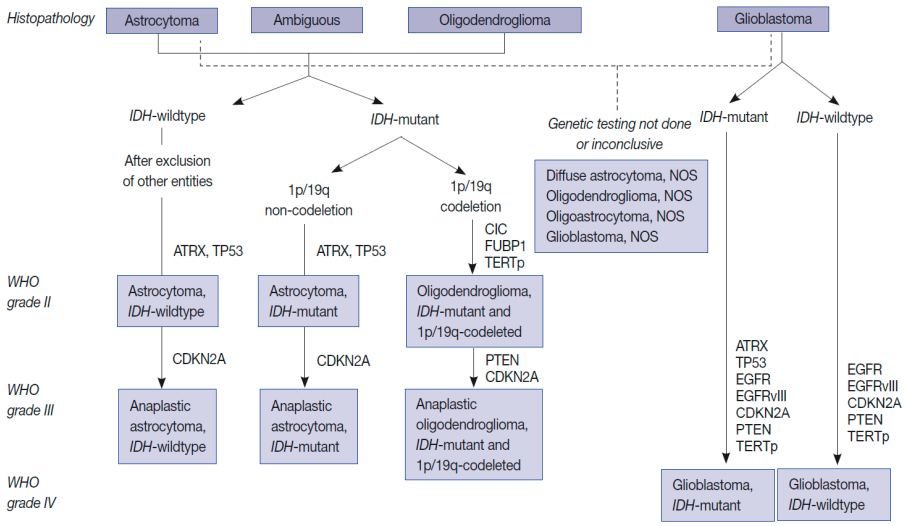

Figure 1. *2016 WHO classification of adult diffuse glioma, from* [28].

Isocitrate Dehydrogenase (IDH) plays a central role in the 2016 WHO classification. The IDH gene exists in 5 variants, encoding 3 enzymes, all of which are involved in the oxidative carboxylation of isocitrate to alfa-keto-glutarate producing nicotinamide adenine dinucleotide phosphate (NADPH). Two of these have been found to be mutated in GBM; IDH1 and IDH2. Both have the same mutagenic effect, and both are validated as prognostic markers for GBM as well as LGG. IDH is found mutated in about 5% of primary GBM and up to 70% in secondary GBM and LGG [29,30].

Further, characterization of genetic pathways has resulted in GBM being subdivided into primary (de novo) developing through multistep tumorigeneses, and those that are secondary, developing through progression from LGG such as astrocytoma or oligodendrogliomas [31-34]. Primary (de novo) GBM are more frequent in older patients, while secondary GBM occur more frequently in younger individuals. Genetically, these two entities carry distinct alterations; TP53 mutations, 19q loss are typical in secondary GBM – while EGFR amplifications, chromosome 10 loss and PTEN mutation are more often seen in primary GBM (**Figure 2**).

Also, more recent genetic profiling has even subdivided GBM further into four different subtypes; classical, proneural, neural, and mesenchymal (**Figure 2**). Each of these has a genetic signature from distinct neural lineages, implying that the expression patterns of the different subtypes may reflect the phenotype of their specific cells of origin [32,33]. The classical subtype is synonymous with high EGFR expression, loss of heterozygosity (LOH) in chromosome 10 is frequently seen as is chromosome 7 amplification. On the other hand, TP53 which is often mutated in GBM, ~~but~~ is rarely mutated in this phenotype. CDKN2A is often deleted which in turn causes inactivation of the Retinoblastoma (Rb) pathway [35]. The mesenchymal subtype is synonymous with mutations and alterations in the NF1 gene, while having fever alterations- and less expression of EGFR than the other three phenotypes. Also, mutations are seen in PTEN which in turn activates the RAS pathway [32,33]. The proneural subtype, often secondary GBMs, is synonymous with high rates of alterations in TP53, IDH1 and PDGFRA (which activates the PI3K and RAS pathways). And finally, the neural subtype, often histopathologically classified as 'normal brain tissue', is synonymous with expression of neuronal markers. Nevertheless, this subdivision, although promising and certainly overcoming some of the heterogeneity in GBM, has currently no role in diagnostics and treatment decisions.

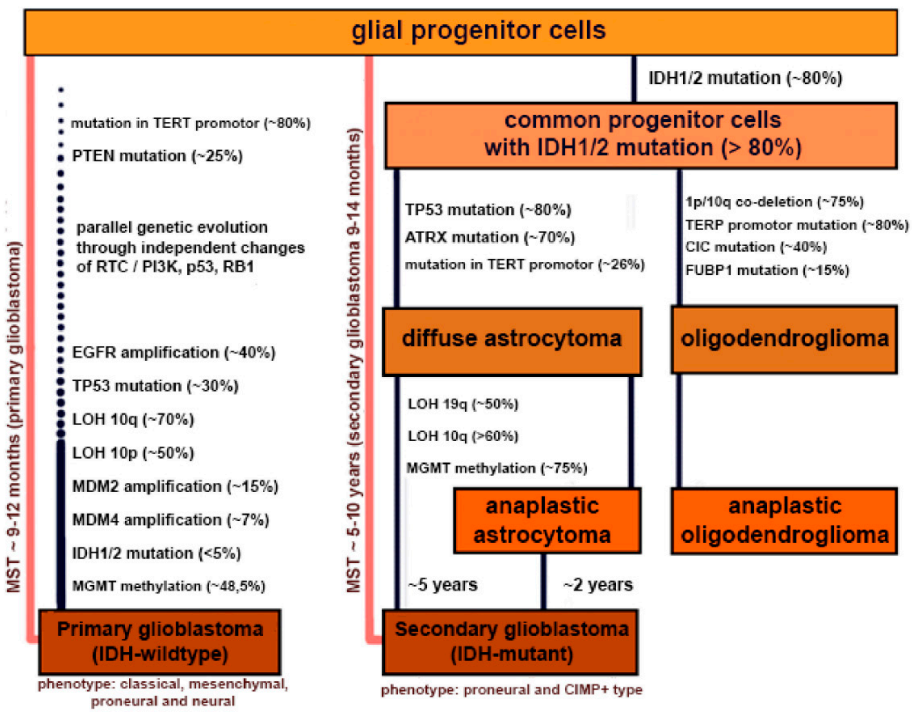

Figure 2. *Glioblastoma carcinogenesis and relevant molecular alterations, from* [36].

Finally, O6-methylguanine DNA methyltransferase (MGMT), a gene encoding a DNA repair enzyme, has recently been found to be of high prognostic value when methylated, since methylation silences DNA transcription, and thus expression of the MGMT enzyme. Since MGMT can repair only one DNA alkylation lesion due to its suicide repair mechanism, methylation of the MGMT gene promotor robustly affects DNA-repair capacity [37], which in turn leads to improved prognosis (improved response) when patients are treated with a DNA-alkylating agent such as Temozolomide (TMZ) [17]. Nevertheless, 60-65% of our patients have an active MGMT enzyme and therefore respond poorly to temozolomide treatment, the mainstay treatment for GBM patients.

1.1.3 Glioblastoma stem-like cells

Recently, a high abundance of cancer cells with stem-like properties have been discovered in several cancer forms, named cancer stem-like cells, or more specifically in GBM the Glioma Stem Cells (GSC) [34,38-41]. The GSC are believed to possess self-renewal and multipotent differentiation abilities, contributing to resistance to conventional treatments, high recurrence rate and high heterogeneity of GBM.

In terms of mechanism of action, GSC have after irradiation shown to activate the DNA Damage Response (DDR) – by activation of ATM, Chk1 and Chk2 – resulting in efficient checkpoint-mediated delay in cell cycle progression to allow active DNA Repair and thus recovery and later recurrent growth of GSC [42]. Notch signaling is another key aspect of GSC biology – often upregulated and promoting self-renewal, while downregulation of Notch increases radio sensitivity [43]. STAT3, involved in cell growth, immunoregulation, cell division and apoptosis is also overexpressed in GBM and GSC where it promotes cell growth and immunosuppression [44]. Transcription factors such as sex determining region Y-Box (SOX2), octamer-binding transcription factor 4 (OCT4), Polycomb complex protein (BMI1) and Nanog homeobox (NANOG) are also known to contribute to the stemness properties of CSC [45], analogous to normal neural stem cells, maintaining self-renewal and undifferentiation as well an ensuring pluripotency.

In terms of surface markers of GSC, Prominin 1 (CD133) is the one most widely recognized, also to be found on the surface of normal neural stem cells, and although the specific role of CD133 is yet to be demonstrated, it was shown to be of prognostic value for some GBM patients [46], with highest concentration of CD133 positive GSC found in the mesenchymal subtype which is highly resistant to radiotherapy and has the poorest prognosis [47,48]. CD133 positive GSC form spheres when grown under conditions permitting stem cell proliferation, with each sphere thought to originate from a single GSC [49]. GSC give rise to more differentiated daughter cells with higher proliferative capacities, contributing to the heterogeneity of the tumor and to tumor recurrence after radio- and chemotherapy [50], which is also confirmed by an increase

of CD133+ cells in glioma xenografts subjected to radiation, suggesting selective survival of GSC. The silencing of CD133 in GBM inhibits self-maintenance (i.e. inhibiting sphere formation) of GSC [51], further strengthening the hypothesis above.

Furthermore, GBM is known for its rich vasculature, with GSC believed to reside near endothelial cells, with easy access to nutrients and signaling ensuring their undifferentiated state. This notion is supported by anti-angiogenic drugs inhibiting GSC population and tumor growth [52], and by the fact that endothelial cells release nitric oxide (NO), which has been shown to maintain GSC through the Notch signaling pathway known to contribute to GBM therapy resistance [53].

Also, we have recently shown that another clinical aspect of GBM treatment, the glucocorticoids administered to GBM patients, result in a stem cell phenotype promotion with increased resistance to chemotherapy in an *in vitro* model. These observations were substantiated with clinical data demonstrating decreased survival correlating to high glucocorticoid administration [54].

All the above mechanisms contribute to the hypothesis that GSC contribute to the therapy resistance of GBM, previously described by us and others as part of the key concepts in Glioblastoma therapy, making GSC a potential target for future therapies [55].

1.1.4 Treatment

Current treatment strategy of GBM consists of a combination of surgery, radio- and chemotherapy, that can prolong OS of a GBM patient up to a medium of 16 months compared to medium 3 months OS if left untreated [16,17].

In terms of surgery, there is increasing evidence that gross total resection (GTR) of more than 90% of the tumor mass results in improved one-year survival compared to those with subtotal resection (< 90% of the tumor mass removed) [56,57]. This leads the research into new technologies that can help the surgeon achieve a GTR safely, such as operational microscope, micro instruments and recently even fluorescent dye in the form of 5-aminolevulinc acid (5-ALA) which stimulates synthesis and accumulation of porphyrins in malignant tissue, making it easily discoverable by the surgeon while looking under a microscope with an ultraviolet filter and resulting in GTR in 65% vs. 36% of GBM patients in a recent randomized study [58].

In terms of radio- and chemotherapy, current treatment protocol (part of the Stupp regimen[17]) consists of fractionated radiotherapy post GTR in 30 fractions a 2 Gray (Gy) resulting in a total of 60 Gy, concomitant with chemotherapy in the form of TMZ. This combined regimen demonstrated a significant 2- and 5-year survival benefit in a recent large multinational trial [18], albeit still very poor.

1.2 Medulloblastoma (MB)

Medulloblastoma (MB) is the most common primary malignant brain tumor in children. As is the case with GBM, despite intensive research for several decades, MB biology is still not fully understood, with the disease characterized by aggressive tumor growth, dissemination through the cerebrospinal fluid, poor response to therapy and overall dismal prognosis.

1.2.1 Epidemiology

Medulloblastoma is a primary malignant brain tumor found in adults and children at a 10:1 ratio, with an incidence of 0,8/100.000 of at-risk children/year. MB is the most common malignant brain tumor in children, and the second-most frequent brain tumor in children after pilocytic astrocytoma [59,60]. MB accounts for up to 20% of primary CNS neoplasms and approximately 40% of all posterior fossa tumors in children. Also, MB are seen more often in younger- than older children, with 40% of patients diagnosed before the age of five [59,60]. In adults, MB is very rare – comprising less than 2% of CNS malignancies [60].

Posterior fossa (cerebellum, brain stem and ventricles) is the predominant tumor location in most cases (90-95%), with occasional dissemination via the cerebrospinal fluid (CSF).

To date, there is no specific risk factor that can explain most CNS malignancies in children, although some hereditary conditions show some association – including neurofibromatosis type 1 (NF1) and tuberculous sclerosis. Also, even though there is a slight association with increased familiar inheritance, it is not consistent. The only clear association has been shown for exposure to ionizing radiation (although this association is mostly historical in nature) and towards male sex, where MB is predominantly seen (62% vs. 38% in females), although this is only seen in children, not in adults [59].

CNS tumors have one of the highest incidence rates among cancer in children and represent the second-most common cause of death from child cancer [61]. With aggressive surgery, craniospinal radiotherapy and chemotherapy, more than 50% of children with medulloblastoma can be expected to be free of disease 5 years later. Nevertheless, the younger the patient is at diagnosis, the worse the prognosis is, with 10-year survival at 48% in those < 1-years old [60].

1.2.2 Pathogenesis and Classification

Pathogenesis

Embryonal tumors (tumors developing from embryonic cells, i.e. medulloblastoma) are all thought to arise from a common precursor cell of the subependymal matrix

in the CNS, nevertheless, as is the case with glioma, this subject is still heavily debated, and several cells are believed to be the possible "cells of origin", including cells of the subependymal layer, external granule layer (EGL), and internal granule layer (IGL). Recent advances targeting the granular cell progenitors (GCP) have revealed subpopulations of cells with distinct properties believed to be contributing to different subgroups of MBs [62,63] – i.e. GCPs from EGL are seen to have SHH mutations, while GCPs in the lower rhombic part of the cerebellum have an activated WNT signaling. Further, large scale studies as is the GENSAT project [64] have shown differences in GCPs at different developmental stages, raising the possibility that these are not a homogenous population as previously believed, but rather represent multiple progenitors with individual capabilities and thus different possibilities of tumorigenesis. Nevertheless, further research including genetic mapping is needed to identify specific GCP subsets responsible for the different subtypes of MB.

Classification

Besides MB, the group termed "embryonal tumors" includes traditionally atypical teratoid/rhabdoid tumors (ATRT), pineoblastoma, ependymoblastoma, cerebral neuroblastoma, ganglioneuroblastoma, medulloepithelioma and supratentorial embryonal tumor. Nevertheless, the newly updated WHO classification [26] has abandoned some of the traditional classification based only on histology and incorporated molecular data in the new classification. Although in the case of MB, a high-grade (WHO grade IV) embryonal neuroepithelial tumor, rather than having a great number of histological-molecular combination, "genetically defined" and "histologically defined" variants have emerged. In the "histologically defined" group, the classic, desmoplastic/nodular, large cell/anaplastic and MB with extensive nodularity have been included. In the "genetically defined" group, MB WNT-activated, MB SHH-activated and TP-53 mutated, MB SHH-activated and TP-53 wildtype as well as MB non-WNT/non-SHH (group 3 and group 4) have been included. Further, those unable to classify as any of the above, a not otherwise specified classification has been made available (Medulloblastoma NOS).

As to the molecular markers/genetically defined subgroups, four main subgroups have been agreed upon; WNT, SHH, Group 3 and Group 4 (**Figure 3**).

WNT is the rarest subgroup accounting for approximately 10% of all MB, typically presenting with somatic mutations in the CTNNB1 gene encoding beta-catenin, which in turn leads to an overexpression in the WNT signaling pathway [63]. These are rarely metastatic and have a favorable outcome compared to the other subgroups [65].

SHH accounts for approximately 30% of all MB, with often seen mutations in the tumor suppressor genes SMO, SUFU and PTCH1 – or amplification of GLI2 or MYCN, resulting in overexpression of the SHH signaling pathway. Further, TP53

mutation seen in approximately 20% defines poorer prognosis in this subgroup, which otherwise has an intermediate prognosis. 20% of patients have metastatic disease at presentation.

Group 3 represents 25% of all MB, and although no specific pathway has been found to be overexpressed (ergo the name Group 3), a frequent MYC amplification and isochromosome 17q alteration. These patients have the worst outcome, and present with meningeal dissemination in almost half the cases at time of diagnosis [66].

Group 4 accounts for most cases, while being the one least defined, without any specific pathway found to be overexpressed (ergo the name Group 4). Isochromosome 17q alteration and MYCN amplification (as in group 3) are often found, besides some other cytogenetic alterations. Group 4 patients often present with metastatic disease at diagnosis, although their prognosis is better than of those classified in Group 3.

	WNT	SHH	Group 3	Group 4
Age group (Infant, Child, Adult)				
Gender ratio (♂:♀)	1 : 1	1 : 1	2 : 1	3 : 1
Outcome	Very good	Infants good, others intermediate	Poor	Intermediate
Anatomic location	Brainstem, 4th ventricle	Cerebellar hemispheres	Midline, 4th ventricle	Midline, 4th ventricle
Metastasis at diagnosis	5-10%	15-20%	40-45%	35-40%
Pattern of recurrence	Rare. Can be local or metastatic	Local	Metastatic	Metastatic
Genetic alterations	*CTNNB1, DDX3X, SMARCA4, TP53* mutation	*PTCH1, SMO, SUFU, TP53* mutation *GLI2, MYCN* amplification	*GLI1, GFL1B* activation, *MYC, OTX-2* amplification, *SMARCA4* mutation	*KDM6A* mutation, *SNCAIP* duplication *CDK6, MYCN* amplification
Cytogenetic aberrations	Monosomy 6	3q gain 9q, 10q, 17p loss	i17q 1q, 7, 18 gain 10q, 11, 16q, 17p loss	i17q 7q, 18q gain 8p,11p, X loss

Figure 3. *Medulloblastoma molecular markers/genetically defined subgroups; WNT, SHH, Group 3 and Group 4, from* [67].

The pathologist generates an integrated diagnosis considering both the "histologically"- and "genetically" defined groups, which can then be used for treatment guidance and risk stratification. The current risk stratification for patients between 3-17 years divides risk classes into "low risk", "standard risk", "high risk" and "very high risk" defining outcome (survival). As such SHH p53 mutated and Grade 3 metastatic and MYC amplified tumors are found on one side of the spectrum (very high risk), while WNT non-metastatic are found on the other side of the spectrum (low risk). This risk stratification helps guiding clinicians in how to treat and inform their patients, i.e. raise the possibility to try more aggressive drugs in patients with otherwise poor prognosis, and/or use less aggressive treatment in those with a good prognosis [67].

1.2.3 Medulloblastoma stem-like cells

As in GBM, cancer cells with properties like stem cells (so called cancer stem-like cells) have also been discovered in MB and are often referred to as Medulloblastoma Stem Cells (MBSC). These cells are characterized mainly by their CD133 and/or CD15 positivity which has been proposed to sort and identify MBSC. MBSC are believed to possess self-renewal and multipotent differentiation abilities, conferring resistance to radio- and chemotherapy, high recurrence rate and heterogeneity of MB. Although less is known about the detailed molecular pathways of MBSC in comparison to GCS, the following are key mechanisms often mentioned when discussing *stemness and therapeutic resistance*;

In terms of *therapeutic resistance*, activated PI3K/Akt signaling pathway and secondary p53 mediated cell-cycle arrest has been found in MBSC after irradiation [68]. This transient p53 induction (propagated through the phosphatase and tensin homologue (PTEN)), enables these cells to reenter the cell cycle later, and as such, inhibition of this signaling pathway (PI3K/Akt) in turn decreases this possibility and sensitizes MBSC to irradiation.

In terms of *stemness*, 2 key mechanisms are often mentioned as contributors; the Notch signaling pathway and the Shh signaling pathway. The Notch signaling pathway, upregulated in MBSC as well as normal brain stem-cells, which if inhibited by the gamma-secretase inhibitors, results in a decrease of CD133 positive MB cells [69,70]. Further, it has been shown that hypoxia, through HIF-1alfa stabilization maintains Notch 1 and stem cell phenotype in MB [71]. These different mechanisms of action, all propagated through Notch, demonstrate a central role of this pathway for MBSC stemness. As to the Shh signaling pathway, it has been reported that an increased Shh pathway activation results in proliferation of CD15 positive MBSC and tumorigenesis in MB [72]. To corroborate the importance of the Shh pathway in MBSC stemness, it has been shown that Gli1/2, the main transcription factors in the SHH pathway, interact with stem-related factors such as MYC-N, Nanog and Bmi1 in the self-renewal regulation of MBSC [73,74]

In general, as in GSC, MBSC are believed to contribute to therapy resistance, with MB patients harboring large number of MBSC having poorer prognosis [38,75,76], thus making MBSC a target for future therapies.

1.2.4 Treatment

Current treatment strategy for MB in adults and children aged 3 and above consists of aggressive surgical resection followed by radiotherapy (craniospinal irradiation) and chemotherapy resulting in a cure rate of up to 70% [77,78]. Although this regimen carries a risk of treatment related toxicity with non-negligible risk of affecting quality of life [79-82].

As with GBM, surgical resection aiming at GTR (in medulloblastoma less than 1,5 cc tumor residual) has been the cornerstone of MB treatment for decades, which has with continuous technical advances decreased the current surgical mortality down to < 1% [83]. Traditionally, to be classed as a "standard risk" MB patient, a successful GTR had to be performed. Historically, patients without GTR have had a poorer outcome [84], although recent studies analyzing the importance of extent of resection taking molecular markers into account did not show any benefit in GTR above sub-total resection (STR, more than 1,5 cc tumor residual) in overall survival, regardless of subgroup [85]. Further, there was no benefit in terms of survival- or progression-free survival in those with near-total versus GTR [85], ultimately leading to the recommendation of maximal safe-resection without any apparent clinical benefit of surgical removal of small volume residual disease that carries a high risk of neurological morbidity [85]. Morbidity wise, the most notable risk is the risk of cerebellar mutism syndrome, which approximately 25% of patients develop after surgery [82], resulting in speech difficulties, hypotonia and ataxia. The difficulties usually last weeks to months but can sometimes even be permanent [82].

In terms of radio- and chemotherapy, patients are now treated differently depending mainly on the "risk" classification and age. Thus, patients > 3-5 years classed as "average" risk are most often treated with 23.4 Gy craniospinal irradiation (adults typically receive 36 Gy) with a boost of 55 Gy to the tumor bed, followed by cytotoxic chemotherapy [86,87], while those classed as "high" risk are treated with a higher dose of craniospinal irradiation, 36-39 Gy. Patients < 3-5 years of age are treated with radiotherapy sparing approaches due to the high risk of serious neurocognitive and endocrine side-effects [88,89]. The chemotherapy regimens typically consist of cisplatin/carboplatin-vincristine-cyclophosphamide, albeit even methotrexate (intravenous and intrathecal/ventricular) and autologous hematopoietic cell rescue have been used in more "high" risk cases [90].

1.3 Cell cycle and checkpoint control

Cell proliferation is a fundamental feature of life, the basis of which at the single cell level is the cell division cycle (*cell cycle* for short), a process that must be very tightly regulated to avoid pathologies such as developmental defects (under-proliferation) or cancer (uncontrolled/excessive proliferation). The two critical events during each cell cycle are: i) the duplication of the genome during the DNA synthesis phase (S phase) and ii) the formation of two daughter cells through cell division at mitosis (M phase). The M and S phases are separated by two other, largely regulatory phases G1and G2, overall in the sequence: G1-S-G2-M (**Figure 4).** Cells that are not proliferating, are commonly referred to as being in a so-called quiescence state (G0 phase). There are many proteins that participate and coordinately control the order, timing and quality of the cell cycle events, however the two major engines that drive the progression through the cell cycle stages are cyclin-dependent kinase complexes (CDK1 with cyclin B, CDK2 with cyclins A or E, and CDK4 and CDK6 with D-type cyclins), and ubiquitin ligases Skp, Cullin, F-box containing complex (SCF) and Anaphase-promoting complex (APC) which timely degrade some cyclins and other regulatory proteins in G1, S or M phases, respectively (**Figure 4**). Additional important elements of the cell cycle control machinery include two families of negative regulators known as CDK inhibitors (the ink4 family: p15, p16, p18 and p19; and the Cip/kip family: p21, p27 and p57) and various positive regulators that activate the cyclin/CDK complexes at the desired times and transitions during the cell cycle, such as the Wee1 kinase or the CDC25 family of phosphatases: CDC25A, B and C (**Figure 4**). Supra-imposed on the basic cell cycle machinery are two layers of the so-called checkpoints, mechanisms that ensure the proper duration and quality of the cell cycle steps. Examples of the first type of checkpoint mechanisms include e.g.: a) the so-called restriction point (R point) in late G1 that allows the entry into S phase only when proper growth factors and mitogenic stimuli are available, and b) the regulatory steps around mitosis, such as the mitotic spindle checkpoint that controls the proper alignment and separation of the mitotic chromosomes. The molecular basis of the R-point mechanism reflects mitogenic signals that activate the cyclin D-CDK4/6 complexes which then phosphorylate the pRB tumor suppressor, thereby releasing the E2F transcription factor and activating a range of genes that allow progression into S phase and completion of the cell division cycle (**Figure 4**). Apart from these intrinsic cell cycle checkpoints, another layer of control encompasses mechanisms that sense and respond to insults such as DNA damage, and through a cascade of kinases can activate the p53-p21 checkpoint and other elements that can transiently delay or block the key cell cycle transitions to allow time for DNA repair or prevent cell division with damaged chromosomes (the G1/S, intra-S and G2/M checkpoints, respectively). More details about these DDR checkpoints including the kinase cascades involved and the effector pathways, are presented in the section 4.2 focusing on the DDR machinery**.**

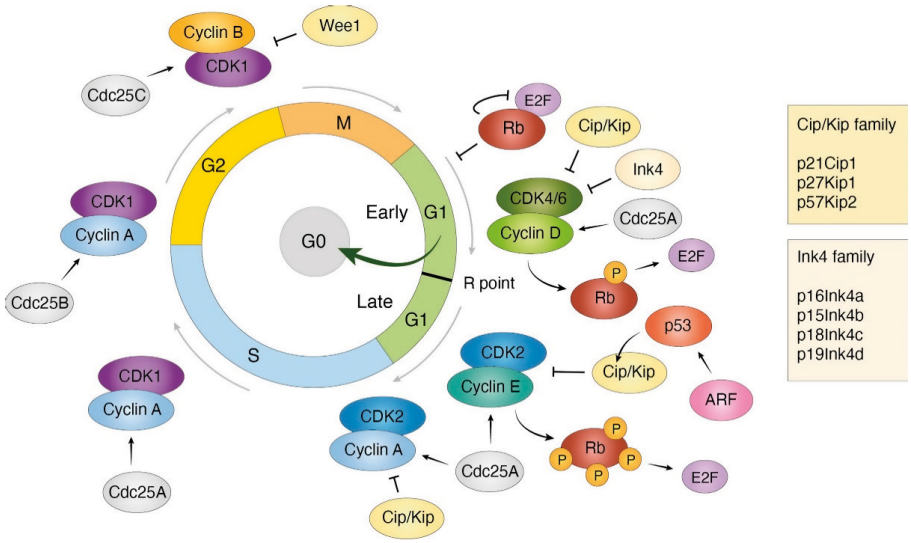

Figure 4. *Cell Cycle regulation mechanisms, from* [91].

1.3.1 Genomic instability, DNA damage response (DDR) and tumorigenesis

Both exogenous and endogenous events or insults, such as faulty DNA replication, ionizing- and ultraviolet radiation and genotoxic substances such as metabolic reactive oxygen species (ROS) can result in the formation of diverse DNA lesions, which, if left unrepaired or mis-repaired, can cause genomic instability. Depending on the type of cells involved (Germ cells vs. somatic cells) the genomic location of mutations, extent and timing, the genomic instability may predispose to (hereditary) or contribute to initiation or progression (somatic) of cancer [92]. In the last decade or so, novel molecular techniques and cancer genome analysis have provided new insights into genomic instability and its roles in tumor evolution as well as response to treatment. The wide spectrum of alterations on a genetic level that together represent the genomic instability and can contribute to tumorigenesis include for example subtle (often point) mutations or chromosomal abnormalities such as deletion-, amplification- or rearrangements of chromosome segments or even gain(s) or loss(es) of whole chromosomes. The latter more gross chromosomal alterations are often referred to as chromosomal instability (CIN) and lead to the state of cancer cells with altered chromosome numbers or structure (aneuploidy). Along with epigenetic changes, the mutations and chromosomal aberrations lead to activation of oncogenes or loss of tumor suppressors, collectively driving tumorigenesis.

In the presence of DNA lesions, an otherwise healthy cell responds by activating the DNA damage response. If unable to repair the lesions, cells will either prematurely stop proliferating (cellular senescence) or undergo cell death, often by apoptosis. As such, the DNA Damage Response (DDR) mechanism(s) is a safeguarding network that senses the presence of single- and double strand DNA breaks, modifications of incorporated nucleotides or aberrant replication fork structures, as well as alterations in higher-order chromatin structure – which all can lead to DDR activation, resulting in activation of cell cycle checkpoints, DNA repair mechanisms and other responses, potentially including apoptosis [93]. These mechanisms are crucial for maintaining genome integrity, and thereby preventing genetic diseases including cancer [94].

As briefly eluded to above, it is important to distinguish between hereditary- and non-hereditary (sporadic) cancers. Hereditary cancers are the consequence of mutations in the germ-line, often affecting DNA repair or checkpoint genes, while the non-hereditary cancers reflect mutations acquired by somatic cells during the lifespan. According to the so-called mutator hypothesis (not generally accepted, however), the initial instability seen already in early precancerous lesions will progressively lead to increase in the spontaneous mutation rate, thus driving tumorigenesis [95,96]. Among examples of hereditary cancers are the mutations in breast cancer susceptibility genes (BRCA1 and BRCA2) which are linked to homologous recombination repair of DNA double strand breaks (DSB), or those mutations that inactivate components of the DNA mismatch repair pathway associated with colon cancer (HNPCC/Lynch Syndrome) and endometrial cancer. The germline mutations are present in every cell in the body, they are largely recessive, and therefore they often manifest fully only when the other allele gets deleted or affected by another mutation in the incipient tumor cells. On the other hand, when it comes to non-hereditary cancers (sporadic), the molecular basis is more variable, there is currently no evidence of any uniform pathway, albeit it is believed that the major contributor to genomic instability is DNA replication stress as discussed in more detail below [97].

In general, one can argue that cellular DNA is under constant attack from both endogenous and exogenous genotoxic insults. As such, even a task as "simple" as maintaining a "healthy" genome requires a repair of more than 10 000 DNA lesions per day [98]. For this purpose, cells have a sophisticated DNA damage response (DDR) machinery in place, with many proteins that sense the diverse types of DNA lesions, initiate signaling pathways that result in activation of checkpoints to assure that damaged DNA is not replicated, and affect many aspects of cell function [99,100]. The checkpoint activation and subsequent effector mechanisms depend on the nature of the damage and cell cycle phase.

1.3.2 The cellular DDR machinery

From a simplified point of view, DNA lesions are found in the form of 1) damaged bases, 2) mis incorporated bases, 3) single strand breaks (SSB), 4) double strand breaks (DSB) and the recently recognized 5) obstacles to DNA replication forks (generally defined as replication stress, discussed separately under 4.3). Depending on the lesion type, different mechanisms are responsible for the response and action in terms of DNA repair: 1) Damaged bases are removed either by nucleotide excision repair (NER) (extra bases added after e.g UV exposure) or base excision repair (BER) (in case of alkylated or oxidized bases), while mis-incorporated bases are removed by mismatch repair (MMR).

The signaling from lesions to diverse checkpoint and repair processes is initiated by activation of different kinases, of which phosphatidylinositol-3-kinase like family (PIKK), including ataxia-telangiectasia Rad-3 related (ATR) and ataxia-telangiectasia mutated (ATM) as central signal transducers (**Figure 5**). Whereas ATM (and another important PIKK member, DNAPKcs) primarily respond to DSBs (and operate throughout the cell cycle), ATR is responding to various forms of damage during DNA replication, often coupled to single strand DNA breaks and stalled replication forks in S-G2 phases. Together with the effector kinases Chk1 and Chk2, the major signaling modules ATR-Chk1 and ATM-Chk2 then phosphorylate hundreds of cellular proteins and thereby trigger the overall multifaceted cellular DNA damage response [94], eventually leading to cell cycle delay, DNA repair, chromatin modulation, transcriptional and metabolic changes, overall designed to survive and repair the damage, or, in case the damage is too severe or irreparable, to permanently arrest or eliminate such potentially unstable cells (**Figure 5)**. For example, the cell cycle delay or arrest are caused by phosphorylation of p53 by the above-mentioned DDR kinases, and thus activation of the p53-p21 axis that blocks the activity of some CDKs, or by degradation/inhibition of the cell cycle-activating CDC25 phosphatases, again through phosphorylations mainly by the effector kinases Chk1 and Chk2. In terms of chromatin response, the ATM (recruited by the MRN complex that senses DSBs), ATR and DNAPKcs kinases can each phosphorylate histone H2AX near DNA breaks, and such modified histone (called gammaH2AX) then serves as a starting point to recruit a cascade of DNA repair proteins (including e.g. the 53BP1, **Figure 5**). In addition, detection of gammaH2AX by antibodies provides a robust marker for overall DNA damage signaling and, if observed in the form of nuclear foci, this marker provides a surrogate for genomic DNA double strand breaks in that cell, or in a clinical specimen.

As indicated above, one prominent substrate of DDR checkpoints is p53, whose activity and abundance increases due to protein stabilization, with the ensuing broad transcriptional response driven by p53. Apart from such moderate and widely cell-to-cell variable increase in p53 abundance, p53 is very frequently mutated in cancer

and the point-mutant forms are commonly very stable, providing a helpful surrogate marker for p53 mutation even when analyzed by immunohistochemistry in archival paraffin samples. This is a very frequent event that is selected for in cancer progression, providing a way for cancer cells to escape the p53-mediated cell death or senescence that is otherwise triggered upon DDR signaling to p53 under conditions of oncogene-induced replication stress or DNA damage. Overall, these and additional antibody-detected markers (such as the 8-oxoguanine (8-OXO) lesion reflecting oxidative DNA damage, relevant for the studies presented in this thesis) can be followed in both clinical samples and cell culture models, and allowed us to obtain a large amount of information about human brain tumors, in this context especially in relation to HCMV, replication stress and stemness phenotypes, and about features important for tumor evolution and responses to treatment [94,101,102].

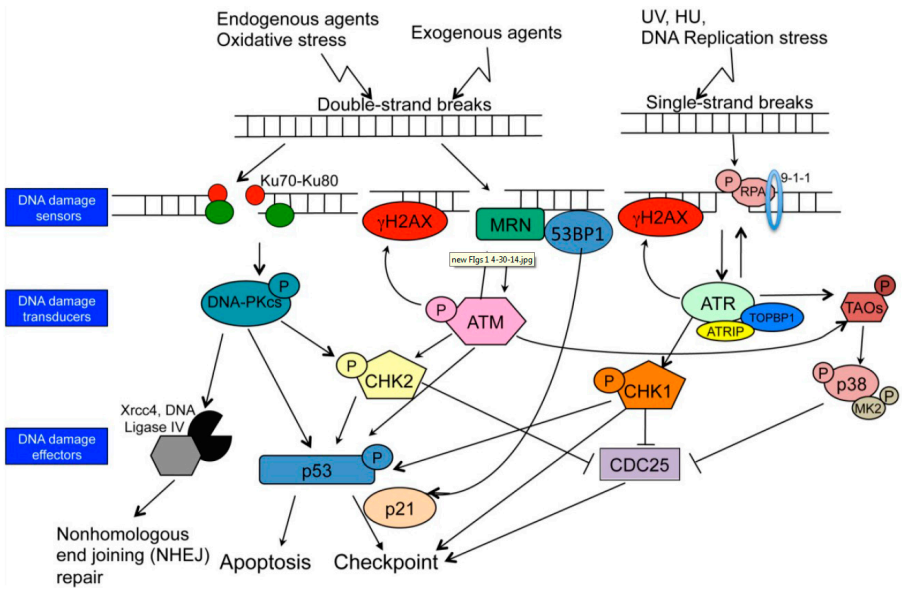

Figure 5. *Cell cycle checkpoint induction through the DDR network, from* [103].

1.3.3 DNA Replication stress (RS): an emerging hallmark of cancer

DNA replication stress is currently defined in broader terms as any obstacles or deregulation of DNA replication process that alter progression and/or fidelity of DNA replication forks. Accumulating evidence indicates that replication stress (RS) represents a very common phenomenon shared by almost all cancer types, leading to a proposed candidacy of RS as an emerging hallmark of cancer. This

concept postulates that activated oncogenes or loss of some tumor suppressors responsible for deregulated cell proliferation and DNA synthesis induce DNA replication stress early in the tumor evolution - often in the preinvasive early lesions. The RS triggers activation of checkpoints responses including the p53, and in this way the activated DDR machinery provides a biological barrier against further tumor progression, mostly leading to cell cycle arrest/senescence or apoptosis. However, this stressful environment under RS at the same time creates conditions for selecting mutations such as in p53 or other DDR checkpoint genes, eventually allowing escape of rare tumor cells from the DDR checkpoints, allowing for cell proliferation and at the same time enhancing genomic instability, which in turn fuels tumor progression and impacts responses to treatment [104].

At the level of replication forks, RS can result in "replication fork stalling" or even fork collapse, the latter then leading to creation of DNA breaks or even cell death, unless prevented by control mechanisms of cell cycle progression. These include mainly S-phase checkpoint responses dependent on the RPA-ATR-Chk1 axis that becomes activated by excessive stretches of single-stranded DNA that form during RS. The ATR signaling prevents new DNA replication origins from 'firing', at the same time stabilizing the affected replication forks and preventing fork collapse. The stalled forks can be repaired by the homologous recombination repair mechanisms that include also the BRCA1/2 proteins and re-started, thereby resuming normal DNA synthesis and ensuring satisfactory replication completion. During tumorigenesis, more severe replication stress conditions can be triggered by diverse oncogenes or loss of tumor suppressors, triggering the DDR anti-cancer barrier as mentioned above, to ensure genome integrity [93].

As in case of DNA lesions caused by other insults (i.e. ionizing or UV radiation), DDR mechanisms that respond to RS ensure maintenance of genome integrity by making sure "damaged" cells are either brought to a state of senescence or forced into apoptosis [105]. Consequently, a non/dys-functional DDR machinery results in genomic instability and potential progression of tumorigenesis [105]. In summary, while hereditary cancers often result from germ-line mutations in DNA repair genes, in non-hereditary cancers, it is commonly the oncogene induced replication stress that is believed to fuel genomic instability and the process of tumorigenesis.

1.3.4 Viruses and the DNA damage response

Recent evidence in the fields of DNA Damage and Virology has demonstrated that mammalian viruses (such as HCMV) use different strategies to disturb host defense systems, including altering the DDR in the host – to facilitate viral replication. More specifically, DNA viruses have been shown to induce and subvert the DDR [106]. RNA viruses may also induce DDR, albeit the mechanism by which

RNA viruses affect the DDR might be indirect. The mechanism through which viruses activate the DDR is usually mediated through the DSB signaling pathways. For example, certain oncoproteins of DNA viruses such as papillomavirus E6 and E7, simian virus Large T antigen or adenovirus E1a and E1b, disable the p53 tumor suppressor or interact with the RB family of tumor suppressors in ways that result in E2F mediated cell cycle stimulation even in the absence of physiological growth stimuli. Such deregulation of DNA synthesis may lead to DNA breakage and activation of the ATM/ATR/DNAPKcs signaling pathways, favoring virus replication through virus-induced modulation in the host cell DDR [107]. In general, the modulation of the DDR is somewhat different from virus to virus, but in all cases, it results in recruitment of host-cell DNA repair proteins to the viral replication compartments [108-111], which then aids the virus with the replication procedure. As such, i.e. EBV induces phosphorylation of ATM, H2AX, CHK2 and p53 during lytic infection, after which these are moved to the EBV replication compartments. As for the interaction between HCMV and DDR – please see chapter 1.5.1.

1.4 Human Cytomegalovirus (HCMV)

Human cytomegalovirus (HCMV), also known as human herpesvirus-5 (HHV-5), is a member of the Herpesviridae (Herpesvirus) family which is carried by 60-100% of the world's population [112]. Following an active primary infection, often asymptomatic, the virus establishes latency in the bone marrow, mainly in the cells of myeloid lineage [113,114].

HCMV structure

The structure of HCMV is typical of that of a herpes virus, being 200-300 nm in diameter, consisting of: i) icosahedral nucleocapsid surrounded by ii) proteinaceous tegument and an iii) outer lipid bilayer membrane. The nucleocapsid proteins assemble into A, B and C capsids of which only one (C) contains the genome and can as such form a "mature" virion, while A and B form non-infectious particles [115]. ii) The tegument is a protein layer surrounding the nuclear capsids before they become enveloped. It consists of more than 30 proteins, of which the ones with the highest concentration are pp65, pp71, pp28 and pp150. pp65 is the most abundant, mainly responsible for early immune system evasion [116] during infection, while pp28 is responsible for the cytoplasmic envelopment of tegument proteins during lytic infection [117]. pp71 activates immediate early genes and as such viral replication during infection [118]. Finally, pp150 is responsible for assembly of virus particles, as well as incorporation of nucleocapsids into these particles [118]. iii) The outer bilayer (envelope) is assembled in the host cell after it has passed through the ER – in Golgi derived vesicles - and released through virus budding [119]. It

contains more than 20 different viral glycoproteins (i.e. glycoprotein B (gB), gH, GM, GN), as well as glycoproteins from the host cell. These play an important role in the life-cycle and spread of the virus infection, where incorporated host glycoproteins contribute to the ability of the virus to spread without the hos immune system recognition [120]. In the viral nucleocapsid, double stranded DNA is packed, which constitutes the HCMV genome, consisting of 235 kbp with 252 open reading frames (ORF) [121]. The HCMV genome however produces about 750 RNAs [121]; most of these are not characterized. The genome is divided into regions named the long (UL) and short (US) – which also gives name to the respective HCMV genes and proteins [122]. Although the genome encodes for over 750 mRNAs, and likely but not yet defined, encode for hundreds of proteins, only about 50 of these are implied to have a role in the viral replication process, the rest are believed to function to aid the virus to coexist with its host [121].

1.4.1 Replication and latency of HCMV

Replication

The HCMV replication takes place mainly in endothelial, epithelial cells and inflammatory activated macrophages, but the virus can also infect neutrophils, fibroblasts, smooth muscle cells (SMC) as well as neurons and glial cells [123]. The virus utilizes surface glycoproteins gB and gH/gL which are essential for cell attachment [124]. After attachment, the viral envelope fuses with the cell membrane leading to the release of nucleocapsids into the cytoplasm. These then travel through the cytoplasm and are eventually translocated into the nucleus, where the viral DNA is released. This leads to viral gene expression in the form of both immediate early (IE)-, early (E)- and late (L) genes, all of which involved in the process of viral replication. IE are produced first, and are responsible for the regulation of transcription, E genes are responsible for the viral DNA replication and L genes encode the structural proteins and as such appears last in the replication cycle [125]. The viral DNA is replicated via HCMVs own functional DNA polymerase in the host cell nucleus and within special viral replication compartments, while the host RNA polymerase II transcribes all the HCMV genes [126]. After the replication process is complete (approximately by 24-48 hours post infection), the viral DNA is packed into newly synthesized capsids and transported from the nucleus to the cytoplasm. During this process, it passes through the cytoplasm and the endoplasmatic reticulum (ER) acquiring initially the lipid envelope and tegument proteins, and finally the secondary envelopment before being released from the cell as a virion – a process that takes about 72 hours (**Figure 6**).

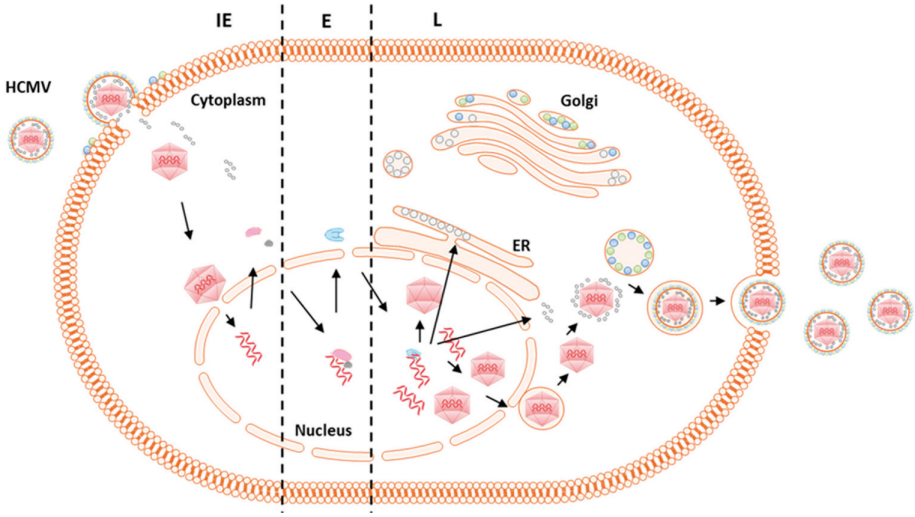

Figure 6. *Human cytomegalovirus (HCMV) life cycle, from* [127].

Latency

As is the case with the other herpes viridae, after primary infection, a life-long latency is established. For HCMV this mainly occurs in cells of the myeloid lineage (i.e. in monocytes and granulocyte-macrophage progenitor cells) and the hematopoietic cell population (CD34+) of the hosts bone marrow [128]. Recent studies have shown that latency, previously thought to be a passive process, is in fact an active process, with different genes being transcribed during latency. For instance, several latency associated proteins are responsible for immune system redirection (US28 and ORF94) and evasion by decreasing the immune recognition (UL44 and UL144) and cmvIL-10 [129]. In general, the latent HCMV gene expression is somewhat heterogeneous, varying between different cell populations [130,131], implying that it is likely the local differences in the cellular milieu that are responsible for the different expression profiles. Further, it has been postulated that HCMV related non-coding RNAs are transcribed during latency [130], making sure that the host cell environment can be modulated to suit the latent infection, without attracting the attention of the immune system. Finally, it is important to keep in mind that latent HCMV can be reactivated by inflammation and stress, often mediated through the initial upregulation of TNFalfa, IFNgamma and GM-CSF [114] which in turn differentiate monocytes into dendritic cells or macrophages. This is important since not all cells permit viral replication – i.e. undifferentiated cells such as monocytes – wherefore it is not until the monocytes are differentiated to macrophages or dendritic cells that the virus is reactivated, and the replication can commence [128]

1.4.2 HCMV epidemiology, transmission and clinical course

Epidemiology

HCMV is generally viewed upon as a very common infection in the general population worldwide, with a seroprevalence of 60-100%, although to a certain degree dependent on the socioeconomic status, geography [112] and patient age, with the seroprevalence increasing with age (up to 90% in the elderly) [132].

Transmission

HCMV has been shown to be transmitted in body fluids exclusively. These include blood, urine and saliva as well as through unprotected sexual contact. Mother to child infection can either be passed along through an intrauterine infection (unborn child) or through breast milk (after child birth) [133].

The clinical course of the HCMV infection

The clinical course varies mostly depending on whether the patient is immunocompromised or not. As such, the primary infection in the immunocompetent individual is often subclinical, although in certain cases the patient does present with mononucleosis-like-symptoms such as fatigue, fever and headache - with even rare cases of adenopathy, hepato- and/or splenomegaly recorded. Mortality is almost never seen, although some cases have been recorded [134]. When the active/lytic infection is over, the virus establishes latency in the bone marrow and circulate in the blood in cells of the myeloid lineage [113,114]. Besides the symptomatology above, evidence has emerged on the presence and possible contributing role of HCMV in several other inflammatory diseases such as atherosclerosis, inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus and psoriasis [135-140]. In principle, since HCMV is reactivated by inflammation, HCMV might represent an epiphenomenon in the above context, or be a contributing factor in the inflammatory process itself as it is able to induce inflammation via for example COX-2 and 5-LO and production of pro-inflammatory cytokines. So far, no consensus has been established on this matter.

In the immunocompromised individuals, such as organ- and stem cell transplant patients, the infection can end in serious developing adverse events resulting in a high risk of morbidity but even mortality [133]. The risk of morbidity and mortality has been inversely associated with the level of immunological competence – i.e. low immunological competence = higher risk of morbidity and mortality in terms of more severe symptoms from the HCMV infection itself, but also higher risk of secondary opportunistic bacterial and fungal infections [141]. Due to this, i.e. organ transplant patients are often treated with antiviral prophylactic therapy (pre- and) post-transplantation to avoid the risk of active and potentially life threatening HCMV infection [142]. This drug regimen also protects against long term effects

of HCMV such as chronic rejection and myocardial infarction. Another exposed patient category is HIV patients – who often are coinfected with HCMV – and at risk of developing HCMV promoted cardiovascular and cerebrovascular inflammatory diseases [143]. Nowadays, anti-retroviral therapy targeting HIV makes sure that the patients CD4+ cell count stays above 50 cells/mm^3; this almost eradicated HCMV retinitis and severe gastroenteritis cases, which previously caused major morbidity and mortality among HIV patients [144].

Finally, the congenital HCMV infection, seen in 0.2-3% of infants born in western countries, is most often asymptomatic, while petechial rash, hepato-splenomegaly, jaundice, chorioretinitis and neurological deficits such as microcephaly, sensorineural hearing loss and retardation is seen in those that are symptomatic [145], while hearing loss is common even in otherwise asymptomatic cases at birth, and may present after several years.

1.4.3 Diagnosis of HCMV

Classically, HCMV infection was for a long time diagnosed thanks to the so-called "owl's eye", or intranuclear inclusion bodies, visible in tissue specimens through the microscope [146]. However, direct microscopy has today been replaced by more advanced diagnostic methods such as a) serology, b) immunohistochemistry, c) quantitative nucleic acid testing (i.e. polymerase chain reaction, PCR) and d) in situ hybridization.

a) Serological techniques are focused on the detection of antibodies, which can tell the examiner if the patient has a primary infection (IgM antibodies present) or if the patient has been infected previously (IgG antibodies present) and usually determined by Enzyme-linked immunosorbent assay (ELISA) [147].

b) IHC uses antibodies to label different HCMV proteins, making it possible to easily detect virus infected cells under a microscope, with the advantage to distinguish- and co-localize these with other cellular proteins. It is important to note that the sensitivity and specificity of the antibodies commercially available varies greatly, necessitating optimization of the IHC protocols [146]. This is in particularly important when detecting HCMV proteins in tumor tissues samples, as a regular IHC method need antigen retrieval steps for detecting HCMV proteins in these specimens.

c) Quantitative nucleic acid estimation by PCR is the most widely used technique for HCMV detection in the clinical setting [147], mainly thanks to the technique's high sensitivity and specificity, as well as the quantitative output, making it easy to ascertain the viral load in patient samples, and to follow treatment responses after initiation of anti-viral therapy [147].

d) In situ hybridization (ISH) uses labeled complimentary DNA strand probe to detect specific HCMV DNA in areas of interest in a tissue sample i.e. HCMV nucleic acids detection in human brain tumor tissue. These can be labeled with biotin or fluorochromes making them easily visible under a light or fluorescent light (FISH) microscope, respectively [148]. This technique can not define whether the virus is active or latent in the tissue specimens.

1.4.4 Treatment of HCMV

The most widely used anti-HCMV treatment(s) available all target the viral DNA polymerase: i) Ganciclovir (GCV) and it´s oral prodrug ii) Valganciclovir, iii) Foscarnet and iv) Cidofovir. Besides drugs targeting the viral DNA polymerase, there are drugs in pipe-line with different HCMV targets, such as Maribavir and Letermovir, both dampering viral DNA synthesis, and both successfully implemented when treating resistant HCMV strains [149,150].

Of the drugs targeting DNA polymerase, i) GCV is the oldest available – and still the drug of choice when it comes to treatment of active HCMV infection. GCV, which is available in both tablet and intravenous formula, is an acyclic nucleoside analog of 2′-deoxyguanosine that is phosphorylated by the viral protein kinase UL97 into a biologically active triphosphate form that inhibits viral DNA synthesis by acting as a nucleoside analogue to block the viral DNA polymerase [151]. Although effective, mutations in the HCMV UL97 gene causing GCV resistance have been observed [152]. Further, even though known side-effects are rare, kidney and liver toxicity cases, as well as hematologic abnormalities have been reported [153]. ii) Valganciclovir (VGCV) is the per oral pro-drug of GCV (available in tablet- and oral mixture form), most frequently used in transplant patients as an effective prophylaxis. VGCV metabolizes to the active form in the intestinal wall [153]. iii) Foscarnet, available in intravenous formula only, is a pyrophosphate analogue, which does not require enzyme activation to exert its effect. Foscarnet is considered a second-line treatment to be used when GCV is not effective due to resistance. The side-effects of Foscarnet treatment include mainly nephrotoxicity, which necessitates close monitoring of patients treated [154]. iv) Cidofovir, an acyclic nucleoside phosphonate analogue, is only available in intravenous formula and is converted to its active form by cellular kinases [155]. The main advantage of Cidofovir is its long intracellular half-life, that allows for enough treatment even in case of treatment delay [155]. As with Foscarnet, nephrotoxicity is a known potential side-effect and Cidofovir is only used as a second line treatment in certain countries (not available in Sweden).

Besides the above, there are currently immunoglobulin preparations (IVIG) available in the form of hyperimmunoglobulin's HCMV IVIG (purified IgG from HCMV seropositive individuals) and standard immunoglobulins polyvalent IVIG

(from donors with unknown HCMV status) [156]. Due to known side-effects such as venous thrombosis and the risk of other disease transmission through blood products, IVIG treatment is not used as standard treatment [156]. Finally, HCMV vaccines, although promising treatment modalities, are still only under development [157]. Finally, the treatment of HCMV in context of brain cancer will be discussed more extensively in chapter 6.2.

1.5 HCMV and (brain) cancer

HCMV and malignant primary brain tumors

Since the early 2000, reports have come out on the presence of HCMV proteins and nucleic acids in multiple solid tumors, such as cervix cancer, ovarian cancer, prostate cancer, breast cancer, colon cancer, sarcomas, medulloblastoma, neuroblastoma and malignant glioma [158,159]. Nevertheless, since the focus of my thesis are primary brain tumors, these will be the focus of the following chapter on HCMV and brain cancer.

After the initial discovery of a high prevalence of HCMV in glioblastoma, further research has led into the charting of more than 90% of patients shown to have an active HCMV infection in multiple malignant primary- as well as secondary brain tumors [148,160,161]. As such, active HCMV infection has also been found in GBM and MB brain tumor tissue [161,162], while at the same time remaining absent or latent in non-brain tumor tissue from the same patients. Further, the levels of active HCMV infection have been found to be of prognostic value in terms of correlation between high HCMV infection positivity and decreased overall survival [162]. Nevertheless, even though active HCMV was found in tumor cells, as well as in endothelial- and inflammatory cells within the tumor, infectious virus that could be cultured *in vitro* has so far not been obtained from any tumor sample. The question thus remains if this HCMV presence in any way contributes to the development of brain tumors, or if active HCMV infection simply represents an epiphenomenon.

Viruses and cancer

Today, the oncogenic (leading to neoplastic transformation) or oncomodulatory (modulating already neoplastic cells) role of certain viruses in tumorigenesis is undisputable; hepatitis C and B virus cause hepatocellular carcinoma [163], human papilloma virus (HPV) causes cervix and oropharyngeal carcinoma [164], Epstein Barr virus (EBV) contributing to nasopharyngeal carcinoma and Hodgkin´s lymphoma [165], HHV-8 virus contributing to Kaposi´s sarcoma [166], and human T-cell leukemia retrovirus (HTLV) is associated with T cell leukemia [167]. About 20% of human cancers are caused by these viruses. As for HCMV, accumulating evidence demonstrating a link between cancer and persistent active HCMV infection [168], but this virus is also not considered as an oncogenic virus. The oncomodulatory

effect of HCMV is believed to be propagated through different viral effects post cell infection, but only rarely transformation of normal cells into cancer cells has been observed [168,169]. In general, HCMV viral proteins are thought to affect cell cycle progression differently in different cells, depending on the cellular differentiation level – believed to promote tumor growth without affecting the surrounding non-tumor cells [170,171]. In the context of the "hallmarks of cancer", as defined by Hanahan and Weinberg [172], I will in the following sections go through the different aspects of HCMV in relation to; *sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activation of invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death and deregulating cellular energetics.*

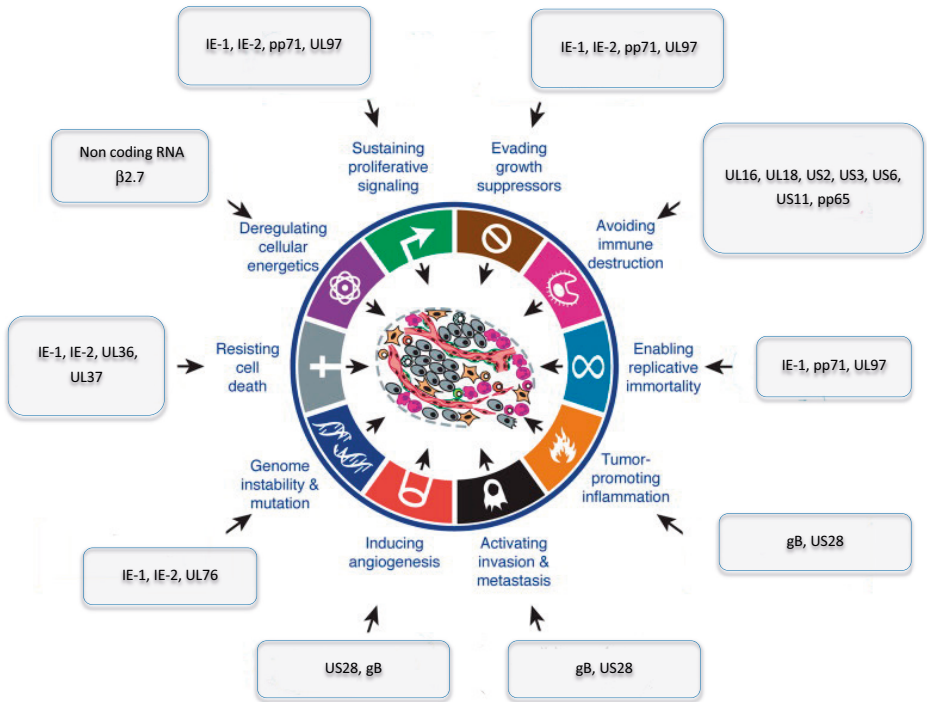

Figure.7. *The "hallmarks of cancer" and HCMV effectors, modified from [172].*

Sustained proliferative signaling and evading growth suppressors

The most renown feature of cancer in general, is its ability to promote sustained proliferation, while this is otherwise strictly controlled by complex signaling mechanisms, mainly consisting of growth factors and cytokines. The two main pathways often dysfunctional and/or overexpressed in cancer are the MAPK- and

PI3K/AKT pathways, that have been shown to stimulate cell growth, proliferation and cell survival [173,174]. In HCMV infected cells, a commonly seen feature is activation of the PI3K pathway, promoting cell proliferation while also initiating viral DNA replication [175]. Similarly, the MAPK, is also activated by HCMV resulting in ensuring viral DNA replication [176]. It has also been shown that HCMV infected GBM cells dysregulate Rb phosphorylation and p53 expression [171]. Related to this, several regulatory proteins of HCMV, such as IE-1, pp71 and UL97 can inactivate pRb promoting cell cycle progression [103]. Also, IE-1 and IE-2 are known to deregulate the cell cycle checkpoint controls by interacting with p53 suppressor proteins [103,177].

Resisting cell death

Programmed cell death, or apoptosis, is essential for maintenance of body homeostasis, making sure damaged cells die and are eliminated in an orderly fashion. This mechanism is believed to be activated by 2 different pathways, known under the terms extrinsic and intrinsic pathways – both leading to induction of cell death and caspase activation making sure the cell is degraded in an orderly fashion [178]. In brief, the extrinsic pathway is initiated outside of the cell, propagating it signaling through the so-called cell death receptors such as TNF-alfa and FAS-ligand receptor. Likewise, the intrinsic pathway is activated inside the cell itself, mainly due to signaling from DNA damage propagated through different proteins such as p53, Rb while being regulated by the Bcl-2 protein family. HCMV has several properties enabling it to inhibit apoptosis, thereby improving the survival of HCMV infected cells. As such, IE-1 and IE-2 block apoptosis mediated by TNF-alfa [179], IE-2 binds p53 and thus inhibits its activation [180] and UL36 and UL37 inhibit apoptosis by inhibiting caspase activation (blocking FAS-ligand mediated apoptosis cascade) [181] and inhibition of the mitochondrial pro-apoptotic proteins Bax and Bac (inhibition of mitochondria mediated apoptosis), respectively, thereby conferring resistance to chemotherapy in chronically infected neuroblastoma cells [182].

Enabling replicative immortality

Normal cells have usually a cell cycle consisting of multiple divisions, although eventually they all undergo senescence and die. The main actors controlling this delicate process are the pRb and p53 proteins, which if inhibited, can result in uncontrolled cell divisions [183]. In this context, telomerase, an enzyme frequently expressed in tumors, makes sure to avoid any shortening of the telomeres, preventing senescence and ensuring endless replication ability [184]. My research group has shown that the activation of telomerase and telomere lengthening through interaction with the hTERT promotor is induced by IE-1 in HCMV infected cells, and IE-1 and hTERT was shown to be co-expressed in GBM cells [185]. Also, in relation to the above paragraph on proliferation, HCMV expresses pp71 and UL97 – both involved in phosphorylation and secondary inactivation of the Rb family tumor suppressor proteins [186,187], modulating proliferation and survival of the infected cell.

Genome instability and mutation

Another hallmark of cancer, called genomic instability and mutation, occurring in case of limitless divisions, as mutations accumulate in the cell genome leading to destabilization. The destabilization itself can then lead to further mutations in important cellular genes controlling gene amplification, deletion, chromosomal segment re-arrangements, gain- or loss of entire chromosomes and others. As such, mutations in p53, pRb and Bcl-2 are known as being responsible of driving several hallmarks of cancer [188], and described in the paragraphs above, HCMV interacts with all of these. Please see chapter 1.5.1 on HCMV and DNA Damage for further details.

Inducing angiogenesis

Angiogenesis is one of the first acknowledged hallmarks of cancer, an essential component of tumor development and enlargement which carries the need for vascularization. The levels of pro-angiogenic (i.e. VEGF and basic fibroblast growth factor) and anti-angiogenic (i.e. thrombospondin-1) factors within the tumor change so that levels of pro-angiogenic factors increase whenever the tumor sees the need for angiogenesis, with significant impact on metastasis and overall tumor progression [172]. In the context of HCMV, promotion of angiogenesis has been demonstrated earlier, with US28 mediated upregulation of VEGF and secondary induction of angiogenesis in GBM tumors by affecting endothelial cells [189]. In our lab, we could show that the infected cells do not exhibit angiogenic properties, but rather promote this in noninfected cells around the infected one through a paracrine effect [190]. Thrombospondins on the other hand, important for anti-angiogenic control, are downregulated in HCMV infected GBMs [191].

Avoiding immune destruction

The loss of anti-tumor immune response in the tumor microenvironment is an important step in tumorigenesis, supported by data showing increasing number of newly diagnosed infection-related cancers such as EBV and Kaposis Sarcoma in immune-compromised individuals [192]. In general, a defect in either the protective function of the immune system against tumor cells (host related) and/or the evasion of the tumor cells from an attack mounted by the immune system (cancer related) can result in a dysfunction in the proper immune response. Multiple mechanisms behind this have been charted, among the more prominent ones is the presence of immunosuppressive signaling and/or increased presence of immunoregulatory cells. An example of immunosuppressive signaling are cytokines such as IL-10, TGF-beta and PGE2 released from the tumor microenvironment [193], while an example of immunoregulatory cells are T regs, which are expressed in higher numbers in infected individuals and suppress cytotoxic lymphocyte function [194]. HCMV inhibits the expression of HLA class I and class II molecules and antigen

presentation (US2, US3, US6, US11, IE-1), it controls T cell activation, inhibits NK cell activation (UL18, UL16), blocks interferon (IFN) signaling (pp65), protects cells from cytolytic peptides that are released from activated T and NK cells [195]. HCMV also produces and controls cellular production of chemokines, cytokines and growth factors [196]. These are examples of strategies that make infected cells invisible to the immune system and may explain why HCMV infected tumors are not controlled by the immune system and express variable response to immunotherapies developed against them, as infected tumor cells will be invisible to the immune system [197].

Tumor-promoting inflammation

At the same time as the virus avoids immune recognition, it is dependent on inflammation. Our group was first to identify cells of the myeloid lineage as the major circulating carriers of latent virus, and that immune activation of T cells and the consequent production of TNF-alfa and IFN-gamma, resulting in macrophage differentiation, is a key element in the reactivation of latent HCMV [114]. HCMV also affects COX-2 and my research group found that the virus also induces 5-LO expression [198], which results in induced inflammation and enhanced virus replication with high relevance in tumor biology. In fact, in our recent work, only HCMV positive cells in medulloblastoma expressed COX-2 [161].

It is well established that chronic inflammation both contributes- as well as predisposes to cancer, with anti-inflammatory drugs such as aspirin linked to risk reduction of suffering from cancer, as well as lowering the risk of metastasis [199]. As such, tumor micro environment including among other inflammatory cells (i.e. myeloid cells) and fibroblasts functions as a supportive matrix for tumor growth, potentiated by HCMV infection which resides in the inflammatory cells as well as in tumor cells, as has been shown in among other colon, breast and glioma patients [200] [201].

Similarly, Cyclooxygenase (COX) has been associated with poor survival in cancer patients when overexpressed [202], as has increased PGE2 [203] – both of which have been target for recent trials with non-steroidal anti-inflammatory drugs such as COX inhibitors [204]. Since COX inhibitors are also good anti-HCMV drugs, the effect observed in cancer patients may involve effects on HCMV. Also, our group has recently reported that HCMV infection in medulloblastoma induces COX-2 expression, - with the COX-2 inhibitor in combination with Valganciclovir inhibiting HCMV replication and PGE2 production *in vitro*, with reduced cell- and tumor growth reported [161].

Activation of invasion and metastasis

To metastasize, tumors must invade the supportive tissue around the tumor mass itself, and breach through to the blood- or lymphatic vessels. Then it must travel to a distant site, where it extravasates from the vessel and initiates cell division eventually forming a metastasis. In relation to HCMV, several studies have reported on the effect HCMV might have on promoting activation of invasion and metastasis; in prostate cancer, HCMV infection increased adhesion to the endothelium, leading to disruption in membrane integrity, aiding in tumor cell migration [205], while another group found that HCMV infected renal tubular epithelial cells underwent epithelial-mesenchymal transition, while increasing the expression of TGF-beta and MMP-2 – believed to be initiated by IE [206]. When it comes to primary brain tumors, HCMV infection has been shown to increase tumor cell adhesion and migration through endothelial cells in neuroblastoma [207] and cell migration through US28 in glioma [208], possibly enhancing invasiveness. In a clinical context, our group has reported on HCMV being expressed in both colon- and breast cancer patients, in lymph node metastases and brain metastases with high-viral load being associated with shorter time to tumor progression and shorter survival.

Deregulating cellular energetics

HCMV has been associated with several mechanisms involving cellular energetics. The metabolic alterations observed in HCMV infected cells are strikingly like those observed in cancer cells and most likely influence on HCMV mediated oncomodulation. For example, i) HCMV induces the Warburg effect [209], enhances glycolysis, fatty acid synthesis and glutamine uptake. ii) HCMV stabilizes ATP production through binding of the non-coding RNA beta2.7 to complex I in the mitochondrial respiratory chain – and simultaneously inhibit apoptosis in HCMV infected glioma cells [182], and iii) HCMVs affects the intermediary metabolism in infected cells –similarly to tumor cells in general, HCMV promotes incomplete glucose degradation in the tricarboxylic acid (TCA) cycle for energy: glucose is metabolized to citrate, leaves the mitochondria and is used for fatty acid synthesis – which in turn promotes glutaminolysis to maintain ATP production [210].

Also, although not directly a part of the hallmarks of cancer, ***epigenetics*** is an emerging field describing regulations of gene expression, without any changes to the underlying DNA sequence. As such, for instance DNA methylation and histone modifications are included among these mechanisms of action [211], i.e. with frequent hyper- and hypomethylations often present among tumor suppressor genes and genome wide hypomethylations in many cancer types affecting gene expression [212]. There is accumulating evidence that viral control of epigenetic mechanisms is important for maintenance and life-cycle control of viruses. This was recently supported by our own group´s findings, with HCMV shown to re-localize DNMT1

and DNMT3a from the nucleus to the cytoplasm to interfere with DNA methylation resulting in hypomethylation in MB and in HUVECs [213,214]. Further, inhibition of HCMV by ganciclovir prevented the cytoplasmic localization of DNMT-1, while treatment of HCMV- infected MB cells as well as HUVECs with the methylation inhibitor 5-Azacytidine (5AZA), significantly increased HCMV-IE and HCMV-gB gene transcription and protein expression. Increased viral protein synthesis in 5AZA-treated cells suggests that HCMV replication may benefit from a DNA methyltransferase-free cellular environment with a hypomethylated DNA and high gene transcription. Our results indicate that epigenetic mechanisms are important for viral interplay with the cellular genome of the infected cells, and that epigenetic mechanisms can impair protective mechanisms of the infected cell while allowing the virus to replicate its genome. Further, these findings also emphasize the importance of assessing potential viral activation in the treatment of MB patients with epigenetic drugs.

1.5.1 HCMV and DDR

Current knowledge in the field indicates that when HCMV infects cells such host cells are forced to adopt a persistent G1/S-phase state, thereby subverting the cell cycle machinery to divert the cell's metabolites and enzymes necessary for viral DNA replication [215-217], while suppressing the cells own DNA synthesis [218-221]. Further, HCMV has been reported to induce DDR to perform tasks for the virus, as such, some host DDR proteins have been reported to be localized to viral replication centers, believed to be aiding in virus genome replication and proof-reading in preparation for viral propagation. Nevertheless, the emerging 'oncomodulatory' role of HCMV in general, and the interplay between HCMV and host DDR potentially contributing to the role of HCMV in tumorigenesis has so far been largely uncharted. Nevertheless, the following is now known in the field.

As discussed earlier, DDR can be activated not only by DNA damage caused by exogenic sources such as radiation, but also by intracellular factors, including viral infections, the latter as part of the viral strategy to gain benefit by exploiting or actively inhibiting different parts of the DDR pathways [107]. It has been stated that HCMV activates DDR through ATM and downstream signaling during infection, which is accompanied by recruitment of ATM and other repair proteins to sites of viral DNA replication [108-111]. Further, HCMV also alters the cell cycle while inducing DDR, with infected permissive cells being driven into the G1/S state that assures necessary enzymes and metabolites for viral DNA replication [215-217], while at the same time suppressing competitive cellular DNA synthesis of the host [218-221]. In general, DDR activation mediated by HCMV includes the activation of ATM, ATR, CHK1 and CHK2 kinases, with the ensuing phosphorylations of e.g. histone H2AX and p53. This DDR activation reflects, at least in part, phosphorylation or

binding of the host RB family proteins through 4 known HCMV proteins; IE1, IE2, pp71 and pUL97. (**Figure 8**). These events may, at least in nonpermissive cells that do not stop cycling upon infection, release the E2F proteins, lead to unscheduled replication events, and thereby cause replication stress with fork stalling and colapse, leading to DNA breakage. The resultant ATM activation and its downstream phosphorylation targets, including H2AX and p53, contribute to checkpoint-mediated modulation of the cell cycle, in permissive cells causing a prolonged delay at the G1/S border, with limited DNA replication [108]. In addition, HCMV pUL35 can activate DDR, causing gamma H2AX and 53BP1 foci formation and induction of cell cycle arrest, further supporting viral replication [222].

More specifically, the permanent G1/S cell cycle arrest due to HCMV in permissive cells is mainly channeled through p53 – the activation of which is increased during HCMV infection - functioning as a transcriptional activator aimed at activating several downstream genes such as the CDK inhibitor p21, Mdm2 and others [103]. The activation of the p53 in a normal state leads to cell cycle arrest (when p21 is the dominant target gene activated by p53) or to apoptosis (if p53-regulated pro-apoptotic genes such as Puma or Noxa become activated), but HCMV prohibits the p53-Mdm2 interaction, leading to p53 stabilization and the above mentioned G1/S phase delay/arrest through p21 activation [103]. Generally, one can say that the complex interaction between HCMV and the HCMV-permissive cell results in the cell being in a state of arrest, albeit still with active mechanisms that favor HCMV replication over the host cell's own DNA synthesis.

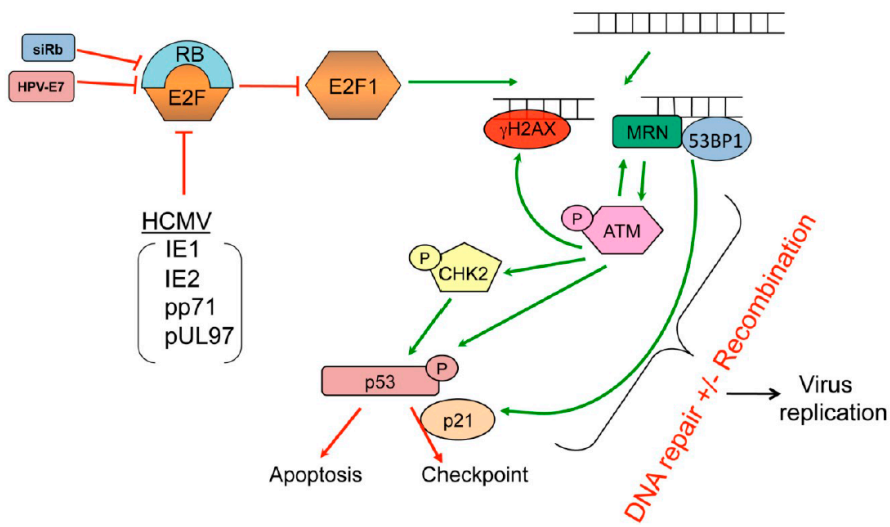

Figure. 8. *DDR and HCMV interplay, from* [103].

1.5.2 HCMV as treatment target in brain tumor patients?

Since the discovery of HCMV infection in GBM and other brain tumors, and its association to poor survival of GBM patients as shown by our group [162], there have been, and are currently ongoing, numerous clinical treatment efforts to exploit this potential target [223]. The rationale for clinical trials is supported by animal data from several groups, among other our own data demonstrating HCMV potentiated growth of neuroblastoma and medulloblastoma in immunocompromised mice, the growth of which was correspondingly decreased when treated with valganciclovir [161,148]

Similarly, data from GBM animal models by Heukers et al demonstrates growth of glioblastoma cells in an orthotopic intracranial GBM-model in mice increased by HCMV US28 and was effectively blocked by US-28 targeting nanobodies – resulting in decreased tumor growth [224].

The focus of the clinical trials is currently in the areas of systemic anti-HCMV treatment, DC vaccines and adoptive T cell therapy. As such, our group was the first to initiate a randomized clinical trial on the use of add-on systemic anti-viral treatment in GBM patients [225]. The primary end-point of decreased tumor growth at 6-months follow up was not achieved, nevertheless, exploratory data demonstrated overall survival of median 24 months in the treatment group (in comparison to 13 months in the control group with similar standard treatment) [226]. Further, this trial did not demonstrate any major adverse events associated with the anti-viral treatment for this indication. Due to these promising results, a randomized trial with the hopes of demonstrating an overall survival benefit in GBM patients treated with anti-viral therapy in a controlled setting was recently initiated (EudraCT: 2019-001083-3).

More recently, HCMV has also become a target in immunotherapy trials for GBM patients. One group from Duke University has successfully targeted HCMV pp65, where patients receiving HCMV pp65-expressing dendritic cells (pp65-DCs) in combination with vaccine site pre-conditioning using tetanus-diphtheria toxoid, demonstrated significantly improved progression-free survival (PFS) and overall survival (OS) compared to controls [227].

Yet another strategy, that of adoptive T cell transfer, based on the discovery of HCMV specific T cells aimed at pp65 recognize and kill autologous GBM cells [228], has recently shown promising clinical results in terms of increased survival in recurrent GBM patients, and without any significant side-effects [229].

Overall, there is increasing evidence that HCMV is a promising new target in the treatment of Glioblastoma.

2 AIMS OF THE THESIS

- To investigate whether, and to what extent is the HCMV capable of inducing features of stemness in primary GBM cell lines (1st study)
- To investigate the presence of HCMV proteins, DDR and replication stress markers in human medulloblastomas, and to assess the response of normal (permissive) human cells *versus* medulloblastoma cells to HCMV infection (2nd study)
- To investigate the DDR and HCMV in the context of human medulloblastoma stem cells, and to asses responses to experimental genotoxic treatments. (3rd study)
- To investigate the mechanisms through which HCMV (or IE72) subverts the host-cell DDR fueling genomic instability and hence likely promoting tumorigenesis (4th study)

3 RESULTS AND DISCUSSION

In recent years, there has been an ongoing debate in the literature on the potential presence and role of HCMV in brain tumors, i.e. GBM and MB. The initial observation of HCMV presence in GBM tissue was made by Cobbs et al. in 2002 [159], and was confirmed by several other groups including our own, in the years that followed. Our research group was the first to demonstrate a high prevalence of HCMV also in MBs [161]. Importantly, HCMV nucleic acids and proteins were found not to be present in the surrounding tissue (only in the tumor tissue), and they were found to be present in different primary intracranial tumor types [148,161,230,231] as well as secondary tumors (metastases) [160]. Nevertheless, some research groups have failed to detect HCMV in brain tumor tissue [232,233], possibly due to the use of suboptimal detection protocols for HCMV in tumor tissue specimens. The variable results have made the question of presence of HCMV in brain cancer a controversial subject [234]. As to the potential role of HCMV in MB and GBM, several mechanisms supporting the role of HCMV as a pro-oncogenic (oncomodulatory) factor have been investigated as discussed previously. Further, there is mounting experimental evidence in vivo (animal models) that anti-HCMV therapy reduces tumor growth in both MB and GBM [161,235].

Clinically, besides identification of HCMV in MB and GBM patient samples, the quantity of these has proven to be of prognostic significance [162]. Our group performed a hypothesis generating phase I/II randomized controlled trial on the use of Valganciclovir as add-on to standard radio-chemotherapy in GBM patients. The study was underpowered, and we found trends but no significant difference in tumor growth at 6 months after diagnosis [225]. However, in an exploratory analysis we observed increased survival in patients treated with Valganciclovir as compared to those that received similar standard of care [226]. Still, efficacy needs to be proven in a controlled setting, which is the aim of a randomized trial recently initiated (EudraCT: 2019-001083-3) in Sweden.

Overall, the literature so far suggests a possible pro-oncogenic (oncomodulatory) role of HCMV in GBM and MB, the action thought to be potentially mediated through, among other aspects, HCMV-subverted or deregulated DDR. This potentially opens for new treatment possibilities in the future, and demonstrates the importance of further research conducted in this area. To investigate this promising topic further, we conducted 4 studies which are part of my thesis.

3.1 1st study: Cytomegalovirus infection induces a stem cell phenotype in human primary glioblastoma cells: prognostic significance and biological impact.

Background: In the 1st study, we turned our attention towards Glioblastoma. GBM is associated with poor prognosis despite aggressive surgical resection, chemotherapy, and radiation therapy. Unfortunately, this standard therapy does not target glioma cancer stem cells (GCSCs), a subpopulation of GBM cells that is known for their ability to self-renew, proliferate and differentiate into heterogenous tumor cells [41,236]. Considering the previously discussed oncomodulatory abilities of HCMV, we sought out to investigate the potential role of HCMV in GBM – more specifically in the GSC subpopulation of cells.

Results and discussion: First, we defined our GCS population as those positive for CD133, a renowned surface stem cell marker, while HCMV IE was used to denote those cells positive for HCMV. We found that 95% or our clinical GBM samples (n=21) expressed HCMV-IE (flow-cytometry), while 91% expressed CD133, and approximately 80% were double-positive. When analyzed with respect to overall survival versus HCMV IE and/or CD133 positivity, these markers were independently as well as combined predicative of shorter overall survival in our patient cohort (n=21). This suggests that HCMV might induce an increase an CD133 GBM phenotype with shorter patient survival, although it can only be speculated upon how this is done; by a paracrine manner with HCMV positive cells maintaining CD133 GCS. This virus may alter the tumor microenvironment favoring GSC to expand, or by direct infection of GSC, HCMV may induce transcription factors regulating CD133 expression [237].

Second, we further searched for an interaction between HCMV and stem cell marker expression in GSC, by infecting adherent GBM cell lines with HCMV and analyzing the stem cell marker expression. After infection, we saw an upregulation of CD133 (surface marker), Nestin (cytoskeletal protein), SOX-2, OCT-4, Notch and Nanog (all transcription factors) but not BMI-1 (transcriptional (repressor) suppressor) [238]. While surface markers are important for recognition, transcription factors are mainly important for proliferation and survival [38]. Our experiments imply that HCMV plays an active role in the regulation of stem cell marker expression.

Third, we studied the ability of HCMV to induce neurospehere formation in GBM. It has been previously reported that GSC form neurospheres when cultured *in vitro*, with tumor growth upon injection *in vivo* [38]. We observed a similar pattern of neurosphere formation in our primary GBM cell lines grown under non-adherent conditions, with a markedly increased sphere formation when these cells were infected with HCMV, suggesting that HCMV seems to induce a more aggressive GSC phenotype in GBM.

Fourth, we wanted to see if inhibition of the Notch pathway, known for its importance in neural stem cell formation [239] and previously shown to be upregulated in GBM [240], in any way affected sphere formation. To interfere with the Notch pathway, we used a gamma secretase inhibitor, which inhibits the final step in the cleavage process leading to Notch activation [43]. Our experiment resulted in reduced sphere formation of GSC in our HCMV infected GBM cell lines, indicating an importance of this pathway in HCMV regulated GBM sphere formation. We observed the same response pattern (inhibition of sphere formation) when the HCMV polymerase itself was blocked by Ganciclovir.

Finally, previous reports from our group showed that HCMV has an ability to keep neural precursor cells (NPCs) in an undifferentiated state and even induce apoptosis [241,242]. Here, we sought out to investigate this aspect of HCMV and GSC, by inducing differentiation of HCMV infected GSC into neuronal or astrocytic differentiation. We then evaluated neuronal (Tuj1) and astrocytic (GFAP) marker expression on uninfected and HCMV infected cells. We observed that only a small population of HCMV positive cells had differentiated, while uninfected GCS differentiated robustly to neuronal cells or astrocytes, respectively. Thus, our observations suggest that HCMV maintains GCS in an undifferentiated state.

In summary, our findings imply that HCMV infection induces phenotypic plasticity of GBM cells to promote GSC features and may thereby increase the aggressiveness of GBM.

3.2 2nd study: Replication stress, DNA damage signaling, and cytomegalovirus infection in human medulloblastomas

Background: In the **2nd study**, we switched our focus to medulloblastoma. We have previously reported on the potential role of HCMV in medulloblastomas [161]. Our research group earlier demonstrated HCMV presence in medulloblastoma specimens and cell lines, and showed that Ganciclovir inhibited tumor growth in vitro, and valganciclovir in vivo growth of xenografted human medulloblastoma tumors. Also, we have previously investigated DNA damage pathways in different primary brain tumors such as gliomas and intra-cranial germ-cell tumors [243,244], suggesting that oncogenic events are probable causes behind activated DDR. Nevertheless, no reports have so far focused on analyzing activation of DNA damage response pathways and potential replication stress in medulloblastomas. And albeit the role of HCMV in the context of DNA damage signaling has been previously discussed in literature [103], none have looked at the interaction between DNA damage signaling and HCMV in medulloblastomas. To investigate this, we have combined immunohistochemical analysis of paediatric medulloblastomas with medulloblastoma cell culture models including experimental HCMV infection.

Results and discussion: First, using eight established immunohistochemical markers previously applied to assess the DNA damage signaling in gliomas and germ-cell tumors [243,244], we looked at the DNA damage signaling pathways, which demonstrated an overall high level of DNA damage signaling (as defined by gammaH2AX) in Medulloblastoma in comparison to germ-cell tumors, but not as high as in glioblastomas. Notably, both ATM-Chk2 and ATR-Chk1 DNA damage checkpoint kinase cascades were active, with the latter, in combination with formation of the so-called 53BP1 bodies in G1 cells [245], indicating endogenous replication stress. These results indicate an ongoing active endogenous DDR signaling in medulloblastomas without any previous genotoxic treatments (i.e. radiation and / or chemotherapy), confirming that as in gliomas, these tumors show features of chronic replication stress that most likely reflects oncogenic events and ensuing DDR activation in medulloblastomas.

Second, p53, a key tumor suppressor in the DDR signaling pathways [246], demonstrated a somewhat unusual expression, with low detection rate in especially molecular subtype 3 and 4 medulloblastomas, in cells that otherwise demonstrated a high DDR signaling activation. We propose that this could be due to a combination of chromosome 17p deletion (where p53 gene resides) [247] and Wip1 phosphatase overexpression dephosphorylating p53 [248], leading to a high proliferation rate despite the ongoing checkpoint signaling.

Third, in literature HCMV immediate early (IE) and Late antigen detection by IHC is often debated, with some groups reporting being unable to detect HCMV IE and Late proteins in i.e. childhood brain tumors [233], questioning positive findings previously made by others [161]. To investigate this, we used three different IHC techniques in parallel, with varying results – i.e. the IHC technique usually implemented for detection of DDR signaling and used in some papers aiming at HCMV protein detection in human tumors (based on citrate buffer pH 6) – proved suboptimal for HCMV protein detection, while the protocol employed by our laboratory previously [161], as well as a protocol utilizing tris buffer (pH 9) demonstrated clear HCMV protein detection in medulloblastoma clinical specimen, in contrast to the lack of staining in normal cerebellum (control). As such, this demonstrates that HCMV detection depends on the tissue processing and staining protocol used.

Fourth, a human medulloblastoma cell line - and a normal diploid fibroblast cell line from ATCC were infected with HCMV and analyzed with immunofluorescence (pre- and post-infection) with respect to cyclin A (to identify cells in S/G2 phase) and 53BP1 bodies in cyclin A-negative/G1 cells (marker of replication stress, see above). The analysis showed 53BP1 mis-localization to the cytosol in HCMV infected fibroblasts permissive for viral production, while HCMV infected medulloblastoma cells (which are non-permissive and survive HCMV infection) did not remove their 53BP1 from the nucleus. While the reason for this can only be speculated upon, two main scenarios can be discussed; 1) In fibroblasts infected by

HCMV, viral factors remove 53BP1from the viral replication centers so that the DDR machinery does not negatively impact viral replication, especially through error-prone repair mediated by the host cell's non-homologous end joining (NHEJ) pathway of DSBs, an error-prone mechanism that is favored by 53BP1 and might therefore compromise the integrity of the nascent HCMV genome; and 2) medulloblastoma cells are non-permissive, hence no viral replication takes place, yet such tumor cells infected with HCMV feature a high degree of chromosomal instability, in that 53BP1 presence and its subsequent effect in terms of double-strand break repair could be beneficial for the survival of the infected tumor cells. In other words, the major difference is that unlike the permissive fibroblasts, the medulloblastoma cells are non-permissive, i.e. they do not support HCMV replication and this likely underlies the observed difference in localization of 53BP1.

Finally, we observed an overall increase in 53BP1 expression and 53BP1 bodies formation in the HCMV infected specimens, unexpectedly even in cells that were themselves not expressing HCMV but were adjacent to HCMV-infected cells in the same culture. This surprising phenomenon suggests a possible cell non-autonomous mechanism impacting the surrounding cells, resulting in enhanced replication stress even in the cells not directly expressing the HCMV proteins, and thereby leading to more frequent formation of 53BP1 bodies in G1 cells.

In summary, our results indicate a constitutively active DDR machinery and presence of HCMV in medulloblastomas, with unorthodox p53 defects allowing for high proliferation despite the ongoing checkpoint signaling. HCMV-associated alteration of 53BP1 is indicative of an HCMV-triggered replication stress. These events in combination likely contribute to genomic instability and thereby also to selection of tumor cell subclones resistant to genotoxic therapies.

3.3 3rd study: Cancer cell stemness, responses to experimental genotoxic treatments, cytomegalovirus protein expression and DNA replication stress in pediatric medulloblastomas

Background: In the **3rd study**, we continued working on medulloblastomas. In this study, we aimed to examine in more detail, two aspects of medulloblastoma biology, namely the possible presence of medulloblastoma stem cells (MBSC), thought to be responsible for treatment resistance towards standard-of-care radiation and chemotherapy, and that of replication stress.

To address this issue, we examined the known cancer stem cell markers (CD133, CD15) as well as VEGFR2 – suggested to be associated with GSC previously [249] [236] – in combination with HCMV protein expression in human medulloblastoma specimens. We complemented our IHC experiments with 3 different medullo-

blastoma cell lines exposed to radiation and hydroxyurea treatment, analyzing the DDR pathways for signaling differences while looking at the phenotypes of the individual cell lines in the context of HCMV and stemness properties.

Finally, we examined the replication fork in terms of speed and symmetry between these cell lines, to estimate the level of replication stress and analyzed this in the context of our previous findings.

Results and discussion: First, our immunohistochemistry analysis on clinical material identified expression of VEGFR2, CD133 and more widely CD15 in all clinical samples. This demonstrates the presence, although in a varying degree, of MBSC in our clinical material. The varying expression and small sample size did not allow us to draw any correlations to clinical outcome or molecular subclass. As such, larger and more advanced studies using double-fluorescence staining methods and cell sorting of fresh surgical tissue samples in combination with biological cell experiments will be needed to investigate this further.

Second, we took advantage of ATCC available medulloblastoma cell lines D324, DAOY (both showing growth and surface marker profiles indicative of largely non-stem cell populations) and D283, a cell line that we showed is robustly positive for the CD133 stemness marker and grows in a non-adherent and sphere-forming manner – resembling a stemness phenotype under standard cell culture conditions. Upon experimental infection by HCMV, the CD133-positive D283 cells showed a high positivity of the viral IE72/86 protein (up to 60% of the cell population) almost comparable with the high degree of infectivity seen in the permissive control human BJ fibroblast-like fibroblasts. In contrast, the other two cell lines D324 and DAOY only expressed low frequency of IE72/86 protein positivity, indicating that in contrast to the stem-like D283 cells, the non-stem cell lines support much lower expression of HCMV. Thus, the D283 stem cell phenotype appears to allow for a non-permissive infection with abundant viral protein expression while not causing cell death despite ongoing proliferation of such HCMV-infected tumor cells. This is especially interesting in the context of our previous results on GBM and HCMV, with HCMV potentiating a GSC (stemness) phenotype which could also be correlated to patient outcome [250].

Third, we moved on to externally induce genotoxic stress in experiments via irradiation and drug-induced replication stress; achieved by exposing all three cell lines to 3 Gy irradiation and adding hydroxyurea (known to result in drug-induced replication stress), respectively. When irradiated, all three cell lines responded by increasing their activity of Chk1 and Chk2, while only the stem-like D283 cells showed unexpectedly low phosphorylation on RPA Ser33 and Kap1, overall indicating an aberrantly re-wired signaling under replication stress. When exposed to hydroxyurea, the D283 cells responded by exceptionally high degree of

RPA Ser4/8 phosphorylation, which in combination with constitutively increased gammaH2AX, suggest a high constitutive degree of endogenous replication stress that is further exaggerated upon treatment with hydroxyurea. The notion of abnormally high replication stress was confirmed by using DNA fiber assays, whereby replication forks in the D283 cells did not recover properly upon a transient exposure to hydroxyurea (in contrast to responses in the non-stem DAOY medulloblastoma cells). These observations suggest that replication stress is constitutively enhanced in the D283 cells, resulting in impaired DNA replication and fueling genomic instability. This adapted rewiring of the DNA damage signaling would allow the MBSC to survive and proliferate, while on the other hand enhancing genomic instability, thus broadening variation within such tumor cell population, and overall increasing the risk of tumor recurrence due to enhanced resistance to radio- and chemotherapy [251,252].

In summary, this study addresses the connections between MBSC (tumor cell stemness), DDR machinery and HCMV by examination of clinical samples corroborated by functional experiments with model cell lines, leading to new insights into the interplay between the above factors. While demonstrating the presence of MBSC in clinical samples and certain cell lines, associated susceptibility to HCMV infection as well as the presence of aberrant signaling pathways and increased endogenous replication stress – the obtained data also open for new research into the role of HCMV in MBSC, with potential implications for better understanding tumor development and targetable vulnerabilities of especially the medulloblastoma stem cells [161].

3.4 4th study: HCMV triggers replication stress and subverts host-cell's DNA damage response fueling genomic instability

Background: In the **4th study**, we turned our attention back to GBM and DDR. It is known, that when a virus has entered a host cell(s), a competition arises between the host DNA and that of the virus – resulting in DDR activation for viral replication benefit, which has previously been observed with different viruses, among them HCMV [103]. A subset of DDR activated proteins have been observed to be mis-localized to the viral replication centers (VRCs) in HCMV infected cells – believed to be used by the HCMV to proofread it´s genome [108]. Nevertheless, the mechanism behind the HCMV misuse of DDR is still not well-defined, with contradictory evidence in literature; i.e. activation of ATM has been reported by some to be essential for HCMV replication [253], while others report this not to be the case [254,255]. Another possibility is that HCMV uses a "hit-and-run" mechanism to exert it´s oncomodulatory effect and promote tumorigenesis. Thus, there is still no consensus on the molecular mechanism behind the interplay between HCMV and DDR.

To investigate this in further depth, we used GBM patient samples before- and after they received radio- and chemotherapy as well as several different cell models including permissive and non-permissive human cells analyzed for HCMV and DDR - with the application of extensive laboratory techniques including IHC, immunofluorescence, immunoblotting, FACS, DNA fiber assays and PCR.

Results and discussion: First, we infected permissive (fibroblasts) cells and analyzed the aspects of viral replication and DDR. We observed that HCMV triggered the cellular DDR response and induced mis localization of error prone non-homologous end joining (NHEJ) repair proteins to the cytosol, while attracting the proteins that participate in the error-free homologous recombination (HR) repair in the VRC.

Second, we looked at another dogma mentioned previously – postulating that ATM/ATR is necessary for viral production [254]. We added inhibitors of ATR, ATM and a general PIKK inhibitor in different concentrations to cell cultures of virally infected fibroblasts. We observed that in low inhibitor concentrations, the viral titer went up while at the same time lowering apoptosis. High inhibitor concentrations resulted in diminished viral production while at the same time already decreasing cell viability. These observations suggest that ATR/ATM are not necessary for viral production, and that the previous studies suggesting the essential role of these kinases were confounded, as the lower virus production in fact reflected the compromised fitness of such cells cause by the high doses of ATR/ATM inhibitors.

Third, we investigated if a direct relationship exists between HCMV and DDR – and assessed the functional status of DDR when cells were infected with HCMV. By treating cells with i) neocarzinostatin, ii) temozolomide or iii) cisplatin, we induced DNA lesions in infected and non-infected fibroblasts. We observed increasing levels of gammaH2AX in all cells, albeit the non-infected cells demonstrated a diminishing level of gammaH2AX over time, while the infected did not – in other words, the kinetics of repairing the DNA lesions caused by exogenous insults such as drugs was clearly delayed in the HCMV-infected cells compared with their non-infected counterparts, a finding consistent with an earlier report [256]. One possibility is that the DDR protein mis localization to cytosol and sequestration into VCR might have resulted in the observed slower DNA repair of the hosts cell own DNA lesions, possibly contributing to genomic instability.

Apart from the results above, the two additional, major and novel findings of this study were as follows:

A. We discovered a new mechanism through which HCMV might benefit from DDR activation in the host cell. We noticed that the viral IE promoter contains transcription-factor binding sites, that resembles promotors of host genes activated by stress – i.e. including sites for the transcription factors NF-kB,

Sp1, AP1 and CREB. As such, stressful insults including DNA damage could activate/enhance IE72/86 viral gene expression. More specifically, we could show that experimentally induced replication stress increased IE72/86 expression – which, through IE72/86 causing more replication stress/DNA damage in the host cell, generates a positive feedback loop between host cell stress response and HCMV IE production. Based on our findings, we propose that the IE promotor has been evolutionally shaped to exploit the host-cell DNA damage response.

B. We provided a detailed and novel insight into how HCMV, or even ectopic IE proteins, indeed evoke robust replication stress at the level of replication forks (forks stalling and collapse as well as DNA breaks) and destabilize the host cell genome, including chromosomal aberrations. While permissive cells died when the viral particles lyse the cells, the non-permissive tumor cells survived with signs of chromosomal instability that are commonly associated with tumorigenesis, despite no signs of virus production. Thus, a non-permissive infection in GBM would fuel DDR mechanism and further enhance HCMV IE promoter activity, which may also promote the oncomodulatory, genome-destabilizing effects in the absence of virus production.

Finally, the intriguing finding of the feedback loop between the host DDR response and HCMV IE expression led us to assess IE 72/86 protein expression in human clinical specimens from GBM patients, from whom we obtained tumor tissue before- and after recurrence. Since all patients were treated with radio-chemotherapy, comparing these matched samples from the same patient gave us insights as to the HCMV and DDR interplay in a clinical setting – demonstrating elevated HCMV IE72/86 in tumor recurrence cases, overall consistent with our concept that chemo/radio-therapy may induce viral re-activation due to the positive feedback-loop described above (A).

Overall, in cell models, we were able to demonstrate that HCMV infection in both permissive and non-permissive cells induces replication stress and DNA damage, indicating one mechanism of how HCMV may plausibly destabilize the host-cell genome and thereby fuel/modulate tumorigenic transformation or tumor progression, as cancer cells are largely non-permissive for the viral production and hence survive the infection and chronically express IE proteins.

Our findings provide novel findings in the quest for elucidating the molecular mechanism behind HCMV and DDR interplay, why providing yet another argument supporting the role in future clinical trials of combining standard-of-care genotoxic treatments (radio- and chemotherapy) with anti-viral drugs in GBM patients.

4 CONCLUSION

Currently, treatment of GBM (the most common primary malignant tumor in adults) and Medulloblastoma (the most common primary malignant tumor in children) may include some combination of surgery, radiation therapy, and chemotherapy. The evolution of most brain tumors is unknown, although varying degree of genomic instability and infection by human cytomegalovirus (HCMV) is suspected. Nevertheless, the causes of the chromosomal instability/DDR changes and its potential links with HCMV infection and/or resistance to genotoxic therapies (i.e. radiation and chemotherapy) remain largely unknown. I build the thesis on the recent discoveries of HCMV being present in malignant brain tumors. Although it is accepted that activation of host DDR occurs in the context of HCMV infection, this has so far not been linked to replication stress, and such mechanism, its links with cancer cell stemness and its consequences for genomic instability have so far not been described in malignant brain tumors, which was the focus of my thesis. Briefly, the main findings can be summarized as follows;

- We found HCMV-IE proteins to be co-expressed with CD133 in GBM. Upon infection with HCMV *in vitro*, primary GBM cell lines were induced into neuro sphere formation while differentiation into neurons, in combination with upregulation of CD133, SOX-2, Notch-1, OCT-4 and Nestin, all of which are characteristic of GSC. These results were corroborated by demonstrating that GCS stayed in a more undifferentiated state when infected with HCMV. Finally, addition of gamma-secretase inhibitor and/or ganciclovir to HCMV infected GBM cell lines inhibited sphere formation. In summary, these findings suggest that HCMV infection induces a more potent GSC phenotype in GBM.
- We found pronounced activation of DNA damage signaling pathways and HCMV presence in clinical medulloblastomas specimen. Further, we demonstrated that HCMV infection leads to selective 53BP1 mis localization, believed to favor viral survival and replication despite replication stress in human fibroblasts and medulloblastomas. In summary, these findings suggest wide activation of DDR in human medulloblastomas, with HCMV fueling genomic instability.
- We found a wide expression of stem cell markers CD133, CD15 and VEGFR2 in clinical specimens of medulloblastomas. Complemented by functional experiments, we were able to demonstrate differences between MB cell lines, with medulloblastoma cells featuring a stemness phenotype being more susceptible to HCMV infection, and high level of endogenous DNA damage. In cell lines with a stemness phenotype, we noted a slow replication fork progression – a hallmark of chronic replication stress. In summary, these finding contribute to the evidence of genomic instability in medulloblastomas, with the cell line expressing a stemness phenotype associated to HCMV and slow replication speed, a surrogate marker correlated with genome destabilization.

- We found that cellular stress response genes and viral IE gene promotor shares the same transcription factor bindings sites, which enables an enhanced IE72/86 expression when DDR is upregulated. These results supported a model that HCMV infection fuels replication stress and genomic instability, overall consistent with the concept of HCMV's oncomodulatory effects. This notion was further substantiated by our findings with clinical GBM specimen, where HCMV expression was elevated after previous genotoxic therapies (in recurrent GBM tumors). In summary, these findings provide insights into the mechanisms by which HCMV evokes a replication stress response, subverting the cellular DDR resulting in genomic instability and thereby likely promoting therapy resistance and tumor progression.

In terms of clinical trial design, the results of my thesis further support the rationale for the already ongoing clinical trial with valganciclovir in human recurrent glioblastoma (EudraCT: 2019-001083-3), a scenario which we now know from one of my studies is associated with an enhanced load of HCMV IE72/86 proteins, possibly contributed by the feedback loop from the genotoxic treatment to the HCMV IE gene promoter stimulation via host cell transcription factors responding to DNA damage and other stresses.

Another relevant and already ongoing clinical trial called DIRECT (EudraCT: 2016-000167-16) focuses on recurrent glioblastoma patients in Sweden and Norway. I am a contributor to design of this multicenter randomized trial [257]. In this trial, standard-of-care treatment by temozolomide is being compared against a temozolomide and add-on combination of disulfiram with copper supplement. Disulfiram is an old and safe alcohol aversion drug and a candidate for repurposing in oncology, since combined with copper it becomes metabolized in vivo into an anti-cancer compound copper-dithiocarb (CuET, for short), which effectively kills diverse types of cancer cells including glioblastomas in preclinical trials. CuET also enhances replication stress in human tumor cells, and since glioblastomas already feature a high degree of replication stress (further enhanced by HCMV, as shown in my thesis), the hope is that the disulfiram+copper combination will boost the replication stress in glioblastoma cells beyond a tolerable threshold and kill these cancer cells, while sparing normal cells. The first information as to whether this strategy could provide any benefit for patients suffering from recurrent glioblastoma will become available to the DIRECT trial team during the summer of 2020, based on the interim analyses.

Furthermore, the results from my thesis also support the notion that glioblastomas and medulloblastomas might be sensitive to drugs such as inhibitors of ATR or Chk1 kinases which normally provide a checkpoint response that might help the tumor cells to stabilize their replication forks and avoid excessive chromosomal instability and cell death under the high degree of chronic replication stress as seen in these brain tumors. Inhibitors of ATR, Chk1 and additional checkpoint kinases are being evaluated in many pre-clinical settings, and clinical trials may soon follow.

Besides the clinical trials mentioned above (which also include HCMV vaccines being applied in small groups of human glioblastoma patients), there are very important issues to be addressed at the basic, mechanistic level to better understand the emerging oncogenic/oncomodulatory effects of HCMV infection in humans. Here, I just mention two such issues which I regard as very important to be addressed in the near future:

First, arguably the most critical, and so-far rather frustrating issue in the field has been the inability to reliably detect and then precisely determine the CMV nucleic acid sequences present in human brain tumors. While or lab has obtained preliminary evidence for an unorthodox variant HCMV sequence from human brain tumors, this issue requires a major effort to be conclusively elucidated and confirmed. If identified and validated, such HCMV sequences and tools to express them experimentally, would then be explored to directly test the notion of HCMV as a candidate oncogenic or oncomodulatory virus, through direct transformation approaches by such viral sequences in cell culture and animals. If confirmed to be oncogenic, as current data suggest but still do not prove beyond doubt, one could then assess the precise mechanisms of such HCMV-mediated transformation or progression of precancerous cells to full malignancy. In addition, these sequence-defined tools would open the long-awaited avenue to also better exploit this knowledge for diagnostic and therapeutic purposes in neuro-oncology and beyond.

Second, while our own work including data in this thesis document the fact that HCMV causes replication stress, DNA damage and thereby genomic/chromosomal instability, much less is known about any potential impact of HCMV on the spectrum of point mutations in human cancer in general, and tumors of the brain in particular. Here, we and likely other researchers in the field intend to address the possibility that HCMV might trigger a robust mutagenic wave by inducing the host endogenous APOBEC family enzymes. These are enzymes whose role is primarily to attack any invading virus genomes and mutate them into a state that is incapable of proper viral propagation and function, thus protecting the host. However, viruses such as HIV are known to partly neutralize this APOBEC defense and in fact hijack the mutagenic potential for their own benefit, mutating the virus into new variants resistant to anti-viral drugs and host immune system. It is not known whether or to what extent APOBEC enzymes may be activated by the presence of HCMV in human tumors, however the mutation signatures that are attributable to APOBEC family's mutagenic impact are rather common in human tumors in general, and therefore it is plausible that HCMV may contribute to this widespread APOBEC-mediated mutation loads in human cancer.

Overall, the work of my thesis has complemented the current knowledge about the potential role of HCMV and DDR/replication stress in Medulloblastoma and Glioblastoma, while opening new avenues to explore, giving hope for new and improved therapies for future patients.

5 ACKNOWLEDGMENTS

It has been a long journey, and a lot of people have helped me to finalize my PhD-thesis.

I would like to thank my main supervisor **Cecilia Söderberg-Naucler** for many years of passionate discussions – for all her energy, enthusiasm and support.

I would like to thank **my parents** for introducing me to their research field already many years ago – I remember when I started working in your lab when still in high-school – and now even a part of my PhD-thesis was coined there.

I would like to thank **all members** of the involved laboratories in Stockholm and Copenhagen for their help and assistance. A special thanks to **Olesja Fornara** for many years of friendship and team-work in the lab.

I would also like to thank **all the staff** at the Departments of Neurosurgery and Neuropathology, both in Stockholm and in Copenhagen, that contributed to my thesis. A special thanks to **Simon Skyrman** for drawing such a nice front-page picture.

And finally, I would like to thank **my wife** for many years of support and understanding.

6 REFERENCES

1. Ostrom QT, Gittleman H, Farah P, et al. CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006-2010. *Neuro Oncol.* 2013;15 Suppl 2:ii1-56.

2. Thakkar JP, Dolecek TA, Horbinski C, et al. Epidemiologic and molecular prognostic review of glioblastoma. *Cancer Epidemiol Biomarkers Prev.* 2014;23(10):1985-1996.

3. Larjavaara S, Mantyla R, Salminen T, et al. Incidence of gliomas by anatomic location. *Neuro Oncol.* 2007;9(3):319-325.

4. Wrensch M, Fisher JL, Schwartzbaum JA, Bondy M, Berger M, Aldape KD. The molecular epidemiology of gliomas in adults. *Neurosurg Focus.* 2005;19(5):E5.

5. Schwartzbaum JA, Fisher JL, Aldape KD, Wrensch M. Epidemiology and molecular pathology of glioma. *Nat Clin Pract Neurol.* 2006;2(9):494-503; quiz 491 p following 516.

6. Brenner AV, Butler MA, Wang SS, et al. Single-nucleotide polymorphisms in selected cytokine genes and risk of adult glioma. *Carcinogenesis.* 2007;28(12):2543-2547.

7. Hamilton SR, Liu B, Parsons RE, et al. The molecular basis of Turcot's syndrome. *N Engl J Med.* 1995;332(13):839-847.

8. Lynch HT, McComb RD, Osborn NK, et al. Predominance of brain tumors in an extended Li-Fraumeni (SBLA) kindred, including a case of Sturge-Weber syndrome. *Cancer.* 2000;88(2):433-439.

9. Frei P, Poulsen AH, Johansen C, Olsen JH, Steding-Jessen M, Schuz J. Use of mobile phones and risk of brain tumours: update of Danish cohort study. *BMJ.* 2011;343:d6387.

10. Lagorio S, Roosli M. Mobile phone use and risk of intracranial tumors: a consistency analysis. *Bioelectromagnetics.* 2014;35(2):79-90.

11. Krex D, Klink B, Hartmann C, et al. Long-term survival with glioblastoma multiforme. *Brain.* 2007;130(Pt 10):2596-2606.

12. Scheurer ME, Amirian ES, Davlin SL, Rice T, Wrensch M, Bondy ML. Effects of antihistamine and anti-inflammatory medication use on risk of specific glioma histologies. *Int J Cancer.* 2011;129(9):2290-2296.

13. Lachance DH, Yang P, Johnson DR, et al. Associations of high-grade glioma with glioma risk alleles and histories of allergy and smoking. *Am J Epidemiol.* 2011;174(5):574-581.

14. Lamborn KR, Chang SM, Prados MD. Prognostic factors for survival of patients with glioblastoma: recursive partitioning analysis. *Neuro Oncol.* 2004;6(3):227-235.

15. Ening G, Osterheld F, Capper D, Schmieder K, Brenke C. Charlson comorbidity index: an additional prognostic parameter for preoperative glioblastoma patient stratification. *J Cancer Res Clin Oncol.* 2015;141(6):1131-1137.

16. Ronning PA, Helseth E, Meling TR, Johannesen TB. A population-based study on the effect of temozolomide in the treatment of glioblastoma multiforme. *Neuro Oncol.* 2012;14(9):1178-1184.

17. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med.* 2005;352(10):987-996.

18. Stupp R, Hegi ME, Mason WP, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol.* 2009;10(5):459-466.

19. Visvader JE. Cells of origin in cancer. *Nature.* 2011;469(7330):314-322.

20. Chow LM, Endersby R, Zhu X, et al. Cooperativity within and among Pten, p53, and Rb pathways induces high-grade astrocytoma in adult brain. *Cancer Cell.* 2011;19(3):305-316.

21. Jacques TS, Swales A, Brzozowski MJ, et al. Combinations of genetic mutations in the adult neural stem cell compartment determine brain tumour phenotypes. *EMBO J.* 2010;29(1):222-235.

22. Alcantara Llaguno S, Chen J, Kwon CH, et al. Malignant astrocytomas originate from neural stem/progenitor cells in a somatic tumor suppressor mouse model. *Cancer Cell.* 2009;15(1):45-56.

23. Zong H, Parada LF, Baker SJ. Cell of origin for malignant gliomas and its implication in therapeutic development. *Cold Spring Harb Perspect Biol.* 2015;7(5).

24. Friedmann-Morvinski D, Bushong EA, Ke E, et al. Dedifferentiation of neurons and astrocytes by oncogenes can induce gliomas in mice. *Science.* 2012;338(6110):1080-1084.

25. Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* 2007;114(2):97-109.

26. Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol.* 2016;131(6):803-820.

27. Louis DN, Perry A, Burger P, et al. International Society Of Neuropathology--Haarlem consensus guidelines for nervous system tumor classification and grading. *Brain Pathol.* 2014;24(5):429-435.

28. Park SH, Won J, Kim SI, et al. Molecular Testing of Brain Tumor. *J Pathol Transl Med.* 2017;51(3):205-223.

29. Yan H, Parsons DW, Jin G, et al. IDH1 and IDH2 mutations in gliomas. *N Engl J Med.* 2009;360(8):765-773.

30. Cancer Genome Atlas Research N, Brat DJ, Verhaak RG, et al. Comprehensive, Integrative Genomic Analysis of Diffuse Lower-Grade Gliomas. *N Engl J Med.* 2015;372(26):2481-2498.

31. Ohgaki H, Kleihues P. The definition of primary and secondary glioblastoma. *Clin Cancer Res.* 2013;19(4):764-772.

32. Verhaak RG, Hoadley KA, Purdom E, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell.* 2010;17(1):98-110.

33. Cancer Genome Atlas Research N. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* 2008;455(7216):1061-1068.

34. Murat A, Migliavacca E, Gorlia T, et al. Stem cell-related "self-renewal" signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. *J Clin Oncol.* 2008;26(18):3015-3024.

35. Sasmita AO, Wong YP, Ling APK. Biomarkers and therapeutic advances in glioblastoma multiforme. *Asia Pac J Clin Oncol.* 2018;14(1):40-51.

36. Silantyev AS, Falzone L, Libra M, et al. Current and Future Trends on Diagnosis and Prognosis of Glioblastoma: From Molecular Biology to Proteomics. *Cells.* 2019;8(8).

37. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med.* 2005;352(10):997-1003.

38. Singh SK, Clarke ID, Terasaki M, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res.* 2003;63(18):5821-5828.

39. Yuan X, Curtin J, Xiong Y, et al. Isolation of cancer stem cells from adult glioblastoma multiforme. *Oncogene.* 2004;23(58):9392-9400.

40. Seymour T, Nowak A, Kakulas F. Targeting Aggressive Cancer Stem Cells in Glioblastoma. *Front Oncol.* 2015;5:159.

41. Chen R, Nishimura MC, Bumbaca SM, et al. A hierarchy of self-renewing tumor-initiating cell types in glioblastoma. *Cancer Cell.* 2010;17(4):362-375.

42. Bao S, Wu Q, McLendon RE, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature.* 2006;444(7120):756-760.

43. Wang J, Wakeman TP, Lathia JD, et al. Notch promotes radioresistance of glioma stem cells. *Stem Cells.* 2010;28(1):17-28.

44. Cao Y, Lathia JD, Eyler CE, et al. Erythropoietin Receptor Signaling Through STAT3 Is Required For Glioma Stem Cell Maintenance. *Genes Cancer.* 2010;1(1):50-61.

45. Yi Y, Hsieh IY, Huang X, Li J, Zhao W. Glioblastoma Stem-Like Cells: Characteristics, Microenvironment, and Therapy. *Front Pharmacol.* 2016;7:477.

46. Grosse-Gehling P, Fargeas CA, Dittfeld C, et al. CD133 as a biomarker for putative cancer stem cells in solid tumours: limitations, problems and challenges. *J Pathol.* 2013;229(3):355-378.

47. Zarkoob H, Taube JH, Singh SK, Mani SA, Kohandel M. Investigating the link between molecular subtypes of glioblastoma, epithelial-mesenchymal transition, and CD133 cell surface protein. *PLoS One.* 2013;8(5):e64169.

48. Nakano I. Stem cell signature in glioblastoma: therapeutic development for a moving target. *J Neurosurg.* 2015;122(2):324-330.

49. Azari H, Millette S, Ansari S, Rahman M, Deleyrolle LP, Reynolds BA. Isolation and expansion of human glioblastoma multiforme tumor cells using the neurosphere assay. *J Vis Exp.* 2011(56):e3633.

50. Lee J, Kotliarova S, Kotliarov Y, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. *Cancer Cell.* 2006;9(5):391-403.

51. Brescia P, Ortensi B, Fornasari L, Levi D, Broggi G, Pelicci G. CD133 is essential for glioblastoma stem cell maintenance. *Stem Cells.* 2013;31(5):857-869.

52. Calabrese C, Poppleton H, Kocak M, et al. A perivascular niche for brain tumor stem cells. *Cancer Cell.* 2007;11(1):69-82.

53. Charles N, Ozawa T, Squatrito M, et al. Perivascular nitric oxide activates notch signaling and promotes stem-like character in PDGF-induced glioma cells. *Cell Stem Cell.* 2010;6(2):141-152.

54. Kostopoulou ON, Mohammad AA, Bartek J, Jr., et al. Glucocorticoids promote a glioma stem cell-like phenotype and resistance to chemotherapy in human glioblastoma primary cells: Biological and prognostic significance. *Int J Cancer.* 2018;142(6):1266-1276.

55. Bartek J, Jr., Ng K, Bartek J, Fischer W, Carter B, Chen CC. Key concepts in glioblastoma therapy. *J Neurol Neurosurg Psychiatry.* 2012;83(7):753-760.

56. Lacroix M, Abi-Said D, Fourney DR, et al. A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival. *J Neurosurg.* 2001;95(2):190-198.

57. Orringer D, Lau D, Khatri S, et al. Extent of resection in patients with glioblastoma: limiting factors, perception of resectability, and effect on survival. *J Neurosurg.* 2012;117(5):851-859.

58. Stummer W, Pichlmeier U, Meinel T, et al. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol.* 2006;7(5):392-401.

59. Smoll NR, Drummond KJ. The incidence of medulloblastomas and primitive neurectodermal tumours in adults and children. *J Clin Neurosci.* 2012;19(11):1541-1544.

60. Khanna V, Achey RL, Ostrom QT, et al. Incidence and survival trends for medulloblastomas in the United States from 2001 to 2013. *J Neurooncol.* 2017;135(3):433-441.

61. Ostrom QT, Gittleman H, Xu J, et al. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2009-2013. *Neuro Oncol.* 2016;18(suppl_5):v1-v75.

62. Gilbertson RJ, Ellison DW. The origins of medulloblastoma subtypes. *Annu Rev Pathol.* 2008;3:341-365.

63. Gibson P, Tong Y, Robinson G, et al. Subtypes of medulloblastoma have distinct developmental origins. *Nature.* 2010;468(7327):1095-1099.

64. Gong S, Zheng C, Doughty ML, et al. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature.* 2003;425(6961):917-925.

65. Shih DJ, Northcott PA, Remke M, et al. Cytogenetic prognostication within medulloblastoma subgroups. *J Clin Oncol.* 2014;32(9):886-896.

66. Kool M, Korshunov A, Remke M, et al. Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, Group 3, and Group 4 medulloblastomas. *Acta Neuropathol.* 2012;123(4):473-484.

67. Miranda Kuzan-Fischer C, Juraschka K, Taylor MD. Medulloblastoma in the Molecular Era. *J Korean Neurosurg Soc.* 2018;61(3):292-301.

68. Hambardzumyan D, Becher OJ, Rosenblum MK, Pandolfi PP, Manova-Todorova K, Holland EC. PI3K pathway regulates survival of cancer stem cells residing in the perivascular niche following radiation in medulloblastoma in vivo. *Genes Dev.* 2008;22(4):436-448.

69. Fan X, Matsui W, Khaki L, et al. Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. *Cancer Res.* 2006;66(15):7445-7452.

70. Garzia L, Andolfo I, Cusanelli E, et al. MicroRNA-199b-5p impairs cancer stem cells through negative regulation of HES1 in medulloblastoma. *PLoS One.* 2009;4(3):e4998.

71. Pistollato F, Rampazzo E, Persano L, et al. Interaction of hypoxia-inducible factor-1alpha and Notch signaling regulates medulloblastoma precursor proliferation and fate. *Stem Cells.* 2010;28(11):1918-1929.

72. Hatten ME, Roussel MF. Development and cancer of the cerebellum. *Trends Neurosci.* 2011;34(3):134-142.

73. Po A, Ferretti E, Miele E, et al. Hedgehog controls neural stem cells through p53-independent regulation of Nanog. *EMBO J.* 2010;29(15):2646-2658.

74. Marino S. Medulloblastoma: developmental mechanisms out of control. *Trends Mol Med.* 2005;11(1):17-22.

75. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature.* 2004;432(7015):396-401.

76. Hemmati HD, Nakano I, Lazareff JA, et al. Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci U S A.* 2003;100(25):15178-15183.

77. Gajjar A, Chintagumpala M, Ashley D, et al. Risk-adapted craniospinal radiotherapy followed by high-dose chemotherapy and stem-cell rescue in children with newly diagnosed medulloblastoma (St Jude Medulloblastoma-96): long-term results from a prospective, multicentre trial. *Lancet Oncol.* 2006;7(10):813-820.

78. Packer RJ, Zhou T, Holmes E, Vezina G, Gajjar A. Survival and secondary tumors in children with medulloblastoma receiving radiotherapy and adjuvant chemotherapy: results of Children's Oncology Group trial A9961. *Neuro Oncol.* 2013;15(1):97-103.

79. Edelstein K, Spiegler BJ, Fung S, et al. Early aging in adult survivors of childhood medulloblastoma: long-term neurocognitive, functional, and physical outcomes. *Neuro Oncol.* 2011;13(5):536-545.

80. Laughton SJ, Merchant TE, Sklar CA, et al. Endocrine outcomes for children with embryonal brain tumors after risk-adapted craniospinal and conformal primary-site irradiation and high-dose chemotherapy with stem-cell rescue on the SJMB-96 trial. *J Clin Oncol.* 2008;26(7):1112-1118.

81. Merchant TE, Happersett L, Finlay JL, Leibel SA. Preliminary results of conformal radiation therapy for medulloblastoma. *Neuro Oncol.* 1999;1(3):177-187.

82. Wibroe M, Cappelen J, Castor C, et al. Cerebellar mutism syndrome in children with brain tumours of the posterior fossa. *BMC Cancer.* 2017;17(1):439.

83. Sutton LN, Phillips PC, Molloy PT. Surgical management of medulloblastoma. *J Neurooncol.* 1996;29(1):9-21.

84. Albright AL, Wisoff JH, Zeltzer PM, Boyett JM, Rorke LB, Stanley P. Effects of medulloblastoma resections on outcome in children: a report from the Children's Cancer Group. *Neurosurgery.* 1996;38(2):265-271.

85. Thompson EM, Hielscher T, Bouffet E, et al. Prognostic value of medulloblastoma extent of resection after accounting for molecular subgroup: a retrospective integrated clinical and molecular analysis. *Lancet Oncol.* 2016;17(4):484-495.

86. Lafay-Cousin L, Smith A, Chi SN, et al. Clinical, Pathological, and Molecular Characterization of Infant Medulloblastomas Treated with Sequential High-Dose Chemotherapy. *Pediatr Blood Cancer.* 2016;63(9):1527-1534.

87. Friedrich C, von Bueren AO, von Hoff K, et al. Treatment of adult nonmetastatic medulloblastoma patients according to the paediatric HIT 2000 protocol: a prospective observational multicentre study. *Eur J Cancer.* 2013;49(4):893-903.

88. Law N, Smith ML, Greenberg M, et al. Executive function in paediatric medulloblastoma: The role of cerebrocerebellar connections. *J Neuropsychol.* 2017;11(2):174-200.

89. Moxon-Emre I, Bouffet E, Taylor MD, et al. Impact of craniospinal dose, boost volume, and neurologic complications on intellectual outcome in patients with medulloblastoma. *J Clin Oncol.* 2014;32(17):1760-1768.

90. Ramaswamy V, Taylor MD. Medulloblastoma: From Myth to Molecular. *J Clin Oncol.* 2017;35(21):2355-2363.

91. Strauss R, Hamerlik P, Lieber A, Bartek J. Regulation of stem cell plasticity: mechanisms and relevance to tissue biology and cancer. *Mol Ther.* 2012;20(5):887-897.

92. Coleman WB, Tsongalis GJ. The role of genomic instability in human carcinogenesis. *Anticancer Res.* 1999;19(6A):4645-4664.

93. Bartek J, Lukas J. Cell cycle. Order from destruction. *Science.* 2001;294(5540):66-67.

94. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. *Nature.* 2009;461(7267):1071-1078.

95. Nowell PC. The clonal evolution of tumor cell populations. *Science.* 1976;194(4260):23-28.

96. Loeb LA. Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res.* 1991;51(12):3075-3079.

97. Negrini S, Gorgoulis VG, Halazonetis TD. Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol.* 2010;11(3):220-228.

98. Lindahl T. Instability and decay of the primary structure of DNA. *Nature.* 1993;362(6422):709-715.

99. Bakkenist CJ, Kastan MB. Phosphatases join kinases in DNA-damage response pathways. *Trends Cell Biol.* 2004;14(7):339-341.

100. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem.* 2004;73:39-85.

101. Bartkova J, Horejsi Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature.* 2005;434(7035):864-870.

102. Bartek J, Bartkova J, Lukas J. DNA damage signalling guards against activated oncogenes and tumour progression. *Oncogene.* 2007;26(56):7773-7779.

103. Xiaofei E, Kowalik TF. The DNA damage response induced by infection with human cytomegalovirus and other viruses. *Viruses.* 2014;6(5):2155-2185.

104. Hills SA, Diffley JF. DNA replication and oncogene-induced replicative stress. *Curr Biol.* 2014;24(10):R435-444.

105. Bartkova J, Rezaei N, Liontos M, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature.* 2006;444(7119):633-637.

106. Lilley CE, Schwartz RA, Weitzman MD. Using or abusing: viruses and the cellular DNA damage response. *Trends Microbiol.* 2007;15(3):119-126.

107. Chaurushiya MS, Weitzman MD. Viral manipulation of DNA repair and cell cycle checkpoints. *DNA Repair (Amst).* 2009;8(9):1166-1176.

108. E X, Pickering MT, Debatis M, et al. An E2F1-mediated DNA damage response contributes to the replication of human cytomegalovirus. *PLoS Pathog.* 2011;7(5):e1001342.

109. Lilley CE, Carson CT, Muotri AR, Gage FH, Weitzman MD. DNA repair proteins affect the lifecycle of herpes simplex virus 1. *Proc Natl Acad Sci U S A.* 2005;102(16):5844-5849.

110. Lu CC, Chen YC, Wang JT, Yang PW, Chen MR. Xeroderma pigmentosum C is involved in Epstein Barr virus DNA replication. *J Gen Virol.* 2007;88(Pt 12):3234-3243.

111. Boichuk S, Hu L, Hein J, Gjoerup OV. Multiple DNA damage signaling and repair pathways deregulated by simian virus 40 large T antigen. *J Virol.* 2010;84(16):8007-8020.

112. Staras SA, Dollard SC, Radford KW, Flanders WD, Pass RF, Cannon MJ. Seroprevalence of cytomegalovirus infection in the United States, 1988-1994. *Clin Infect Dis.* 2006;43(9):1143-1151.

113. Sinclair J. Human cytomegalovirus: Latency and reactivation in the myeloid lineage. *J Clin Virol.* 2008;41(3):180-185.

114. Soderberg-Naucler C, Fish KN, Nelson JA. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell.* 1997;91(1):119-126.

115. Tandon R, Mocarski ES, Conway JF. The A, B, Cs of herpesvirus capsids. *Viruses.* 2015;7(3):899-914.

116. McLaughlin-Taylor E, Pande H, Forman SJ, et al. Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. *J Med Virol.* 1994;43(1):103-110.

117. Smith RM, Kosuri S, Kerry JA. Role of human cytomegalovirus tegument proteins in virion assembly. *Viruses.* 2014;6(2):582-605.

118. Tomtishen JP, 3rd. Human cytomegalovirus tegument proteins (pp65, pp71, pp150, pp28). *Virol J.* 2012;9:22.

119. Homman-Loudiyi M, Hultenby K, Britt W, Soderberg-Naucler C. Envelopment of human cytomegalovirus occurs by budding into Golgi-derived vacuole compartments positive for gB, Rab 3, trans-golgi network 46, and mannosidase II. *J Virol.* 2003;77(5):3191-3203.

120. Crough T, Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. *Clin Microbiol Rev.* 2009;22(1):76-98, Table of Contents.

121. Stern-Ginossar N, Weisburd B, Michalski A, et al. Decoding human cytomegalovirus. *Science.* 2012;338(6110):1088-1093.

122. Kalejta RF. Tegument proteins of human cytomegalovirus. *Microbiol Mol Biol Rev.* 2008;72(2):249-265, table of contents.

123. Britt W. Manifestations of human cytomegalovirus infection: proposed mechanisms of acute and chronic disease. *Curr Top Microbiol Immunol.* 2008;325:417-470.

124. Vanarsdall AL, Johnson DC. Human cytomegalovirus entry into cells. *Curr Opin Virol.* 2012;2(1):37-42.

125. Wathen MW, Stinski MF. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. *J Virol.* 1982;41(2):462-477.

126. Penfold ME, Mocarski ES. Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis. *Virology.* 1997;239(1):46-61.

127. Manandhar T, Ho GT, Pump WC, Blasczyk R, Bade-Doeding C. Battle between Host Immune Cellular Responses and HCMV Immune Evasion. *Int J Mol Sci.* 2019;20(15).

128. Sinclair JH, Reeves MB. Human cytomegalovirus manipulation of latently infected cells. *Viruses.* 2013;5(11):2803-2824.

129. Dupont L, Reeves MB. Cytomegalovirus latency and reactivation: recent insights into an age old problem. *Rev Med Virol.* 2016;26(2):75-89.

130. Rossetto CC, Tarrant-Elorza M, Pari GS. Cis and trans acting factors involved in human cytomegalovirus experimental and natural latent infection of CD14 (+) monocytes and CD34 (+) cells. *PLoS Pathog.* 2013;9(5):e1003366.

131. Kondo K, Xu J, Mocarski ES. Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. *Proc Natl Acad Sci U S A.* 1996;93(20):11137-11142.

132. Ahlfors K. IgG antibodies to cytomegalovirus in a normal urban Swedish population. *Scand J Infect Dis.* 1984;16(4):335-337.

133. Griffiths P, Baraniak I, Reeves M. The pathogenesis of human cytomegalovirus. *J Pathol.* 2015;235(2):288-297.

134. Kocak ED, Sherwin JC, Hall AJ. Cytomegalovirus disease in immunocompetent adults. *Med J Aust.* 2015;202(8):419.

135. Nieto FJ, Szklo M, Sorlie PD. Cytomegalovirus infection and coronary heart disease. *Circulation.* 1999;100(25):e139.

136. Epstein SE, Speir E, Zhou YF, Guetta E, Leon M, Finkel T. The role of infection in restenosis and atherosclerosis: focus on cytomegalovirus. *Lancet.* 1996;348 Suppl 1:s13-17.

137. Vega R, Bertran X, Menacho M, et al. Cytomegalovirus infection in patients with inflammatory bowel disease. *Am J Gastroenterol.* 1999;94(4):1053-1056.

138. Rothe K, Quandt D, Schubert K, et al. Latent Cytomegalovirus Infection in Rheumatoid Arthritis and Increased Frequencies of Cytolytic LIR-1+CD8+ T Cells. *Arthritis Rheumatol.* 2016;68(2):337-346.

139. Choo HMC, Cher WQ, Kwan YH, Fong WWS. Risk factors for cytomegalovirus disease in systemic lupus erythematosus (SLE): a systematic review. *Adv Rheumatol.* 2019;59(1):12.

140. Kirby B, Al-Jiffri O, Cooper RJ, Corbitt G, Klapper PE, Griffiths CE. Investigation of cytomegalovirus and human herpes viruses 6 and 7 as possible causative antigens in psoriasis. *Acta Derm Venereol.* 2000;80(6):404-406.

141. Nichols WG, Corey L, Gooley T, Davis C, Boeckh M. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. *J Infect Dis.* 2002;185(3):273-282.

142. Humar A, Snydman D, Practice ASTIDCo. Cytomegalovirus in solid organ transplant recipients. *Am J Transplant.* 2009;9 Suppl 4:S78-86.

143. Lichtner M, Cicconi P, Vita S, et al. Cytomegalovirus coinfection is associated with an increased risk of severe non-AIDS-defining events in a large cohort of HIV-infected patients. *J Infect Dis.* 2015;211(2):178-186.

144. Adland E, Klenerman P, Goulder P, Matthews PC. Ongoing burden of disease and mortality from HIV/CMV coinfection in Africa in the antiretroviral therapy era. *Front Microbiol.* 2015;6:1016.

145. Marsico C, Kimberlin DW. Congenital Cytomegalovirus infection: advances and challenges in diagnosis, prevention and treatment. *Ital J Pediatr.* 2017;43(1):38.

146. Mattes FM, McLaughlin JE, Emery VC, Clark DA, Griffiths PD. Histopathological detection of owl's eye inclusions is still specific for cytomegalovirus in the era of human herpesviruses 6 and 7. *J Clin Pathol.* 2000;53(8):612-614.

147. Ross SA, Novak Z, Pati S, Boppana SB. Overview of the diagnosis of cytomegalovirus infection. *Infect Disord Drug Targets.* 2011;11(5):466-474.

148. Wolmer-Solberg N, Baryawno N, Rahbar A, et al. Frequent detection of human cytomegalovirus in neuroblastoma: a novel therapeutic target? *Int J Cancer.* 2013;133(10):2351-2361.

149. Prichard MN. Function of human cytomegalovirus UL97 kinase in viral infection and its inhibition by maribavir. *Rev Med Virol.* 2009;19(4):215-229.

150. Bogner E. Human cytomegalovirus terminase as a target for antiviral chemotherapy. *Rev Med Virol.* 2002;12(2):115-127.

151. Littler E, Stuart AD, Chee MS. Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. *Nature.* 1992;358(6382):160-162.

152. Tan BH. Cytomegalovirus Treatment. *Curr Treat Options Infect Dis.* 2014;6(3):256-270.

153. Biron KK. Antiviral drugs for cytomegalovirus diseases. *Antiviral Res.* 2006;71(2-3):154-163.

154. Mercorelli B, Lembo D, Palu G, Loregian A. Early inhibitors of human cytomegalovirus: state-of-art and therapeutic perspectives. *Pharmacol Ther.* 2011;131(3):309-329.

155. Ahmed A. Antiviral treatment of cytomegalovirus infection. *Infect Disord Drug Targets.* 2011;11(5):475-503.

156. Ohlin M, Soderberg-Naucler C. Human antibody technology and the development of antibodies against cytomegalovirus. *Mol Immunol.* 2015;67(2 Pt A):153-170.

157. Anderholm KM, Bierle CJ, Schleiss MR. Cytomegalovirus Vaccines: Current Status and Future Prospects. *Drugs.* 2016;76(17):1625-1645.

158. Soderberg-Naucler C. Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer? *J Intern Med.* 2006;259(3):219-246.

159. Cobbs CS, Harkins L, Samanta M, et al. Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res.* 2002;62(12):3347-3350.

160. Taher C, Frisk G, Fuentes S, et al. High prevalence of human cytomegalovirus in brain metastases of patients with primary breast and colorectal cancers. *Transl Oncol.* 2014;7(6):732-740.

161. Baryawno N, Rahbar A, Wolmer-Solberg N, et al. Detection of human cytomegalovirus in medulloblastomas reveals a potential therapeutic target. *J Clin Invest.* 2011;121(10):4043-4055.

162. Rahbar A, Orrego A, Peredo I, et al. Human cytomegalovirus infection levels in glioblastoma multiforme are of prognostic value for survival. *J Clin Virol.* 2013;57(1):36-42.

163. Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol.* 2006;45(4):529-538.

164. Munoz N, Castellsague X, de Gonzalez AB, Gissmann L. Chapter 1: HPV in the etiology of human cancer. *Vaccine.* 2006;24 Suppl 3:S3/1-10.

165. Chua MLK, Wee JTS, Hui EP, Chan ATC. Nasopharyngeal carcinoma. *Lancet.* 2016;387(10022):1012-1024.

166. Blackadar CB. Historical review of the causes of cancer. *World J Clin Oncol.* 2016;7(1):54-86.

167. Giam CZ, Semmes OJ. HTLV-1 Infection and Adult T-Cell Leukemia/Lymphoma-A Tale of Two Proteins: Tax and HBZ. *Viruses.* 2016;8(6).

168. Michaelis M, Doerr HW, Cinatl J. The story of human cytomegalovirus and cancer: increasing evidence and open questions. *Neoplasia.* 2009;11(1):1-9.

169. Geder L, Sanford EJ, Rohner TJ, Rapp F. Cytomegalovirus and cancer of the prostate: in vitro transformation of human cells. *Cancer Treat Rep.* 1977;61(2):139-146.

170. Cobbs CS, Soroceanu L, Denham S, et al. Human cytomegalovirus induces cellular tyrosine kinase signaling and promotes glioma cell invasiveness. *J Neurooncol.* 2007;85(3):271-280.

171. Cobbs CS, Soroceanu L, Denham S, Zhang W, Kraus MH. Modulation of oncogenic phenotype in human glioma cells by cytomegalovirus IE1-mediated mitogenicity. *Cancer Res.* 2008;68(3):724-730.

172. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144(5):646-674.

173. Downward J. Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer.* 2003;3(1):11-22.

174. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer.* 2002;2(7):489-501.

175. Johnson RA, Wang X, Ma XL, Huong SM, Huang ES. Human cytomegalovirus up-regulates the phosphatidylinositol 3-kinase (PI3-K) pathway: inhibition of PI3-K activity inhibits viral replication and virus-induced signaling. *J Virol.* 2001;75(13):6022-6032.

176. Johnson RA, Huong SM, Huang ES. Activation of the mitogen-activated protein kinase p38 by human cytomegalovirus infection through two distinct pathways: a novel mechanism for activation of p38. *J Virol.* 2000;74(3):1158-1167.

177. Hwang ES, Zhang Z, Cai H, et al. Human cytomegalovirus IE1-72 protein interacts with p53 and inhibits p53-dependent transactivation by a mechanism different from that of IE2-86 protein. *J Virol.* 2009;83(23):12388-12398.

178. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell.* 2004;116(2):205-219.

179. Zhu H, Shen Y, Shenk T. Human cytomegalovirus IE1 and IE2 proteins block apoptosis. *J Virol.* 1995;69(12):7960-7970.

180. Tanaka K, Zou JP, Takeda K, et al. Effects of human cytomegalovirus immediate-early proteins on p53-mediated apoptosis in coronary artery smooth muscle cells. *Circulation.* 1999;99(13):1656-1659.

181. Skaletskaya A, Bartle LM, Chittenden T, McCormick AL, Mocarski ES, Goldmacher VS. A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. *Proc Natl Acad Sci U S A.* 2001;98(14):7829-7834.

182. Goldmacher VS, Bartle LM, Skaletskaya A, et al. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. *Proc Natl Acad Sci U S A.* 1999;96(22):12536-12541.

183. Wright WE, Pereira-Smith OM, Shay JW. Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. *Mol Cell Biol.* 1989;9(7):3088-3092.

184. Deng Y, Chang S. Role of telomeres and telomerase in genomic instability, senescence and cancer. *Lab Invest.* 2007;87(11):1071-1076.

185. Straat K, Liu C, Rahbar A, et al. Activation of telomerase by human cytomegalovirus. *J Natl Cancer Inst.* 2009;101(7):488-497.

186. Kalejta RF, Bechtel JT, Shenk T. Human cytomegalovirus pp71 stimulates cell cycle progression by inducing the proteasome-dependent degradation of the retinoblastoma family of tumor suppressors. *Mol Cell Biol.* 2003;23(6):1885-1895.

187. Hume AJ, Finkel JS, Kamil JP, Coen DM, Culbertson MR, Kalejta RF. Phosphorylation of retinoblastoma protein by viral protein with cyclin-dependent kinase function. *Science.* 2008;320(5877):797-799.

188. Shen Z. Genomic instability and cancer: an introduction. *J Mol Cell Biol.* 2011;3(1):1-3.

189. Maussang D, Verzijl D, van Walsum M, et al. Human cytomegalovirus-encoded chemokine receptor US28 promotes tumorigenesis. *Proc Natl Acad Sci U S A.* 2006;103(35):13068-13073.

190. Gustafsson RK, Jeffery HC, Yaiw KC, et al. Direct infection of primary endothelial cells with human cytomegalovirus prevents angiogenesis and migration. *J Gen Virol.* 2015;96(12):3598-3612.

191. Cinatl J, Jr., Bittoova M, Margraf S, et al. Cytomegalovirus infection decreases expression of thrombospondin-1 and -2 in cultured human retinal glial cells: effects of antiviral agents. *J Infect Dis.* 2000;182(3):643-651.

192. Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet.* 2007;370(9581):59-67.

193. Gomez GG, Kruse CA. Mechanisms of malignant glioma immune resistance and sources of immunosuppression. *Gene Ther Mol Biol.* 2006;10(A):133-146.

194. Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. Regulatory T cells in cancer. *Adv Cancer Res.* 2010;107:57-117.

195. Soderberg-Naucler C. Human cytomegalovirus persists in its host and attacks and avoids elimination by the immune system. *Crit Rev Immunol.* 2006;26(3):231-264.

196. McSharry BP, Avdic S, Slobedman B. Human cytomegalovirus encoded homologs of cytokines, chemokines and their receptors: roles in immunomodulation. *Viruses.* 2012;4(11):2448-2470.

197. Soderberg-Naucler C, Fish KN, Nelson JA. Interferon-gamma and tumor necrosis factor-alpha specifically induce formation of cytomegalovirus-permissive monocyte-derived macrophages that are refractory to the antiviral activity of these cytokines. *J Clin Invest.* 1997;100(12):3154-3163.

198. Qiu H, Straat K, Rahbar A, Wan M, Soderberg-Naucler C, Haeggstrom JZ. Human CMV infection induces 5-lipoxygenase expression and leukotriene B4 production in vascular smooth muscle cells. *J Exp Med.* 2008;205(1):19-24.

199. Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol.* 2012;13(5):518-527.

200. Harkins LE, Matlaf LA, Soroceanu L, et al. Detection of human cytomegalovirus in normal and neoplastic breast epithelium. *Herpesviridae.* 2010;1(1):8.

201. Soroceanu L, Matlaf L, Bezrookove V, et al. Human cytomegalovirus US28 found in glioblastoma promotes an invasive and angiogenic phenotype. *Cancer Res.* 2011;71(21):6643-6653.

202. Shono T, Tofilon PJ, Bruner JM, Owolabi O, Lang FF. Cyclooxygenase-2 expression in human gliomas: prognostic significance and molecular correlations. *Cancer Res.* 2001;61(11):4375-4381.

203. Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer.* 2010;10(3):181-193.

204. Cuzick J, Otto F, Baron JA, et al. Aspirin and non-steroidal anti-inflammatory drugs for cancer prevention: an international consensus statement. *Lancet Oncol.* 2009;10(5):501-507.

205. Blaheta RA, Weich E, Marian D, et al. Human cytomegalovirus infection alters PC3 prostate carcinoma cell adhesion to endothelial cells and extracellular matrix. *Neoplasia.* 2006;8(10):807-816.

206. Shimamura M, Murphy-Ullrich JE, Britt WJ. Human cytomegalovirus induces TGF-beta1 activation in renal tubular epithelial cells after epithelial-to-mesenchymal transition. *PLoS Pathog.* 2010;6(11):e1001170.

207. Cinatl J, Jr., Blaheta R, Bittoova M, et al. Decreased neutrophil adhesion to human cytomegalovirus-infected retinal pigment epithelial cells is mediated by virus-induced up-regulation of Fas ligand independent of neutrophil apoptosis. *J Immunol.* 2000;165(8):4405-4413.

208. Streblow DN, Soderberg-Naucler C, Vieira J, et al. The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. *Cell.* 1999;99(5):511-520.

209. Warburg O. On the origin of cancer cells. *Science.* 1956;123(3191):309-314.

210. Chambers JW, Maguire TG, Alwine JC. Glutamine metabolism is essential for human cytomegalovirus infection. *J Virol.* 2010;84(4):1867-1873.

211. Jones PA, Baylin SB. The epigenomics of cancer. *Cell.* 2007;128(4):683-692.

212. Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev Cancer.* 2004;4(2):143-153.

213. Esteki-Zadeh A, Karimi M, Straat K, et al. Human cytomegalovirus infection is sensitive to the host cell DNA methylation state and alters global DNA methylation capacity. *Epigenetics.* 2012;7(6):585-593.

214. Estekizadeh A, Landazur N, Bartek J, Jr., et al. Increased cytomegalovirus replication by 5-Azacytidine and viral-induced cytoplasmic expression of DNMT1 in medulloblastoma and endothelial cells. *Int J Oncol.* 2018;52(4):1317-1327.

215. Fortunato EA, Spector DH. p53 and RPA are sequestered in viral replication centers in the nuclei of cells infected with human cytomegalovirus. *J Virol.* 1998;72(3):2033-2039.

216. Fortunato EA, McElroy AK, Sanchez I, Spector DH. Exploitation of cellular signaling and regulatory pathways by human cytomegalovirus. *Trends Microbiol.* 2000;8(3):111-119.

217. Kalejta RF, Shenk T. Manipulation of the cell cycle by human cytomegalovirus. *Front Biosci.* 2002;7:d295-306.

218. Biswas N, Sanchez V, Spector DH. Human cytomegalovirus infection leads to accumulation of geminin and inhibition of the licensing of cellular DNA replication. *J Virol.* 2003;77(4):2369-2376.

219. Wiebusch L, Uecker R, Hagemeier C. Human cytomegalovirus prevents replication licensing by inhibiting MCM loading onto chromatin. *EMBO Rep.* 2003;4(1):42-46.

220. Bresnahan WA, Boldogh I, Thompson EA, Albrecht T. Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1. *Virology.* 1996;224(1):150-160.

221. Dittmer D, Mocarski ES. Human cytomegalovirus infection inhibits G1/S transition. *J Virol.* 1997;71(2):1629-1634.

222. Salsman J, Jagannathan M, Paladino P, et al. Proteomic profiling of the human cytomegalovirus UL35 gene products reveals a role for UL35 in the DNA repair response. *J Virol.* 2012;86(2):806-820.

223. Sampson JH, Mitchell DA. Is cytomegalovirus a therapeutic target in glioblastoma? *Clin Cancer Res.* 2011;17(14):4619-4621.

224. Heukers R, Fan TS, de Wit RH, et al. The constitutive activity of the virally encoded chemokine receptor US28 accelerates glioblastoma growth. *Oncogene.* 2018;37(30):4110-4121.

225. Stragliotto G, Rahbar A, Solberg NW, et al. Effects of valganciclovir as an add-on therapy in patients with cytomegalovirus-positive glioblastoma: a randomized, double-blind, hypothesis-generating study. *Int J Cancer.* 2013;133(5):1204-1213.

226. Soderberg-Naucler C, Rahbar A, Stragliotto G. Survival in patients with glioblastoma receiving valganciclovir. *N Engl J Med.* 2013;369(10):985-986.

227. Mitchell DA, Batich KA, Gunn MD, et al. Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. *Nature.* 2015;519(7543):366-369.

228. Nair SK, De Leon G, Boczkowski D, et al. Recognition and killing of autologous, primary glioblastoma tumor cells by human cytomegalovirus pp65-specific cytotoxic T cells. *Clin Cancer Res.* 2014;20(10):2684-2694.

229. Schuessler A, Smith C, Beagley L, et al. Autologous T-cell therapy for cytomegalovirus as a consolidative treatment for recurrent glioblastoma. *Cancer Res.* 2014;74(13):3466-3476.

230. Ranganathan P, Clark PA, Kuo JS, Salamat MS, Kalejta RF. Significant association of multiple human cytomegalovirus genomic Loci with glioblastoma multiforme samples. *J Virol.* 2012;86(2):854-864.

231. Libard S, Popova SN, Amini RM, et al. Human cytomegalovirus tegument protein pp65 is detected in all intra- and extra-axial brain tumours independent of the tumour type or grade. *PLoS One.* 2014;9(9):e108861.

232. Baumgarten P, Michaelis M, Rothweiler F, et al. Human cytomegalovirus infection in tumor cells of the nervous system is not detectable with standardized pathologico-virological diagnostics. *Neuro Oncol.* 2014;16(11):1469-1477.

233. Sardi I, Lucchesi M, Becciani S, et al. Absence of human cytomegalovirus infection in childhood brain tumors. *Am J Cancer Res.* 2015;5(8):2476-2483.

234. Johnsen JI, Baryawno N, Soderberg-Naucler C. Is human cytomegalovirus a target in cancer therapy? *Oncotarget.* 2011;2(12):1329-1338.

235. Hadaczek P, Ozawa T, Soroceanu L, et al. Cidofovir: a novel antitumor agent for glioblastoma. *Clin Cancer Res.* 2013;19(23):6473-6483.

236. Hamerlik P, Lathia JD, Rasmussen R, et al. Autocrine VEGF-VEGFR2-Neuropilin-1 signaling promotes glioma stem-like cell viability and tumor growth. *J Exp Med.* 2012;209(3):507-520.

237. Soroceanu L, Matlaf L, Khan S, et al. Cytomegalovirus Immediate-Early Proteins Promote Stemness Properties in Glioblastoma. *Cancer Res.* 2015;75(15):3065-3076.

238. Sundar SJ, Hsieh JK, Manjila S, Lathia JD, Sloan A. The role of cancer stem cells in glioblastoma. *Neurosurg Focus.* 2014;37(6):E6.

239. Hitoshi S, Seaberg RM, Koscik C, et al. Primitive neural stem cells from the mammalian epiblast differentiate to definitive neural stem cells under the control of Notch signaling. *Genes Dev.* 2004;18(15):1806-1811.

240. Kanamori M, Kawaguchi T, Nigro JM, et al. Contribution of Notch signaling activation to human glioblastoma multiforme. *J Neurosurg.* 2007;106(3):417-427.

241. Odeberg J, Wolmer N, Falci S, et al. Late human cytomegalovirus (HCMV) proteins inhibit differentiation of human neural precursor cells into astrocytes. *J Neurosci Res.* 2007;85(3):583-593.

242. Odeberg J, Wolmer N, Falci S, Westgren M, Seiger A, Soderberg-Naucler C. Human cytomegalovirus inhibits neuronal differentiation and induces apoptosis in human neural precursor cells. *J Virol.* 2006;80(18):8929-8939.

243. Bartkova J, Hamerlik P, Stockhausen MT, et al. Replication stress and oxidative damage contribute to aberrant constitutive activation of DNA damage signalling in human gliomas. *Oncogene.* 2010;29(36):5095-5102.

244. Bartkova J, Hoei-Hansen CE, Krizova K, et al. Patterns of DNA damage response in intracranial germ cell tumors versus glioblastomas reflect cell of origin rather than brain environment: implications for the anti-tumor barrier concept and treatment. *Mol Oncol.* 2014;8(8):1667-1678.

245. Lukas C, Savic V, Bekker-Jensen S, et al. 53BP1 nuclear bodies form around DNA lesions generated by mitotic transmission of chromosomes under replication stress. *Nat Cell Biol.* 2011;13(3):243-253.

246. Halazonetis TD, Gorgoulis VG, Bartek J. An oncogene-induced DNA damage model for cancer development. *Science.* 2008;319(5868):1352-1355.

247. De Smaele E, Di Marcotullio L, Ferretti E, Screpanti I, Alesse E, Gulino A. Chromosome 17p deletion in human medulloblastoma: a missing checkpoint in the Hedgehog pathway. *Cell Cycle.* 2004;3(10):1263-1266.

248. Castellino RC, De Bortoli M, Lu X, et al. Medulloblastomas overexpress the p53-inactivating oncogene WIP1/PPM1D. *J Neurooncol.* 2008;86(3):245-256.

249. Cojoc M, Mabert K, Muders MH, Dubrovska A. A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms. *Semin Cancer Biol.* 2015;31:16-27.

250. Fornara O, Bartek J, Jr., Rahbar A, et al. Cytomegalovirus infection induces a stem cell phenotype in human primary glioblastoma cells: prognostic significance and biological impact. *Cell Death Differ.* 2016;23(2):261-269.

251. Burrell RA, McClelland SE, Endesfelder D, et al. Replication stress links structural and numerical cancer chromosomal instability. *Nature.* 2013;494(7438):492-496.

252. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. *Nature.* 2013;501(7467):338-345.

253. E X, Savidis G, Chin CR, et al. A novel DDB2-ATM feedback loop regulates human cytomegalovirus replication. *J Virol.* 2014;88(4):2279-2290.

254. Luo MH, Rosenke K, Czornak K, Fortunato EA. Human cytomegalovirus disrupts both ataxia telangiectasia mutated protein (ATM)- and ATM-Rad3-related kinase-mediated DNA damage responses during lytic infection. *J Virol.* 2007;81(4):1934-1950.

255. Zavala AG, O'Dowd JM, Fortunato EA. Infection of a Single Cell Line with Distinct Strains of Human Cytomegalovirus Can Result in Large Variations in Virion Production and Facilitate Efficient Screening of Virus Protein Function. *J Virol.* 2015;90(5):2523-2535.

256. O'Dowd JM, Zavala AG, Brown CJ, Mori T, Fortunato EA. HCMV-infected cells maintain efficient nucleotide excision repair of the viral genome while abrogating repair of the host genome. *PLoS Pathog.* 2012;8(11):e1003038.

257. Jakola AS, Werlenius K, Mudaisi M, et al. Disulfiram repurposing combined with nutritional copper supplement as add-on to chemotherapy in recurrent glioblastoma (DIRECT): Study protocol for a randomized controlled trial. *F1000Res.* 2018;7:1797.