Oncomodulatory properties of the human cytomegalovirus-encoded receptors US28 and UL33

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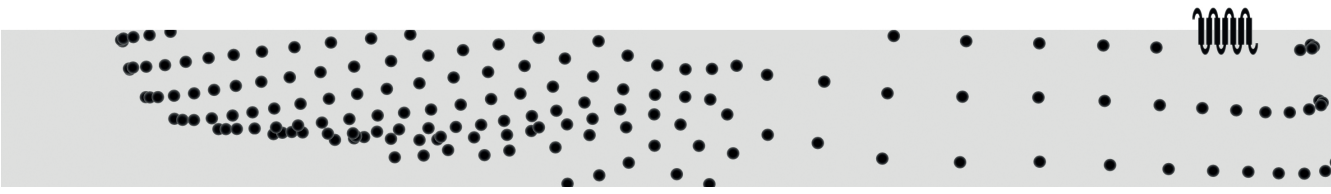
chapter 6

Discussion



This thesis describes how the HCMV-encoded receptors US28 and UL33 orchestrate several proliferative, anti-apoptotic and angiogenic signaling pathways. In this final chapter, the oncomodulatory properties of US28 and UL33 are summarized (Figure 6.1) and put in context with other herpesvirus-encoded receptors described in literature.

6.1 US28 induces COX-2 gene expression

In the early 2000's, extensive studies by our lab and various other research groups, resulted in the pharmacological characterization of the HCMV-encoded receptor US28 [82, 151, 156, 169-171, 219, 248]. The constitutive activity of US28, stably expressed in NIH-3T3 mouse fibroblasts, as well as the availability of the G-protein uncoupled mutant US28-R^{129}A, have proven to be valuable tools in deciphering several US28-induced cellular signaling pathways. Using these tools, we showed that the constitutively active chemokine receptor US28 has the ability to promote tumor formation and therefore might promote or contribute to HCMV-associated malignancies [6]. **Chapter 2** of this thesis describes the role of cyclooxygenase 2, or COX-2, in US28-mediated angiogenesis and tumor formation. US28-induced upregulation of the expression of the COX-2 gene via activation of NF-κB, resulting in enhanced angiogenic responses as exemplified by VEGF promoter activation. COX-2 is of specific interest because it is a key mediator of inflammation and it is now well-established that it contributes to the pathogenesis of several forms of cancer [173, 175]. Other potentially interesting genes that were found differentially expressed in the US28-expressing NIH-3T3 cells will be discussed later-on in Section 6.6. Also, in HCMV-infected cells, US28 contributed to the viral induction of

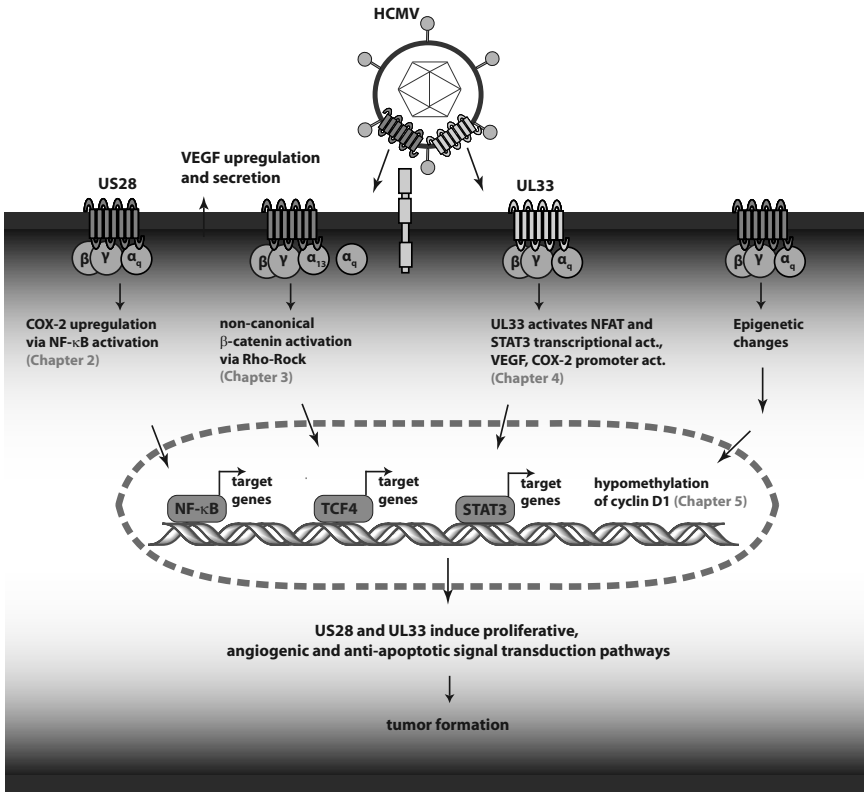


Figure 6.1: Overview of US28- and UL33-induced signal transduction pathways described in this thesis. US28 activates COX-2 upregulation via NF-κB (Chapter 2), non-canonical β-catenin activation via Rho-Rock (Chapter 3) and epigenetic demethylation of the cell cycle regulator cyclin D1 gene (Chapter 5). Additionally, HCMV-encoded receptor UL33 induced various signal transduction pathways, including NFAT and STAT3 transcriptional activity, VEGF and COX-2 promoter activity. These proliferative, angiogenic and anti-apoptotic signal transduction pathways play a role in US28 and UL33-related oncomodulation, leading to tumor formation when expressed in NIH-3T3 cells, and are associated with a poor prognosis for HCMV positive glioblastoma patients.

COX-2. US28-dependent increases of at least COX-2 early after infection might be sufficient to catalyze inflammatory processes, which may contribute to or enhance tumor formation.

As was recently shown by Baryawno *et al.*, a large proportion of primary medulloblastomas, the most common malignant brain tumors in children, as well as medulloblastoma cell lines are infected with HCMV and show increases in COX-2 expression [234]. Both HCMV immediate-early proteins and late proteins are present in the majority of primary medulloblastomas. When engrafted into immunocompromised mice, human medulloblastoma cells induced expression of HCMV proteins. HCMV and COX-2 expression correlated in primary tumors, cell lines, and medulloblastoma xenografts. Additionally, COX-2 expression and increased PGE2 levels in medulloblastomas were shown to be directly modulated by the HCMV virus. In **Chapter 2** we describe the US28-mediated induction of COX-2 gene expression and its link to tumor formation. Treatment of US28-positive cells with Celecoxib, a specific COX-2 inhibitor, reduced the tumor formation in the xenograft model. In the study by Baryawno *et al*. the antiviral drug valganciclovir and the specific COX-2 inhibitor Celecoxib prevented HCMV replication *in vitro*. Both compounds inhibited PGE2 production and reduced medulloblastoma tumor cell growth both *in vitro* and *in vivo*. Ganciclovir did not affect the growth of HCMV-negative tumor cell lines.

Additionally, Baryawno *et al*. found that US28 mRNA could be detected in *in vitro* infected medulloblastoma cells already at 15 minutes after infection: low US28 mRNA levels were also detected in UV-treated HCMV (UVHCMV)–infected cells, in which no transcription of viral DNA takes place and no *de novo* RNA molecules are synthesized. This US28 expression was consistent with the increased expression of COX-2 mRNA observed in both HCMV- and UVHCMV–infected cells at 1 and 3 hours after infection. The COX-2 expression was detected in cells positive for US28, but earlier than IE mRNA could be detected. Baryawno et al. speculate that US28 (protein and RNA) delivered by the virus particle results in the early expression of COX-2 protein, while newly transcribed US28 RNA molecules produced during viral replication were responsible for the later induced COX-2 expression (48 and 72 hours after infection). In **Chapter 4**, we provide evidence that US28 is expressed readily after infection, as detected by enhanced CCL5 binding on HCMV as well as UVHCMV-infected of cells, suggesting its presence in virions. Altogether, these findings suggest an important role for HCMV, US28 and HCMV-induced activation of COX-2 in medulloblastoma and may put HCMV encoded receptors forward as potential therapeutic targets for this brain tumor [234].

6.2 US28 induces β-catenin signaling

Chronic activation of Wnt/β-catenin signaling is found in a variety of human malignancies including melanoma, colorectal and hepatocellular carcinomas [221, 222]. Recently, a study with transgenic mice, demonstrated that expression of US28 induces intestinal neoplasia, via activation of this well-known tumorigenesis-related classical β-catenin signaling [87]. **Chapter 3** of this thesis describes a novel mechanism of β-catenin activation by US28, involving the Rho-Rock pathway, which results in Tcf-Lef activation. Mechanistically the classical Wnt-induced β-catenin pathway does not seem to be involved in the US28-expressing NIH-3T3 cells, as the characteristic Wnt-induced LRP6 phosphorylation of serine 1490 does not occur upon US28-induced activation of the pathway. Moreover, GSK-3β, a component of the destruction complex, is not phosphorylated at serine 9 (data not shown), as is the case in the US28 transgenic mice [87]. After exclusion of the well-studied canonical β-catenin pathway, we found a novel, non-canonical pathway to be involved in US28-mediated β-catenin activation. Analysis of US28-mediated signaling, indicated the involvement of the Rho-Rho kinase (ROCK) pathway in the activation of β-catenin when expressed in NIH-3T3 or HEK293 cells. Coupling of US28 to both $G\alpha_q$ and $G\alpha_{12/13}$ proteins is essential for the observed activation of β-catenin signaling. Overexpression, scavenging and/or downmodulation of either G protein greatly affect US28-mediated β-catenin signaling. Moreover, cells infected with HCMV show significant increases in β-catenin stabilization and signaling. This is mediated to a large extent by expression of US28, as shown by infection of cells with the US28-deletion virus. The modulation of the β-catenin signal transduction pathway by a viral chemokine receptor provides alternative regulation of this pathway. A potential role for the constitutive active US28 in the development of colon cancer, as well as brain cancers in combination with activation of the classical β-catenin pathway [87], is of high interest and requires further investigation.

Besides Wnt and mutations of activators of the Wnt pathway, also viruses appear to interfere with β-catenin signaling (Table 6.1). The Epstein-Barr virus (EBV) e.g. activates β-catenin in latently infected B lymphocytes [242]. The human papillomavirus (HPV) E6 and E7 oncogenes appear to contribute to activation of β-catenin signaling in HPV16-positive oropharyngeal squamous carcinoma cells [243] and the hepatitis C virus (HCV) encoded core protein potentiates Wnt/β-catenin signaling in hepatocellular carcinoma cells [244]. For the human immunodeficiency virus (HIV), however, active Wnt/β-catenin signaling plays a significant role in repression of HIV-1 replication in multiple cell targets [245, 246].

Table 6.1: Viruses interfering with β-catenin signaling. N.S. indicates Not Specified

Virus	Viral gene	Action	Cells	Ref
EBV	N.S.	Activation of β-catenin signaling	latently infected B lymphocytes	[242]
HPV	E6 and E7 oncogenes	Activation of β-catenin signaling	HPV16-positive oropharyngeal squamous carcinoma cells	[243]
HCV	core protein	Potentiation of Wnt/β-catenin signaling	hepatocellular carcinoma cells	[244]
HIV	N.S.	repression of HIV-1 replication	multiple cell targets	[245, 246]
HCMV	US28UL33	Activation of β-catenin signaling, both classical and new mechanism	intestinal stem cells, NIH-3T3 stable expressing cells	[87], Chapter 3

Hence, by expression of a viral receptor or other proteins, viruses, like HCMV, EBV, HPV and HCV might be able to rewire β-catenin signaling, contributing to malignant phenotypes.

6.3 UL33, a second HCMV-encoded oncomodulatory GPCR

US28's capacity for proliferative signaling has been studied in detail [6, 80, 81, 87]. Like US28, we have previously shown that UL33 signals in a constitutive manner via $G\alpha_q$, $G\alpha_i$, and $G\alpha_s$ [156]. In **Chapter 4** we describe that UL33 displays oncogenic potential as well. Previously, UL33 localization was examined using a HCMV-TB40-BAC4 derived recombinant virus containing a flag-tagged UL33 [146, 265], Here we used the flag-tagged version of UL33 to compare the kinetics of expression to that of US28. Analysis of the UL33 expression pattern by fluorescent imaging as well as Western blotting revealed different kinetics for UL33 compared to those of US28. Expression of UL33 is observed at 96, 120 and 144 hours post infection (h.p.i.), while US28 expression is already detecTable 24 h.p.i. In addition, PLC activation at 48 h.p.i. is reduced in cells infected with recombinant HCMV lacking US28, and not UL33, suggesting that US28 is the important player in the early phase after infection. Moreover, we show evidence of US28 receptor expression readily after infection, suggesting its presence on the virion,. In view of UL33's expression pattern, UL33-mediated activation of proliferative signaling pathways is expected to occur in late stages after infection.

We show UL33 activates similar pathways as US28, including NFAT and STAT3

transcriptional activation, VEGF and COX-2 promoter activation. UL33 also induces Tcf-Lef dependent transcriptional activity (**Chapter 3**), which may account for the remaining β-catenin activation in cells infected with the HCMV strain lacking US28 (ΔUS28). With UL33-expressing NIH-3T3 cells the proliferative potential of UL33 was confirmed by tumor formation in nude mice (**Chapter 4**). The level of proliferation seen with UL33-expressing cells in e.g. foci formation assay, PLC activation and thymidine incorporation, was in all cases lower compared to US28-induced proliferation.

Finally, UL33 expression has been detected in glioblastoma samples, hinting at a potential role in tumor formation for this receptor as well. While US28 was expressed in the vascular niche of the tumor, UL33 appeared cytoplasmic in patches of tumor cells throughout the tissue. One patient out of 25 was negative, while 15/25 showed UL33 expression in <10% of the tumor cells (grade 1) and the remaining 9/25 showed UL33 expression in >10%-50% of the tumor cells (grade 2). However, these studies showed no positive correlation between patient prognosis and UL33 expression level, as was earlier found for US28 expression [80]. The fact that the UL33 receptor is expressed in the glioblastoma and not outside the tumor, suggests this could be a novel, non-endogenously expressed therapeutic target for glioblastoma treatment. In view of defining potential HCMV-related therapeutic targets, UL33 can therefore be added to the list of HCMV proteins that are present in human glioblastoma tissue samples [260], as a potential drug target.

6.4 Interplay vGPCRs

Data presented in this thesis are obtained from *in vitro* experiments, in which DNA of the viral receptor or the laboratory (AD169) or clinical strains (TB40/E) of the HCMV and mutant viruses are used to show the potential influence of the viral receptors on the signal transduction pathways in cellular systems. We have used mouse and human cell lines like NIH-3T3, HEK293 cells, human foreskin fibroblasts (HFF) or glioma cancer cell line U373. Using US28-deletion viruses we validated the importance of the US28 in activating signal transduction pathways like COX-2, NF-κB and β-catenin (**Chapter 2** and **3**). These virus experiments validated the importance of the activation of the PLC pathway, as measured by inositol phosphate formation. The US28 deletion mutant virus showed a signif-

icant reduction in PLC activation 48 hrs post infection (**Chapter 4**). For future experiments it is recommended to continue the use of (clinical) HCMV viruses as well as mutant viruses and infect cell types that are natural hosts for the HCMV virus. One can think of endothelial or epithelial cells, or macrophages. Effects of HCMV on cellular signaling and the role of US28 and UL33 therein should be further investigated in these cells.

The UL33 protein was described to be present on the virion [146]. Since both US28 and UL33 might be expressed on the virus particles or 96 h.p.i., as described in **Chapter 4**, we speculate that dimerization of these GPCRs might occur. Recently, dimerization of US28 and UL33 has been reported and was found to reduce constitutive signaling of US28 [89]. Interestingly, this was associated with a marked decrease of NF-κB activation, while no effect was seen on PLC activation [89]. Conformational changes introduced by the heteromerization of these vGPCRs, might result in these altered signaling capacities. The mechanisms that are potentially involved require further attention.

Recently, also the role of the HCMV-encoded receptor US27 was studied in more detail. Mutant virus strains lacking US27 rely primarily on direct cell-to-cell spread, and the viral GPCR homologue is thought to act at a late stage of the HCMV replication cycle to support spread of virus by the extracellular route [91]. The fact that US27 is able to dimerize with US28 [89] might indicate that expression of US27 influences the membrane expression of US28, thereby interfering in US28-mediated signaling. Also HCMV-driven signal transduction aimed at virus dissemination and replication [293] requires further attention.

6.5 US28 is involved in epigenetic regulation of cyclin D1

Besides the analysis of gene expression profiles of US28-expressing NIH-3T3 cells, epigenetic changes were studied by means of methylation arrays. From the considerable amount of interesting data the NimbleGen technology provided, we focused on one particularly interesting gene, Ccnd1, encoding the cell cycle progression protein Cyclin D1. Previously, we showed increased expression of CyclinD1 upon expression of US28 [6]. In **Chapter 5** we examined potential epigenetic regulation by US28. In this study we describe US28-mediated epigenetic changes in the third CpG island of the Ccnd1 gene. US28-induced changes in methylation

of the Ccnd1 gene were validated by methylation-specific melting curve analysis (MS MCA), using inhibitors and bisulfite sequencing. Sequence analyses suggested several CG-pairs in the Ccnd1 promoter region to be partially hypomethylated in US28-expressing NIH-3T3 cells. Treatment with the demethylating agent DAC showed induction of both Cyclin D1 (Ccnd1) mRNA level as well as Cyclin D1 protein level. Hence, the US28-induced demethylation of Ccnd1 might play a role in tumor onset. This is the first report in which a virally encoded GPCR is linked to the specific epigenetic alterations of a Cyclin D1 CpG island, eventually resulting in upregulation of the Ccnd1 cell cycle protein. Our study indicates that the cell cycle gene Cyclin D1 is regulated via an epigenetic mechanism, suggesting HCMV interference in the host cell at the DNA, RNA and protein level.

Since HCMV encodes for other potential viral oncogenes like the viral receptor UL33 described in **Chapter 4**, the question of epigenetic aberrations induced by HCMV needs to be studied in a broader way; using HCMV-infected cells. In a recent paper, the sensitivity of HCMV infection to the host cell DNA methylation state as well as the altered global DNA methylation state upon infection is described [283]. In general the data by Esteki-Zadeh [283] demonstrate that: (i) DNA methylation influences cellular susceptibility to HCMV infection; (ii) HCMV inhibits the host cell DNA methylation machinery and; (iii) infection by HCMV results in the re-localization and accumulation of DNMTs from the nucleus to the cytoplasm. Additionally, analysis at 3 days post infection (d.p.i.) of DNA extracted from MRC-5 cells infected with the endothelial cell adapted clinical isolate VR1814 revealed a profound hypomethylation compared with uninfected cells. However, the techniques used to detect this hypomethylation, Luminometric methylation assay (LUMA) and Nearest Neighbor Analysis (NNA) do not involve host gene methylation specifically. The authors conclude that the main source of hypomethylated DNA in infected cells is derived from the newly replicated viral genome, since it is not detected upon treatment with Foscavir, which inhibits viral DNA replication. As such, in late stages after infection US28 and UL33 might play a role in this process.

Recently, several double-stranded DNA viruses that are associated with human cancers, were tested by bisulfite sequencing [294]. The dynamic DNA methylomes of HPV16 and 18, Hepatitis B and EBV evolved from an unmethylated to a highly methylated genome in association with the progression of the related disease [294]. This ranged from asymptomatic healthy carriers, through chronically infected tissues and pre-malignant lesions, to the full-blown invasive tumors. A similar approach could be applied to HCMV infected cells, as well as HCMV positive glioblastoma, medulloblastoma and other tumor material. This might provide important clues to help understand the molecular reasons why infection is controlled

or instead progresses to subsequent stages of tumorigenesis. It would be interesting to compare the epigenetic regulation in infected cells caused by several HCMV strains with other herpes viruses (KSHV and EBV), to find common denominators of genes that are regulated at this transcriptional level. Additionally, besides having effects on the host cell methylome, HCMV might also influence its own methylation status and thereby be of influence on latency or immune evasion.

6.6 Transcriptional control of oncomodulatory signaling proteins by US28

Transcriptional profiling of US28-expressing NIH-3T3 cells generated a lot of interesting data, of which some results have been described in the **Chapters 2, 3 and 5**. The proliferative potential of US28 was also observed when the vGPCR was stably expressed in the mouse microvascular endothelial SVEC cells [170]. However, not in all cell lines, tested, US28 mediates transformation. Studies in HEK293 and HeLa cells indicated that caspase-dependent apoptosis occurred upon high expression levels of US28 [78]. The cellular context, therefore, appears to be important for US28-mediated responses. Nevertheless, transcriptional profiling in US28-expressing NIH-3T3 cells hinted at additional pathways potentially involved in US28-mediated oncomodulation. Several interesting genes are differentially regulated. Previously, the US28-induced apoptosis in HEK293 and HeLa cells, was shown to involve the apoptosis-regulating gene cFLIP [78], also known as Cflar (CASP8 and FADD-like apoptosis regulator). Cellular proteins like Cflar, and their viral homologues, inhibit the activation of caspases 10 and 8. Pleskoff *et al.* showed that the US28-induced apoptosis can be prevented by expression of Cflar. In our gene profiling data obtained with the US28-expressing NIH-3T3 cells, Cflar is shown to be upregulated. This suggests that Cflar plays a role in the US28-expressing NIH-3T3 cells, retaining them from undergoing apoptosis. Thus, in HCMV-infected cells, US28 may function either as a pro-apoptotic factor or a transforming agent, depending on expression levels of HCMV and/or cellular anti-apoptotic proteins like Cflar. Interestingly, additional apotosis-related factors are differentially expressed (down-regulated) in US28-expressing NIH-3T3 cells. Several mRNAs encoding for apoptosis regulating proteins, like Bcl2l11 (BCL2-like 11 (apoptosis facilitator)), Bfar (bifunctional apoptosis regulator) and Pawr

(PRKC, apoptosis, WT1, regulator) are down-regulated. Further investigation of Cflar and these other apoptosis regulating protein levels in various cell lines might shed light on the importance of these apoptosis regulating genes on the cellular background in US28-induced apoptosis or proliferation.

Oncogenes that were significantly modulated in US28-expressing NIH-3T3 cells include Fus, Mdm2 and Jarid1a. Fus, a fusion protein derived from human malignant liposarcoma, upregulated in US28-expressing cells, is an important regulator of genome stability [295]. Mdm2, a negative regulator of the tumor suppressor protein p53, is also upregulated by US28. So is the nuclear protein Jarid1a, which binds directly with several other proteins to the retinoblastoma (Rb) protein, which regulates cell proliferation.

Besides upregulation of oncogenes, several tumor suppressor genes, like Fst and Rgs4, were found to be downregulated by US28. Fst has been shown to be downregulated in endometrical and cervical cancer tissues [296]. Rgs4, or regulator of G protein signaling 4, belongs to the family of regulatory RGS proteins that deactivate $G\alpha_i$, $G\alpha_o$ and $G\alpha_q$ protein subtypes by driving G proteins into their inactive GDP-bound forms. Therefore, this protein negatively regulates signaling at the level of the heterotrimeric G protein. Hypothetically, a decrease of Rgs4 in US28-expressing cells could point at loss of a negative regulator of constitutive US28 signaling.

Besides the well studied transcription factors NF-κB, CREB, STAT3 and NFAT that are activated by US28, in gene profiling experiments US28 was found to have an effect on several additional transcription factors. US28 enhances the mRNA level of several interesting transcription factors, like Cebpb, Bclaf1 and Mef2c. Cebpb (CCAAT/enhancer binding protein beta) is a downstream target of Ras signaling, that has been linked to anaplastic large cell lymphoma [297] and oncogenic processes [298]. The Cebpb protein is important in the regulation of genes involved in immune and inflammatory responses. Next, Bclaf1, or BCL2-associated transcription factor 1, is associated with murine lung differentiation and T cell activation. Bclaf1 functions as an inducer of apoptosis, RNA processing and stabilization, lung development, T cell activation, and control of the lytic infection program in KSHV [299]. Transcription factor Mef2c, myocyte enhancer factor 2C, increases the expression of its target gene Hdac9 [300], which was found to be strongly upregulated in US28-expressing cells (**Chapter 5**). A role for Mef2c in proliferation of different cell types like B cells and vSMC, as well as in the development of gastric and breast cancers has been suggested [301, 302]. Altogether, these data clearly suggest US28 is interfering in more cellular signaling pathways. More experiments need to be performed to further validate the role of these proteins in US28- and HCMV-mediated oncomodulation.

6.7 US28 G protein-independent signaling

The constitutive activity of the US28 is highly important, but not solely responsible for tumor formation, as we have demonstrated using the G protein uncoupled mutant US28-R^{129}A in the NIH-3T3 xenograft studies in nude mice [6]. Interestingly, US28-R^{129}A-induced tumor formation is significantly delayed in onset, indicating that activation of $G_{q/11}$ proteins is involved in the (fast) US28-mediated proliferation. However, the residual tumor forming potential of US28-R^{129}A clearly indicates that additional pathways play a role in the effects induced by US28. Onset of tumor growth after an incubation time of 48 instead of 20 days post injection, could be related to modulation of alternative signaling pathways activated by the mutant US28-R^{129}A, like e.g. β-arrestin signaling. US28 does not require β-arrestin for endocytosis, but clathrin-mediated signaling [303]. However, β-arrestins might be involved in biased signaling by virus-encoded receptors. Like the US28-WT, the US28-R^{129}A mutant is not expected to be hampered in β-arrestin recruitment, since this receptor is only altered in the arginine in the DRY motif in TM3, involved in G-protein binding [152, 304]. The C-terminal as well as third intracellular loop of the US28 contain multiple potential phosporylation sites for the GRKs [305] and subsequent β-arrestin binding. GPCR-bound β-arrestin inhibits G protein signaling by hindering GPCR–G protein coupling and (normally) by recruiting proteins involved in receptor endocytosis. However, β-arrestin can also scaffold new signaling cascade components to the activated GPCR, thereby initiating a second wave of intracellular signaling [306]. β-arrestins were also found to be involved in activation of p42/p44 MAPK for several chemokine receptors like CCR5, CCR7 and CXCR4. Whether also G protein-independent signaling pathways are activated by US28 and UL33 requires further research with the non-G-protein coupling mutant US28-R^{129}A and potentially a constitutive inactive mutant of UL33.

6.8 Viral mechanisms in host immune evasion and inflammation

Herpesviruses contain genes that encode for proteins that are involved in mechanisms to evade the host immune system. Besides the four GPCRs encoded by

HCMV, several other immune-response related genes are duplicated by viral genomes. HCMV contains homologs for TNFR (UL144), vCXC-1 (UL146) and vCXC-2 (UL147), both chemoattractants of neutrophils, and vIL-10 (UL111a) which downregulates the Th1 response [307]. During HCMV infection, MHC class I molecules are efficiently down-regulated from the cell surface by the products of four HCMV encoded genes (*US2*, *US3*, *US6*, and *US11*). This down-regulation of endogenous MHC class I combined with the up-regulation of NK activating ligands would be expected to promote a NK cell attack. However, HCMV encodes an impressive array of genes that act in concert to suppress NK cell recognition [308, 309], involving gpUL18, UL141 and UL142, UL16, gpUL40 and UL83 (pp65). Additionally, intervening in cellular antigen presentation mechanisms is also possible. US6 and pp65 prevent peptide generation and transport, resulting in down-regulation of MHC class I molecules [307], which prevents CD8+ T-cell recognition. Clearly, HCMV devotes significant coding capacity to immune modulation.

Besides a role as a chemokine sink [79], US28 was shown to activate NF-κB signal transduction [82]. NF-κB is typically classified as a pro-inflammatory transcription factor, involved in cellular proliferation via induction of a cell type-specific profile of secreted inflammatory factors. Additionally, it is well established that NF-κB induces anti-apoptotic genes [310]. We described the US28-induced and NF-κB-regulated activation of COX-2 gene expression and subsequent increase in COX2 protein level in **Chapter 2**. CMV-mediated inflammation may facilitate viral replication and dissemination. The inflammatory mediator COX-2 is induced by the virus particles upon infection [176, 311]. COX-2 enzymatic activity increases prostaglandin E2 (PGE2) levels, which then positively influences the expression of proteins controlling viral transcription and infectivity [308]. We described NF-κB-mediated IL-6 secretion in US28-expressing cells [80]. As is the case for the tumor-inducing KSHV-encoded constitutive signaling receptor ORF74, US28 induces constitutive activation of this NF-κB transcription factor. ORF74 activated NF-κB controls the expression of pro-angiogenic and pro-inflammatory factors, suggesting it could influence uninfected cells or latently infected cells via autocrine and paracrine mechanisms, thus enhancing KS pathogenesis [104].

Anti-viral genes belonging to the interferon-stimulated gene family (ISGs) and pro-inflammatory genes, like CCL5, IL-6, IL-7, IL-11 and COX-2, were all strongly induced in CMV-infected fibroblasts [176, 308]. Activation of these factors does not require virus replication. US28, which is presumably present at the virus particles, is able to induce signaling immediately upon infection [234]. In the case of UL33, we started detecting the protein 96 hours post infection (**Chapter 4**). In

earlier studies this receptor was also postulated to be present on virus particles [146], suggesting that both US28 and UL33 readily after infection may contribute to activation of these factors. The HCMV glycoprotein B (gB) is an abundant virion envelope protein which is required for virus entry and cell-to-cell spread of the virus. However, HCMV gB is not required for virus attachment or assembly and egress from infected cells [312]. Cells treated with only gB, exhibited an immune marker response similar, but not identical, to that of cells treated with intact virus, suggesting that a signal transduction pathway is activated by cell contact of CMV envelope proteins, resulting in numerous physiological changes that culminate with innate immune activation. A role for both US28 and UL33 in these processes should be further investigated. Which pro-inflammatory factors are secreted by other HCMV-infected physiologically relevant cells, like epithelial cells, endothelial and monocytes, requires more investigation.

6.9 US28 and UL33 as modulators of viral gene transcription?

Transcription factors within host cells that are activated by US28 and/or UL33 might also be involved in regulation of viral gene transcription. To investigate this possibility the presence of Tcf-Lef- and NF-κB binding sites within the HCMV genome was analyzed. Target genes of the TCF4-β-catenin enhancing transcription factor contain the upstream activating sequence (UAS) CCTTTGww with w being A ot T. Interestingly, several HCMV genes were found to contain this UAS. These genes include UL5 (RL11 family), UL33 (vGPCR), UL69 (unknown function), UL75 (Glycoprotein H), UL87 (betagamma gene), UL146 (vCXCL1), UL63 (function unknown) and UL78 (vGPCR). The presence of several of these target sequences were confirmed in multiple viral strains including the TB40 BAC4 strain (EF999921), AD169 (X17403, 2006), the Merlin strain (AY446894) as shown in Table 6.2. Especially the fact that UL33 and UL146 (vCXCL1) contain Tcf-Lef binding sites that are (probably) involved in their transcriptional activation, might point at an additional role for US28. Besides all the previous described roles (see introduction), US28 might as well be involved in the regulation of viral gene expression. Since US28 is expressed early after infection, it might therefore be able to regulate the later UL33 expression.

Table 6.2: HCMV strains containing Tcf-Lef sites present in (close proximity of) genes. Virally-encoded genes containing a Tcf-Lef recognition site CCTTTGww with w being A ot T, potentially involved in their transcriptional regulation, are shown for the three sequenced and annotated HCMV strains TB40-BAC, AD169 and Merlin, with RefSeq annotation number between brackets. Tcf-Lef target site present in at least 2 out of the 3 HCMV strains are indicated in the final column, called Overlap in strains. – indicates site is not found.

Gene (function)	TB40-BAC4 (EF999921)	AD169 (X17403)	Merlin (AY446894)	Overlap in strains
UL75 (glycoprotein H)	Yes	Yes	Yes	Yes
UL87 (betagamma gene)	Yes	-	Yes	Yes
UL33 (vGPCR)	Yes	Yes	-	Yes
UL78 (vGPCR)	-	Yes	Yes	Yes
UL146 (vCXCL1)	Yes	-	Yes	Yes
UL69 (unknown)	Yes	-	Yes	Yes
UL5 (RL11 family)	Yes	IRL11, Yes?	-	Probably
UL63	-	Yes	-	No

The HCMV genome also contains the UAS for NF-κB. NF-κB is known to regulate expression of e.g. the critical major IE proteins of CMV, via the consensus NF-κB elements in their promoter region [308]. US28, constitutively activating the inflammation related NF-kB transcription factor [82] (and **Chapter 2**) was indeed shown to increase major immediate-early promoter/enhancer activity [313]. In the TB40-BAC4 genome (EF999921) the κB recognition peptide is located within the coding sequence of UL18 MHC class I homolog UL18, which inhibits specific NK cells [309]. AD169 (X17403), contains 2 sites present in the proximity of UL18 and US4. The Merlin strain contains 1 NF-κB-binding site between the US3 and US6 genes. Both US3 and US6 proteins are involved in immune regulation. US3 inhibits processing and transport of MHC-I and MHC-II molecules and US6 inhibits TAP-mediated peptide transport, important in antigen presentation. Clearly, both viral genes play a role in disturbing an adequate host immune response. Hence we hypothesize that both US28 and UL33 proteins might contribute to virus-induced pathogenesis by modulating viral-gene expression through activation of host cell transcription factors. This should be more extensively checked for instance by using P-Match software, available through http://www.gene-regulation.com. Using this software the three annotated HCMV genomes described above, can be checked for the presence of multiple transcription factor binding sites, including e.g. STAT3 also shown to be activated by US28 and UL33. This might shed light on other HCMV-encoded genes that potentially can be activated through host transcription factors that may be enhanced in case of infection, proliferation or other pathogenic related processes ongoing in the infected host.

6.10 HCMV-mediated oncomodulation

The hallmarks of cancer comprise multiple biological capabilities acquired during the development of human tumors [210, 275]. Within the HCMV genome several genes have been postulated to be involved in oncomodulation, *i.e.* HCMV may infect tumor cells and increase their malignancy. Tumor cells provide a genetic environment, characterized by disturbances in intracellular signaling pathways, transcription factors, and tumor suppressor proteins. This enables HCMV to exert its oncomodulatory potential, which cannot be manifested in healthy, non-transformed cells [314]. Increasing evidence and open questions concerning the link between HCMV and cancer have been extensively discussed [259]. First of all, many laboratories failed to detect the virus by using non-optimized techniques [315]. The detection of latent HCMV present in tumor material is crucial, but difficult. HCMV was detected first in malignant glioblastomas and colorectal cancers [135, 136]. However, attempts to detect HCMV virus in for example kidney tumors by PCR resulted in no signs of virus. Breast milk is the primary route of transmission of HCMV infection in humans worldwide. Therefore breast epithelium is a likely site of persistent infection and/or reactivation. Persistent HCMV expression (and infection) occurs in breast glandular epithelium in a significant percentage (63%) of normal adult females [258]. HCMV expression is also evident in neoplastic breast epithelium in a high percentage (97%) of normal and neoplastic breast tissues obtained from breast cancer patients. Recently, a causal association between adult HCMV infection and breast cancer was shown in some women from the Janus serum bank cohort [316]. The increased risk observed was present for few women in this study, suggesting that HCMV infection may only be involved in the development of a minority of breast cancers.

Besides the vGPCR US28, HCMV encodes additional genes that are suggested to be involved in rewiring of cellular signaling, and more specifically regulators of tumor cell cycle and/or apoptosis. These include IE1-72, UL97 and pp71 (inhibiting pRb), pUL69 (inhibiting S-phase), IE2-86 (inhibiting p53 and p21 as well), pUL37x1, pUL36, pUL38 and β2.7 RNA (inhibiting apoptosis) [259]. To this list, the viral receptor UL33 can now be added. Understanding signal transduction involved in oncomodulatory regulation by the viral chemokine receptors US28 (**Chapters 2 and 3**) as well as by UL33 (**Chapter 4**), requires further research. Additionally, vGPCR-control of epigenetic regulation of gene expression may be important in oncomodulatory signal transduction. In this work, we studied epigenetic control of cyclin D1 gene expression, in US28-expressing NIH-3T3 cells. As we show in **Chapter 5**, the expression of this cell cycle regulatory gene seems

to be subject to US28-mediated epigenetic control. Further study into global epigenetic changes mediated by HCMV-encoded proteins in the host cell will clarify the importance for this oncomodulatory property of the virus.

An increasing body of evidence, both from genetically engineered mice and from clinical epidemiology, suggests that the immune system operates as a significant barrier to tumor formation and progression, at least in some forms of non-virus induced cancer. However, the great majority of these are virus-induced cancers, suggesting that at least part of the control of this class of cancers normally depends on reducing viral burden in infected individuals, i.e. through eliminating virus-infected cells. Additionally, some immune-suppressed organ transplant recipients have been observed to develop donor-derived cancers, suggesting that in the tumor-free donors, the cancer cells were kept under control in a dormant state, by a fully functional immune system [323].

The 3-fold increase of cancer incidence after solid organ transplantation (mostly kidney transplantation), for which patients become immunosuppressed, and the role of viruses was investigated in a meta-analysis [317]. Up to 20% of cancers show correlation with inflammation, indicating a role for the immune system. Striking increases in occurrence of virus-related cancers, including Kaposi's sarcoma (KSHV), Non-Hodgkin as well as Hodgkin lymphoma (EBV), liver cancer (HPB and HPC) and cancer of the cervix, vulva/vagina, penis, anus and oral cavity and pharynx (HPV), are observed upon decreased activity of the immune responses. The increased risk of these cancers is believed to arise from impaired immune control of viral oncogenes, but the precise biological mechanisms of neoplastic progression are not yet understood. Breast, prostate, ovarian, brain and testicular cancers were not increased in incidence in transplant recipients. Since some of the cancer-associated viruses are ubiquitous, the role of cofactors, including host, behavioral and transplantation-related factors, cannot be underestimated [317]. An etiological role for HCMV infection in post transplant lymphoproliferative disorders (PTLD) is unlikely, given the lack of association between PTLD risk and the receipt of CMV antiviral drugs in a large multicentre study [138].

6.11 Role HCMV in glioblastoma and the link with US28 and UL33

Recently, consensus was reached on the role of HCMV in glioblastoma [260]. Moreover, low-grade HCMV infection was strongly associated with long-term survival in glioblastoma patients [318]. Interestingly, recently the effect of valganciclovir, an anti-CMV agent, has been evaluated in glioblastoma patients [319]. An unexpectedly high survival in patients undergoing radical surgery and receiving long-term treatment with valganciclovir in combination with chemotherapy and radiation was observed. Although the study was small including only 42 patients, requiring well-powered studies to further evaluate the efficacy of valganciclovir in glioblastoma patients, the described studies are encouraging.

The detection of the viral US28 [80] and UL33 (**Chapter 4**) proteins in glioblastoma patient material, as well as the correlation of poor disease prognosis and US28 expression, and their ability to activate oncomodulatory signaling pathways, indicate that the HCMV-encoded chemokine receptor US28 and likely UL33 induce a more cancerous phenotype. In the IL-6-STAT3 activation by US28 [80] a correlation is evident between poor patient prognosis and strongly induced STAT3-IL-6 activation by US28. Correlation was not found for the expression of UL33 in glioblastoma patient material and the patient's prognosis. The detection of both receptors specifically in glioblastoma patient tumor specimen and their oncogenic potential, corroborates with the hypothesis of HCMV-enhanced tumor formation [260].

Controversy still exists whether HCMV is present in a lytic or latent state in glioblastoma [260]. Known tumor viruses are typically latent, some replicate in the host cell using host cellular proteins, but not producing infectious particles [320]. Similar to that, existence of HCMV in glioma does not appear to fit the classic definitions of lytic or latent disease. However, the state of the virus as found in glioblastoma patient material is of interest; US28, co-localized with activated STAT3 in the vascular niche [80], may point at reactivation of the virus. In latency the US28 was not expected to be expressed, previously. However, our data indicate, that US28 is also expressed at later time points (**Chapter 4**). UL33 expression in latency and upon reactivation is of equal interest. The observed expression of these viral receptors in HCMV positive glioblastoma tumors and detection of US28 in primary medulloblastomas, the most common malignant brain tumors in children [234], makes both viral receptors interesting potential therapeutic targets.

6.12 US28 and UL33 as therapeutic targets?

Directly after the start of the pharmacological characterization of US28 some years ago, compounds targeting US28 were discovered and further developed. The small non-peptidergic molecule VUF2274 is an inverse agonist of US28 signaling, binding at an allosteric site when compared to chemokines [169-171]. Besides VUF2274, several other related VUF compounds were tested in the functional β-catenin activation assay. The compound VUF6068 showed significant decrease of the US28-mediated activation of Tcf-Lef reporter gene (**Chapter 3**). This interesting observation requires further optimalization of potential inverse agonists for US28. Besides the VUF compounds, recently dihydroisoquinolinone and tetrahydroisoquinoline scaffolds were found to be promising lead structures for novel US28 allosteric inverse agonists having comparable or improved efficacy over the VUF2274 series [172]. For the characterization of these novel allosteric modulators of US28, the luciferase based PathDetect Elk1 gene reporter assay was used. In order to validate these potential US28 targeting compounds [169, 170] or compounds that act on US28 induced cellular signaling, appropriate *in vivo* model systems are required. Clearly, US28 remains an interesting pharmacological target because of its implication on viral dissemination, cardiovascular diseases [159] and tumorigenesis [136, 161, 234, 257, 260]. With our study showing UL33-induced tumor formation (**Chapter 4**) the orphan vGPCR UL33 becomes an interesting therapeutic target with a different expression profile, compared to US28. As UL33 likely contributes to the oncomodulatory traits of HCMV, targeting this vGPCR may prove to be a promising avenue to interfere with virus-induced pathology.

6.13 *In vivo* model systems

Like other herpesviruses, HCMV has the ability to establish lifelong persistence. The salivary glands are the primary site of both primary and latent infection. A new paradigm is emerging, involving identification and targeting of hijacked host factors [139], in contrast to canonical pathogen-targeting. In order to study HCMV-hijacked host factors, a mouse submandibular gland (SMG) organ culture model to study MCMV-induced dysplasia and the candidate COX-2/AREG/EGFR/ERK

signaling network was recently developed [139]. Inhibitors targeting several key steps in the autocrine loop, indicated that upregulation of ERK phosphorylation is necessary for initial MCMV-induced pathogenesis. Unlike the HCMV, the MCMV contains no homolog for US28, but only for UL33 and UL78. Complementation of viral receptors in an animal model is of importance for evaluation of therapies targeting the HCMV chemokine receptors. Knockout of the mouse CMV M33 protein resulted in substantial attenuation of salivary gland infection and viral replication, and reduced efficiency of reactivation from tissue explants (derived from spleen and lung). M33-mediated G protein-coupled signaling is critical for the salivary gland phenotype. Recently, is was demonstrated that US28 and (to a lesser degree) UL33 restore reactivation of MCMV from tissue explants of spleen and lung and partially restore replication in salivary glands [293] compared to a signaling-deficient M33 mutant $R^{131}Q$. These studies provide a novel small animal model for drug development targeting HCMV. It would be of interest to see whether the signaling-deficient US28 mutant $R^{129}A$ also shows lowered reactivation in spleen and lung. This would imply the involvement of US28 signaling in virus reactivation in these organs and thus tissue specific processes.

In addition, HCMV DNA, RNA, and proteins were detected in several medulloblastoma cell lines, and viral protein expression was highly induced by xenografting of human medulloblastoma cells *in vivo* [234]. Since US28 mRNA has been detected in glioblastoma [234], the medulloblastoma cell lines may also be used to further dissect a role of HCMV and vGPCRs in medulloblastoma.

6.14 Concluding remarks

We have shown that the constitutively signaling vGPCRs US28 and UL33 activate several signal transduction pathways that might be involved in oncomodulation, e.g. in glioblastoma, an HCMV-linked pathology (see Figure 6.1). In **Chapter 2** we describe the US28-induced and NF-κB-regulated upregulation of COX-2 and their involvement in tumor formation. In the study described in **Chapter 3** we show that US28 constitutively activates β-catenin and enhances β-catenin dependent transcription. Our data illustrate that the viral receptor does not activate β-catenin via the classical Wnt/Frizzled signaling pathway, but points to the involvement of the Rho-Rho kinase (ROCK) pathway. Additionally, cells infected with HCMV

stabilize β-catenin thereby inducing β-catenin signaling, which is to a large extent mediated by US28. This modulation of the β-catenin pathway by the HCMV-encoded receptor US28 might be of relevance in (colon) cancer, glioblastoma and virus-associated diseases. **Chapter 4** focuses on signal transduction pathways induced by the HCMV-encoded receptor UL33. We show that UL33, like US28, may act as an oncomodulator by rewiring several cellular signaling pathways in NIH-3T3 expressing cells and promoting tumor formation in a xenograft model. Hence, UL33 could also play a role in proliferative diseases. Finally, in **Chapter 5** we examined epigenetic regulation of gene expression by US28. In this chapter we describe US28-mediated epigenetic changes, i.e. hypomethylation of the cell cycle progression gene Ccnd1, which encodes for cyclin D1. Taken together, our data shed new light on the mechanisms that might potentially be involved in oncomodulatory properties of the human cytomegalovirus-encoded receptors US28 and UL33.