Identification of a Human Cytomegalovirus-encoded Jak1 antagonist using a novel screening strategy

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Table of contents

Abbreviations

AIM2	Absent in melanoma 2
ATF	Activating transcription factor
ATP	Adenosine-tri-phosphate
β2m	β2 microglobulin
BAC	Bacterial artificial chromosome
cGAS	cyclic GMP-AMP synthase
CIITA	Class II transactivator
d	days
DAI	DNA-dependent activator of IRFs
DDB1	DNA-damage DNA-binding protein 1
DNA	Deoxyribonucleic acid
dsRNA	double-stranded RNA
E	Early
E/L	Early/Late
EBV	Epstein-Barr-Virus
E.coli	*Escherichia coli*
ER	Endoplasmatic reticulum
FFU	Focus forming units
g	grams
GAF	Gamma activated factor
GAS	Gamma activated sequences
GBP	Guanylate-binding protein
GCV	Ganciclovir
GvHD	Graft-versus-host disease
h	hour
HCV	Hepatitis C Virus
HCMV	Human Cytomegalovirus
HECT	Homologous to E6AP C-Terminus

HIV	Human Immuno-Deficiency Virus
HSV-1	Herpes Simplex Virus 1
IDO	Indoleamine-2, 3-dioxygenase
IE	Immediate Early
IFI16	Interferon-γ- inducible factor 16
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNGR	Interferon gamma receptor
IKK	IκB kinase
iNOS	Inducible NO-synthetase
IL	Interleukin
IP	Immuno-precipitation
IRF	Interferon regulatory transcription factor
IRL	Internal repeat long
IRS	Internal repeat short
ISG	Interferon stimulated gene
ISRE	Interferon-stimulated response elements
Jak1	Janus kinase 1
Jak2	Janus kinase 2
KSHV	Kaposi Sarcoma-associated Herpesvirus
L	Late
LPS	Lipo-polysaccharide
M	molar
mM	millimolar
µM	micromolar
MAVS	Mitochondrial antiviral-signaling protein
MCMV	Mouse Cytomegalovirus
MHC	Major histocompatibility complex
MIEP	Major Immediate Early Promoter
mg	milligram

µg	microgram
ml	milliliter
µl	microliter
mRNA	messenger RNA
MSC	Mesenchymal stem cells
mTOR	mammalian target of rapamycin
NEMO	NFκB essential modulator
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK	Natural killer
NLRs	NOD-like receptors
NO	nitric oxide
OAS	2'-5'-oligoadenylate synthetase
o/n	over night
ORF	Open Reading Frame
PAMPs	Pathogen-associated molecular patterns
PKR	ds-RNA dependent protein kinase R
PRR	pattern recognition receptors
RIP3	Receptor-interacting protein 3
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
STAT1	Signal transducer and activator of transcription 1
STAT2	Signal transducer and activator of transcription 2
STAT3	Signal transducer and activator of transcription 3
STING	Stimulator of interferon genes
TAP	transporter associated protein
TK	Thymidine Kinase
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factors
TRIF	TIR-domain-containing adapter-inducing interferon-β

TRL	Terminal repeat long
TRS	Terminal repeat short
Tyk2	Tyrosine kinase 2
U	Units
Ub	Ubiquitin
UL	Unique long
UPS	Ubiquitin-proteasome system
US	Unique short
VSV	Vesicular Stomatitis Virus

Figure Summary

Abstract

To alleviate antiviral activities of the immune system, viruses express antagonists which often operate by degradation of specific cellular proteins. Viruses with large genomes (like human cytomegalovirus [HCMV]) often encode several genes each of which counteracts a single component of immunity (e.g. interferon [IFN] signal transduction). HCMV induces selective proteasomal degradation of janus kinase (Jak) 1 – a key factor of IFN signaling. To identify viral gene products which induce degradation of defined target proteins, we devised, established, and validated a broadly applicable forward genetic screening approach. To translate degradative forces into a survival advantage, the HSV thymidine kinase (HSV-TK) was used which activates the prodrug Ganciclovir (GCV), leading to cell death. The target proteins (e.g. Jak1) were fused to HSV-TK. Stable transfection of the respective viral antagonist destabilizes the construct, abrogating TK-activity and allowing cell proliferation in the presence of GCV due to its detoxification effect. Cell lines expressing fusion chimeras composed of TK and Jak1 or STAT2 were generated and found to be highly susceptible towards GCV. The approach was validated using the known IFN signaling inhibitors pM27 and the V protein of parainfluenza virus type 2. The screening was applied using a plasmid-based HCMV ORFeome library and a novel lentivirus-based HCMV cDNA library. Surviving cell lines were selected and enriched genes were identified by PCR. One gene product, gpUL42, reduced Jak1 amounts in co-transfection experiments. A HCMV knock-out mutant lacking the respective gene exhibited delayed Jak1 degradation kinetics compared to wild-type HCMV, indicating on the one hand that the gene product contributes to JAK1 degradation and on the other hand that further HCMV JAK1 antagonists must exist.

Zusammenfassung

Viren exprimieren Antagonisten, die spezifische zelluläre Proteine abbauen um die Aktivitäten des Immunsystems zu verringern. Viren mit großen Genomen (z.B. das humane Zytomegalovirus [HCMV]), kodieren oftmals mehrere Gene, die einem speziellen Teil der antiviralen Immunität (z.B. der Interferon-[IFN] Signaltransduktion) entgegenwirken. HCMV induziert selektiv den proteasomalen Abbau der janus kinase (Jak) 1 – einem entscheidenden Protein des IFN Signalweges. Um virale Genprodukte, die einen Abbau von definierten Zielproteinen veranlassen, zu identifizieren, haben wir einen umfassend anwendbaren genetischen Screen erdacht, etabliert und validiert. Hierfür wurde die herpesvirale Thymidinkinase (HSV-TK) verwendet, welche die Prodrug Ganciclovir (GCV) aktiviert und damit den Zelltod herbeiführt. Hiermit konnten wir den Abbau von Zielproteinen in einen Überlebensvorteil der Zellen umwandeln. Die Zielproteine, z.B. Jak1 wurden mit der HSV-TK fusioniert. Stabile Transfektion des respektiven unbekannten Antagonisten destabilisiert das Fusionskonstrukt, führt zu der Aufhebung der TK Aktivität und erlaubt die Zellproliferation auch während der Anwesenheit von GCV, da der toxischen Wirkung von GCV entgegengewirkt wird. Es wurden Zelllinien generiert, die Fusions-Chimären von TK und Jak1 oder STAT2 stabil exprimieren. Diese Zellen waren hoch empfindlich gegenüber einer GCV-Behandlung. Das Vorgehen, den Abbau des Zielproteins in einen Überlebensvorteil umzuwandeln, wurde mittels der bekannten IFN Signalweg Inhibitoren pM27 von MCMV und dem V Protein von Parainfluenza Virus Typ 2 validiert. Der Screen wurde auf zwei verschiedene Arten angewendet: einerseits wurde eine Plasmid-basierte ORFeome Bibliothek genutzt, auf der anderen Seite eine neuartige Lentivirus-basierte HCMV cDNA Bank. Überlebende Zellklone wurden ausgewählt und angereicherte Gene in diesen Zellen mittels PCR identifiziert. Ein Genprodukt, gpUL42, reduzierte Jak1 Proteinmengen in Co-Transfektionsexperimenten. Ein HCMV Deletionsmutant, dem das respektive Gen fehlte, zeigte eine verzögerte Abbaukinetik von Jak1 im Vergleich zum Wildtyp-Virus. Dies zeigt, dass das Genprodukt gpUL42 zum Jak1 Abbau beiträgt, allerdings auch, dass noch weitere Jak1 Antagonisten von HCMV existieren müssen.

1. Introduction

1.1 Viruses

Throughout its life-time, the human body is constantly challenged by various pathogens like bacteria, viruses, parasites and fungi. While bacteria, parasites and fungi are in principle capable of their own protein biosynthesis, viruses are capsid-encoding 'organisms' that strictly rely on the host's cellular machinery, especially the ribosomes, for the generation of new virus particles (Raoult and Forterre, 2008). Viruses are not only able to infect species of every animal kingdom but they even infect bacteria or other larger viruses as phages. Although the classification of viruses as living organisms is continuously under debate, newly identified virus species which are capable of developing structures outside of host cells and the existence of 'virophages' in large viruses shine new light on the discussion (Haring et al., 2005; Pearson, 2008). Nevertheless, the evolutionary pressure elicited by a virus infection not only on the host but also on the virus itself is undisputable. Viruses harbor their genetic material either as DNA or RNA which is present in the viral capsid either in a sense or anti-sense manner. Some viruses are also surrounded by an envelope that consists mostly of lipids. By the expression of specific receptors on their surface, viruses are able to attach and infect host cells, in which the virus progeny is replicated. Viruses continuously mutate during their replication leading to virus progeny which is positively selected to adapt and adjust to its environment, e.g. by inserting mutations that establish anti-viral drug resistance. Hence, the examination and understanding of viral mechanisms important for replication and evasion, and viral targets for the development of novel drugs, neutralizing antibodies and vaccines is an important field in modern molecular biology.

1.1.1 Herpesviruses

Herpesviruses are large enveloped viruses containing linear un-segmented double-stranded DNA genomes. The virus capsid harboring the viral genome is embedded in the tegument, a protein matrix mostly composed of phosphoproteins, and surrounded by a membrane which contains viral glycoproteins as well as proteins derived from the former host cell. The viral DNA circularizes in the nucleus of infected cells and is replicated by a *rolling circle* mechanism (McVoy and Adler, 1994), giving rise to new virus progeny. Although there can be strong homology between proteins of different *Herpesvirus* species e.g. *Human Cytomegalovirus* and *Chimpanzee Cytomegalovirus*, *Herpesviruses* are usually highly species specific, and in nature only infect their genuine host species. Sterile immunity as seen in other viruses, e.g. *mumps virus*, will not be established as the virus establishes life-long latency in its natural host. However, high numbers of functional B- and T-cells can be found in infected individuals. Upon stress or decrease of the immune competence, the virus is able to reactivate and enter its lytic replication cycle. In most cases, a sufficient anti-viral immune response can be mounted, re-establishing the latent status of herpesviral infection.

There are eight known *Herpesviruses* regularly infecting humans which are subdivided into α-, β- and γ-*Herpesvirinae*, however the *Simian Herpes B virus* is also capable of infecting humans

(Hummeler et al., 1959). The *Human Cytomegalovirus*, a prototypical β-*Herpesvirus*, is subsequently described in more detail.

1.1.1.1 Cytomegaloviruses (CMV)

Cytomegaloviruses were identified in the 1950s as viruses found in salivary glands of mice (Craig et al., 1957; Rowe et al., 1956; Smith, 1954; Smith, 1956; Smith and Vellios, 1950). CMVs have a large double-stranded DNA genome (~230 kbp), which is longer than the genome of all other mammalian herpesviruses (Kilpatrick and Huang, 1977). The genome consists of two unique segments (unique long (*UL*) and unique short (*US*)) that are flanked by a pair of inverted repeats, terminal/internal repeat long (*TRL/IRL*) and internal/terminal repeat short (*IRS/TRS*) (Oram et al., 1982; Sijmons et al., 2014). The genome circularizes in the cell before DNA synthesis producing concatamers and genomic inversions inside this concatameric DNA (McVoy and Adler, 1994).

´Essential´ genes needed for viral genome replication or encoding for structural components of the virion are often located in the 'central' area of the genome (Brocchieri et al., 2005; Rawlinson et al., 1996). It is not possible to create knock-out viruses lacking these genes, as these viruses would not be able to replicate. On the other hand, the deletion of genes located in the genome terminal regions, mostly encoding genes needed for immune evasion or cell tropism, still leads to the production of virus progeny growing to similar viral titers *in vitro* as wild-type viruses (Cicin-Sain et al., 2007).

The major immediate early promoter (MIEP) facilitates the transcription of immediate early genes, representing the first class of viral gene products expressed in the infected cell. As the viral genome is subjected to *alternative splicing*, a process enabling the translation of more than one protein from the same primary transcript, the MIE genes IE18, IE19, IE55, IE72 and IE286 mRNAs are transcribed (Boshart et al., 1985; Shirakata et al., 2002). These proteins initiate transcription of further viral mRNAs, as the transcription of early genes needs trans-activation of the early promoter by viral proteins.

Gene expression is normally divided into functional expression profiles determined by the use of metabolic inhibitors. Hereby, four major classes are differentiated: immediate early (IE), early (E), early-late (E/L) and late (L) genes (Chambers et al., 1999; Honess and Watson, 1977; Wathen and Stinski, 1982; Wathen et al., 1981). A novel study performing a large proteomic screen of HCMV infected cells proposed a fifth temporal class, with a peak expression at 48 h of infection (Weekes et al., 2014).

Determining the precise coding potential of the CMV genome is challenging. The virus uses *alternative splicing* and non-classical start-codons. Also, the genome consists of overlapping open-reading frames (ORFs) and the mRNAs of certain ORFs are transcribed anti-sense to canonical ORFs (Boshart et al., 1985; Rawlinson et al., 1996; Stern-Ginossar et al., 2012). Earlier sequencing and comparative analysis of the HCMV genome with CMVs of other species predicted the coding potential of HCMV to be ~160 to 190 ORFs (Bankier et al., 1991; Brocchieri et al., 2005; Chee et al., 1990; Davison et al., 2003; Murphy et al., 2003; Rawlinson et al., 1996). However, a recent ribosomic profiling and transcript analysis approach increased this number

dramatically, identifying 751 translational products (Stern-Ginossar et al., 2012). Ribosomic profiling enables to analyze all mRNAs present in the cell protected by the ribosome during translation by identification of the translational start site and speed of the translating ribosomes (Ingolia et al., 2009). Hence, the sequences of potentially encoded ORFs can be detected and analyzed even if these proteins are only expressed in very minor quantities during the viral replication cycle. Additionally, 15 canonical genes not identified by ribosomic profiling were quantified using a proteomic approach (Weekes et al., 2014), demonstrating the complexity defining the complete coding capacity of HCMV.

The assignment of certain functions and phenotypes to defined genes was enabled by the cloning of the complete CMV genome into a bacterial artificial chromosome (BAC). This approach was first applied for the MCMV genome followed by successful cloning of the HCMV genome (Borst et al., 1999; Messerle et al., 1997). This method allows mutagenesis of the CMV-BAC in *Escherichia coli* (*E. coli*). After virus reconstitution, mutant viruses generated from these BACs lose the bacterial BAC backbone through passaging thereby acquiring wild type virus properties in terms of viral growth titers (Wagner et al., 1999). The contribution of specific viral gene products to a given phenotype can be assessed using BAC mutagenesis representing an important tool to explore the biology of cytomegaloviruses.

1.1.1.2 The Human Cytomegalovirus (HCMV)

Serological studies show that a majority of the global adult population is infected with HCMV. In immune-competent individuals, the primary infection is mostly asymptomatic or subclinical. Nevertheless, an HCMV infection can also have profound manifestations in apparently immune-competent individuals (Rafailidis et al., 2008). However, active replication of HCMV can have severe effects in immune-compromised (e.g. HIV patients) or neonates. According to the Centers for Disease Control and Prevention (CDC), 1 of 150 children in the United States is born with congenital HCMV infection, as the virus is able to infect and harm the fetus during gestation upon transplacental dissemination (http://www.cdc.gov/cmv/overview.html). Up to 20% of the infected children suffer from lifelong sequelae and handicaps e.g. deafness or mental retardation. Thereby, HCMV constitutes one of the most common non-genetic ‘congenitally’ acquired diseases. Furthermore, HCMV is also discussed as a reason for abortions or stillbirth (Rawlinson et al., 2008). Unfortunately, an effective vaccine against HCMV is not foreseeable in the near future and the commonly used anti-viral drugs e.g. Ganciclovir (GCV) have severe adverse effects (granulocytopenia, neutropenia, anemia and thrombocytopenia) (Nakamae et al., 2011; Venton et al., 2014).

Since HCMV is highly species specific and meaningful *in vivo* data cannot be collected in small animal models, most *in vivo* experiments are performed in the mouse model using MCMV. Here, a hierarchical and redundant anti-viral response comprised of interferons, activation of natural killer (NK) cells, B- and T-cells has to be elicited to control primary and recurrent CMV replication (Polic et al., 1998). Nevertheless, the virus is never completely eliminated from the host, establishing a lifelong latency which can give rise to reactivation of the virus. Examining genetically different HCMV genotypes showed that successive co- or superinfections cannot be prevented by the immune system (Arav-Boger et al., 2002; Baldanti et al., 1998; Deckers et al.,

2009). Astonishingly, patients experiencing HCMV super-infections were found to be infected with up to six different genotypes, highlighting the exquisite, and concomitantly challenging, intrahost genetic diversity of this virus (Renzette et al., 2014). In rhesus macaques, it was also shown that immune evasion genes are essential for superinfection with rhesus CMV (Hansen et al., 2010; Hengel and Koszinowski, 2010).

1.2 Interferons (IFN)

Interferons constitute the first line of defense against viruses and were initially described as bioactivity derived from infected cells to 'interfere' with influenza viruses to infect chick chorio-allantoic membrane (Isaacs and Lindenmann, 1957). IFNs play a pivotal role in initiating the immune response against viral and bacterial infections. IFNs are indispensable to survive primary MCMV infection (Gil et al., 2001; Ramana et al., 2001). On the contrary, interferon treatment of cells *in vitro* is quite inefficient (Trilling and Hengel, 2013). In the following, IFN induction upon encountering pathogen-associated molecular patterns (PAMPs), the IFN signaling cascade and IFN stimulated genes (ISGs) transcribed to facilitate an anti-viral state are described.

1.2.1 Interferon induction

IFNs are cytokines transcribed *de novo* after contact with PAMPs, e.g. LPS of gram-negative bacteria, viral DNA or dsRNA, triggering a potent antibacterial and antiviral status (Mohr et al., 2012; Stark and Darnell, 2012). This response is mediated by cellular pattern recognition receptors (PRRs) that are divided in five major sub-classes: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), cyclic GMP-AMP synthase (cGAS) and the recently identified absent in melanoma 2 (AIM2) (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Janeway and Medzhitov, 2002; Motta et al., 2015; Reikine et al., 2014; Sun et al., 2013). After recognition, several adaptor proteins and kinases are activated that are crucial for the engagement of the transcription factors ATF/cJun, NF-κB and IRFs which in turn facilitate the transcription and expression of IFN-β. TLRs signal via the adaptor proteins MyD88 and TRIF, RNA-sensors activate MAVS and TRAF3 and DNA sensors use the proteins STING or AIM2 for efficient transcription factor activation. MAVS, TRIF and Myd88 lead to NF-κB activation by initiating the degradation of IκBα; IRF-3 activation is initiated by TRIF and STING (Oshiumi et al., 2003; Seth et al., 2005; Zhong et al., 2008). The activation of the transcription factors leads to the translocation of these proteins into the nucleus. Here, the IFN-β enhanceosome is synergistically assembled followed by productive interferon transcription (Thanos and Maniatis, 1995). Transcription of IFN-stimulated genes (ISGs) is activated by the secreted IFNs in an autocrine and paracrine manner.

1.2.2 Jak-STAT signaling pathway

To date there are three different classes of IFNs: type I IFN (IFN-α (12 subtypes in humans), IFN-β, IFN-ε, IFN-κ and IFN-ω), type II IFN (IFN-γ) and type III IFN (IFN-λ1, IFN-λ2, IFN-λ3 and IFN λ4).

After the binding of IFN to the specific IFN receptor complex (IFN α/β receptor, IFN-γ receptor or IFN-λ receptor, respectively) receptor-associated tyrosine kinases, janus kinase 1 (Jak1), janus kinase 2 (Jak2) or tyrosine kinase 2 (Tyk2) are activated. This facilitates the tyrosine phosphorylation of signal transducer and activator of transcription (STAT) molecules. Phosphorylated STATs form homo- or heterodimers to assemble the ISGF3 complex (STAT1, STAT2 and IRF9) after IFN-α/β or IFN-λ stimulation or the gamma activated factor (GAF) complex (STAT1 homo-dimers) after IFN-γ stimulation, respectively. After translocation and binding to promoters or enhancers with specific DNA consensus motifs called interferon-stimulated response elements (ISRE) and gamma activated sequences (GAS), respectively, the expression of ISGs is initiated (Darnell, 1997). A simplified schematic overview of the Jak-STAT signal transduction is shown in Figure 1.

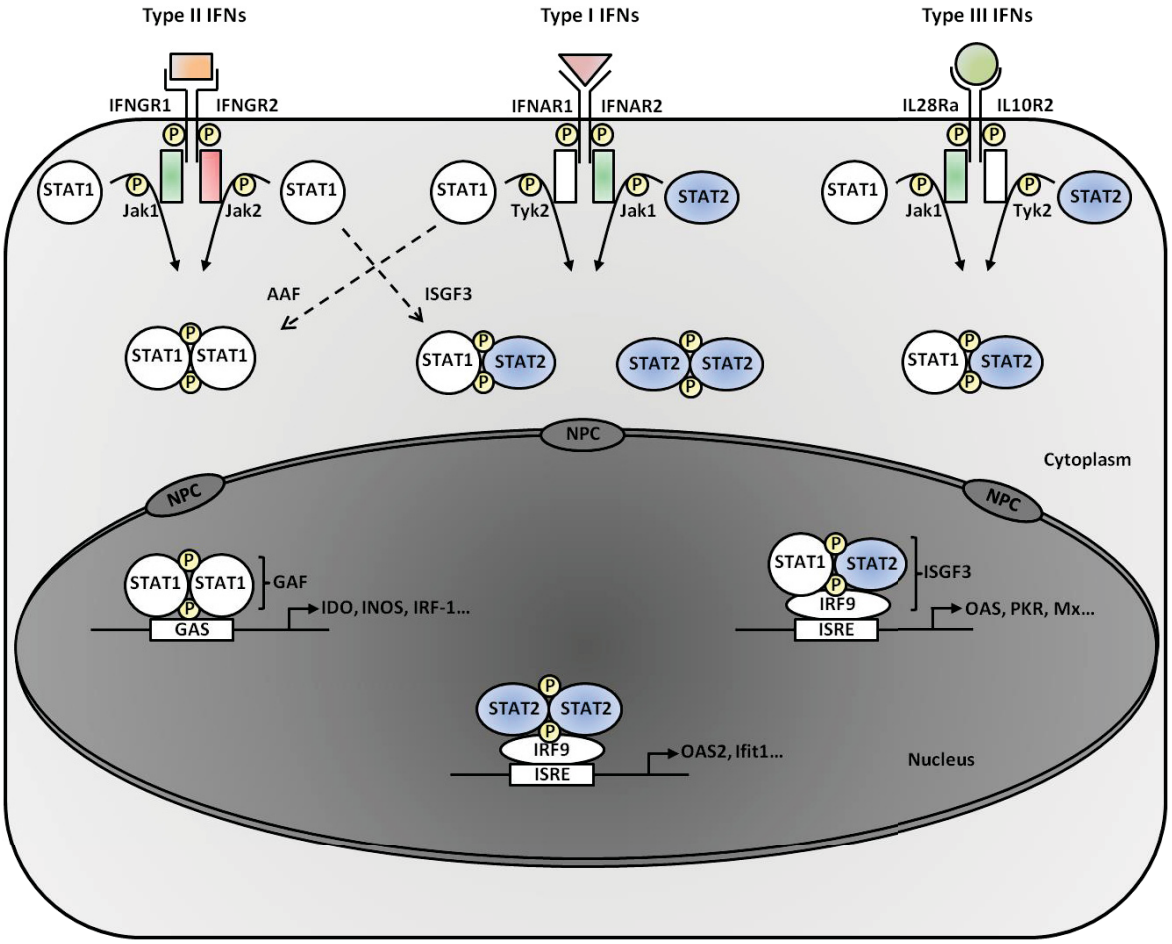

Figure 1: Interferon receptor-signaling pathways. Depicted is a simplified representation of Jak-STAT-signaling cascades. Upon stimulation of specific IFN receptors, Janus kinases are phosphorylated leading to the activation of STAT molecules. These form STAT1 homo-dimers (GAF complex), STAT2-IRF9 hetero-trimers or STAT1-STAT2-IRF9 hetero-trimers (ISGF3 complex) which translocate into the nucleus to initiate transcription of interferon-stimulated genes (ISGs). NPC, nuclear pore complex; AAF, IFN-α activated factor. Adapted from Decker et al.; 1991 and Zimmermann et al.; 2005.

When IFNs I bind to the receptor complex comprised of the IFNAR1 and IFNAR2 chains, the receptor associated kinases Jak1 and Tyk2 become phosphorylated, a process that is essential for a functional signaling cascade (Muller et al., 1993; Velazquez et al., 1992). The activated kinases phosphorylate the receptors followed by STAT2 binding to docking sites on the receptor. The bound STAT2 is then phosphorylated by Jak1. Phosphorylated STAT2 results in subsequent binding and phosphorylation of STAT1 molecules (Li et al., 1997). Consistently, loss of STAT2 will also lead to less abundant levels of phosphorylated STAT1 upon IFN-I treatment (Leung et al., 1995). However, IFN-I signaling by STAT2-mediated ISGF3 complex formation is not essential for the host defense of most childhood viral infections (Hambleton et al., 2013). Phosphorylated STATs interact intermolecularly with their SH2 domains and the phosphorylated tyrosine residues, additionally recruiting IRF-9. This heterotrimer translocates as the ISGF3 complex into the nucleus (Levy et al., 1989; Veals et al., 1992). After binding to ISRE sites, the transcription of more than 300 ISGs (depending on dose, cell type and duration of IFN treatment) can be induced, resulting in the induction of an antiviral state.

A similar pathway is operative in presence of IFNs II. Upon IFN-γ stimulation, the two receptor chains of the IFN-γ receptor IFNGR1 and IFNGR2 and the associated tyrosine kinases Jak1 and Jak2 are phosphorylated, finally leading to phosphorylation and homo-dimerization of STAT1 (Muller et al., 1993; Shuai et al., 1992; Shuai et al., 1993; Silvennoinen et al., 1993; Watling et al., 1993). These activated STAT1 molecules form the GAF (Decker et al., 1991; Shuai et al., 1992; Shuai et al., 1993), translocate into the nucleus and bind to GAS sites within the DNA, subsequently inducing the transcription of downstream ISGs.

IFNs III signal like IFNs I as they transmit responses via Jak1, Tyk2 and the ISGF3 complex (STAT1:STAT2:IRF-9), however the specific receptor complex (comprised of IL28R and IL10R2 chains) is differentially distributed. Receptors are primarily expressed on epithelial cells of the intestine (Gad et al., 2009; Kotenko et al., 2003; Sheppard et al., 2003; Sommereyns et al., 2008). IFN-λ has been shown to efficiently induce an antiviral state against viruses attacking the intestine, e.g. rotavirus or norovirus but also against *Herpesviruses* (Ank et al., 2006; Lopusna et al., 2014; Pott et al., 2011; Sun et al., 2015).

Recent data show that the orchestrated IFN-signaling pathways are much more complex than initially suspected. STAT molecules exist in parallel and antiparallel conformations and change their orientation in presence of IFN from X to Y conformation (Mao et al., 2005; Zhong et al., 2005). Also, STATs are constantly shuttling between the nucleus and the cytoplasm. Interferon stimulation rather shifts the balance between nuclear retention and shuttling than actively initiating nuclear translocation of STATs (Meyer et al., 2003; Vinkemeier, 2004). Furthermore, it was shown that a STAT2/IRF-9 heterodimer elicits ISGF3-like transcriptional response and antiviral activity also in the absence of STAT1 (Blaszczyk et al., 2015). Additionally, STAT2 can also be phosphorylated upon IFN-γ stimulation (Zimmermann et al., 2005).

The biological impact and control of the Jak-STAT signaling pathway is crucial as patients with mutations in genes coding for components of the IFN-Jak-STAT signaling pathway frequently suffer from recurrent infections of otherwise opportunistic pathogens (Casrouge et al., 2006; Dupuis et al., 2003; Jouanguy et al., 1996; Levin and Hahn, 1985; Zhang et al., 2007).

1.3 HCMV-mediated immune evasion

Throughout evolution, pathogens have evolved different ways to counteract recognition by the host immune system to accomplish productive infection. From experiments using rhesus macaque animal models, it was shown that RhCMV superinfections of preimmune animals are especially dependent on the coding capacity of defined immune evasion genes (Hansen et al., 2010). The interplay between HCMV and the immune system is complex and multilayered: On the one hand, a concerted action of intrinsic, innate and adaptive immune responses clears productive CMV replication. On the other hand, HCMV prevents sterile immunity due to the ability to establish latency and the coding capacity of virus-encoded immune evasion mechanisms. As outlined above, HCMV encoding MHC antagonists is able to superinfect pre-immune individuals. Although this is a poor prospect for the development of a potent vaccine (Hengel and Koszinowski, 2010), it shows the strict dependency of the successful expression of viral immune evasion proteins.

The selective pressure of the IFN-system has led to multiple mechanisms of intracellular bacteria, viruses and parasites to inactivate or degrade molecules of the Jak-STAT signaling pathway (Andrejeva et al., 2002; Lebreton et al., 2014; Padrao Jda et al., 2014; Taylor et al., 2014). The potent coding capacity of HCMV to translate active IFN-antagonists is highlighted comparing the antiviral potency of IFN against HCMV and *Vesicular Stomatitis Virus* (VSV). Upon treatment with 500 U/ml IFN-γ, VSV titers are reduced by a factor of 10^5 to 10^8 whereas HCMV titers are only reduced by a factor of 10-100 under the same conditions (Le et al., 2008a; Trilling et al., 2009). A significant anti-viral effect of IFN can only be demonstrated after a long-term pre-treatment (1 – 2 days) of permissive cells in cell culture before HCMV infection. During this time, IFN-induced effector proteins are translated which the virus cannot circumvent. Hardly any anti-viral effect can be measured if the IFN is applied after HCMV entry of cells. This highlights that, at least in highly permissive human fibroblasts, CMV massively affects the induction of IFN-induced effector functions by means of IFN-antagonists.

1.3.1. HCMV-encoded IFN signaling antagonists assigned to a defined HCMV gene

HCMV has evolved multiple ways to evade recognition by the host thereby showing a remarkable redundancy of viral factors interfering with a given pathway.

IFI16 was recently identified as an intracellular DNA sensor that recruits and activates stimulator of interferon genes (STING). This leads to TBK1-dependent phosphorylation of IRF-3 and IFN-I transcription (Thompson et al., 2014; Unterholzner et al., 2010). It also acts via ASC and procaspase-1 to form functional inflammasomes (Kerur et al., 2011). IFI16 is implicated to be a restriction factor against HCMV by suppressing the promoter of HCMV UL54 thereby impairing viral DNA synthesis (Dell'Oste et al., 2014; Gariano et al., 2012). Furthermore, IFI16 and STING have been shown to play important roles in sensing and control of other *Herpesviruses*, namely HSV-1 (Johnson et al., 2013; Kalamvoki and Roizman, 2014; Orzalli et al., 2012), *Epstein-Barr-Virus* (EBV) (Ansari et al., 2013) and *Kaposi Sarcoma-associated Herpesvirus* (KSHV) (Kerur et al., 2011).

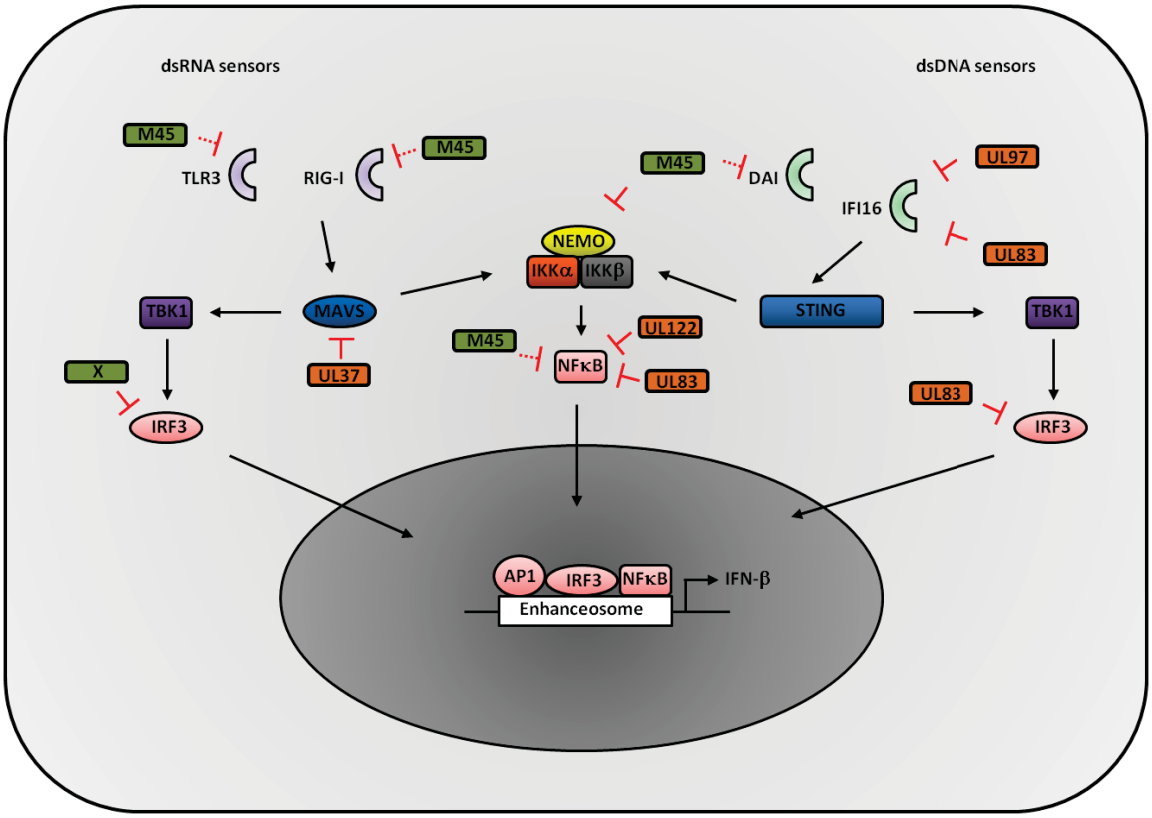

Figure 2: Multi-layered CMV immune evasion of IFN-induction. HCMV and MCMV encode multiple proteins to dampen IFN induction. Continuous lines represent direct cellular targets of the viral proteins; dotted lines represent inhibition of a cellular protein in the signaling pathway, e.g. RIP, leading to inhibition of further downstream signaling events. Proteins encoded by HCMV are depicted in orange, MCMV-encoded proteins in green. HCMV interferes with IFI16, IRF3, MAVS and NF-κB signaling. MCMV inhibits NEMO and IRF3; M45 interacts with RIP1 thereby inhibiting NF-κB signaling and nucleic acid recognition (dotted red line). The viral protein products responsible for these phenotypic changes that have not yet been identified are marked with an X.

Recently, it was shown that HCMV pUL83 interacts with the pyrin domain of the DNA sensor IFI16 blocking its oligomerization (Li et al., 2013). The viral protein recruits IFI16 to the major immediate early promoter (MIEP) exploiting the cellular protein to initiate viral gene transcription (Cristea et al., 2010). Furthermore, IFI16 is phosphorylated by pUL97 following re-localization into the cytoplasm and thereupon entrapped within mature virions (Dell'Oste et al., 2014). This shows a highly sophisticated mechanism of hijacking and misplacing cellular proteins. The tegument protein pUL83/pp65 is able to interfere with IFN-α signaling by preventing the DNA-binding of the NF-κB complexes and subsequent IRF-1 transcription (Browne and Shenk, 2003). Although the NF-κB translocation is comparable between wild-type HCMV and *UL83* knockout virus, there is an impact on the regulation of IRF3 phosphorylation and translocation in the knockout virus. Overexpression of pUL83 alone was sufficient to interfere with IFN induction (Abate et al., 2004). However, it was shown that the *UL83* knockout virus had lower abundance of the viral proteins pUL82/pp71 and pUL122/IE2-pp86. A virus mutant harboring a stop codon

mutation in *UL83* with unaltered protein expression of pUL122 was able to effectively block expression of cytokines and chemokines during infection. Therefore, at least part of the observed effect in the *UL83* knockout virus is due to a lower expression of pUL122 (Taylor and Bresnahan, 2006).

As mentioned before, pUL122/IE2/pp86 alone is able to interfere with IFN-β transcription induced by HCMV infection, or upon experimental Sendai virus infection (Taylor and Bresnahan, 2005). pUL122 efficiently blocks virus- and TNF-induced NF-κB binding to the IFN-β promoter, however, this block is not mediated by a direct interaction of pUL122 with the NF-κB subunits p50 and p65 (Taylor and Bresnahan, 2006).

Another HCMV-encoded IE protein sufficient to interfere with IFN-α signaling is pUL123/pp72. Ectopic expression of pUL123 inhibits ISG induction, while an HCMV mutant lacking the *ie1*-exon 4 has severe growth deficits and is more susceptible towards IFN-α treatment (Greaves and Mocarski, 1998; Paulus et al., 2006). pUL123 is able to interact with STAT2 requiring an acidic domain (aa 421-475); a virus mutant lacking the acidic stretch in pUL123 was shown to have a severe (>1.000 fold) growth defect when cells were pre-treated with IFN-β (Huh et al., 2008; Paulus et al., 2006). Surprisingly, pUL123 also seems to have agonistic IFN signaling capacities as overexpression of the protein elicits a STAT1-dependent ISG transcription that is comparable to an IFN II like host cell response (Knoblach et al., 2011).

HCMV is not only able to efficiently block transcription of ISGs by interfering with IFN induction but also codes for potent inhibitors of adaptor proteins needed for sufficient recognition of viral DNA. The HCMV encoded protein pUL37x1/vMIA was identified as an anti-apoptotic protein localized to mitochondria (Goldmacher et al., 1999). Recent studies showed that its anti-apoptotic capacity is elicited by binding to Bax, a pro-apoptotic protein initializing the permeabilization of the outer mitochondrial membrane. This results in proteasomal degradation of Bax at mitochondria-associated membranes (Ma et al., 2012; Zhang et al., 2013). Next to its anti-apoptotic capabilities, the mitochondrial localization enables pUL37x1 to reduce the association of the adaptor MAVS with STING, thereby inhibiting downstream signaling by IFN I (Castanier et al., 2010).

MHC molecules are central determinants of the adaptive immune system by presenting potentially antigenic peptides to T cells. Type II IFN induces the activation of class II transactivator (CIITA), thereby initiating the expression of MHC-I and MHC-II molecules. In case of $CD8^+$ T cells, this mediates an active anti-viral response against the infected cells. Proteins degraded by the immune-proteasome are transported by the transporter associated protein (TAP) to the endoplasmic reticulum (ER). Here, peptides are loaded on MHC molecules comprised of the MCH class I heavy chain and β2 microglobulin (β2m) to be transported to the cell surface. The ability of IFN to stimulate MHC-I presentation and to induce MHC-II on non-APC cells on the cell surface was already found more than 40 years ago (Attallah and Strong, 1979; Fellous et al., 1982). This increase in MHC/HLA surface expression is crucial in the detection of virus infected cells (Koszinowski et al., 1977; Schrader and Edelman, 1977). The IFN-induced MHC upregulation is decisive, as especially viruses have evolved multiple mechanisms to antagonize this pathway (Hengel et al., 2005).

HCMV encodes for at least six MHC-I antagonists (*US2*, *US3*, *US6*, *US10*, *US11* and *miR-US4-1*) (Ahn et al., 1997; Furman et al., 2002; Hengel et al., 1997; Jones and Sun, 1997; Jones et al., 1996; Kim et al., 2011b; Wiertz et al., 1996) and ten antagonists interfering with NK cell activation (*US9*, *US18*, *US20*, *UL16*, *UL18*, *UL40*, *UL83*, *UL141*, *UL142* and *miR-UL112*) (Arnon et al., 2005; Fielding et al., 2014; Hassan-Walker et al., 1998; Seidel et al., 2015; Stern-Ginossar et al., 2007; Tomasec et al., 2000; Tomasec et al., 2005; Welte et al., 2003; Wills et al., 2005). In the following section, target proteins of the IFN pathway are described that are specifically inhibited by HCMV encoded proteins but for which no specific viral protein has yet been identified.

1.3.2 HCMV-encoded IFN signaling antagonists unidentified

Much is known about virus-encoded proteins interfering with proteins needed for efficient initialization of an anti-viral state. However, various other viral proteins efficiently degrading or inhibiting IFN-signaling have not been assigned a specific gene product.

HCMV is able to degrade Jak1 via the proteasome (Miller et al., 1998; Miller et al., 1999), the mechanism being independent of viral late gene expression as inhibition by phosphonoacetic acid (PAA) does not alter its ability to degrade Jak1. Additionally, the virus is capable to repress the CIITA mRNA expression, thereby inhibiting pUL123 presentation to T cells (Le Roy et al., 1999). By degrading Jak1, following phosphorylation steps in the signaling cascade are abrogated, inhibiting transcription of ISGs. Moreover, HCMV also degrades STAT2 via the proteasome (Le et al., 2008a). IRF-9, which is needed for efficient assembly of the ISGF3 complex, is reduced post-transcriptionally (Miller et al., 1999). Adding another layer of complexity, HCMV exploits the cellular phosphatase SHP2, resulting in dephosphorylation of STAT1 (Baron and Davignon, 2008).

Recently, a systematic quantitative analysis of proteome changes of HCMV-infected cells confirmed previous data and gave new insights in the changes of cellular proteins by HCMV (Weekes et al., 2014). The study could show that HCMV is able to manipulate the expression of > 8.000 cellular proteins establishing productive infection. The virus is not only reducing protein amounts of Jak1, STAT2 and IRF9 but also the protein amounts of IFNAR2, IFNGR1 and Jak2. As CMVs have the potential to be used as vaccine vectors (Hansen et al., 2013), identification of the responsible HCMV gene products might be helpful to gain better understanding of its ability to modulate the host immune system.

1.3.3 HCMV-encoded ISG antagonists assigned to a defined HCMV gene

PKR senses viral dsRNA resulting in the serine phosphorylation of the eukaryotic translation initiation factor 2 alpha subunit (eIF-2α). This inhibits polypeptide chain initiation and shuts down translation (Kimchi et al., 1979; Wu and Kaufman, 1996). PKR is also able to activate the NF-κB pathway (Jiang et al., 2003) and is responsible for IFN-γ-induced up-regulation of high affinity receptors for IgG antibodies (CD64) (Karehed et al., 2007). Recent data reveal important interactions of PKR with various compartments of the inflammasome, thereby activating the release of caspase activation-dependent cytokines (Lu et al., 2012). Hence, PKR resembles a very

important protein needed for efficient control of viral infections. PKR knockout mice are strongly impaired in the anti-viral response upon IFN-γ stimulation, however the induction of IFN I can be restored by priming with IFNs (Yang et al., 1995).

HCMV encodes for two PKR antagonists, pTRS1/pIRS1, blocking the shut-off of cellular translation (Cassady, 2005). The viral proteins directly interact with dsRNA and PKR, retaining them in the nucleus. Thereby the phosphorylation of eIF-2α is prevented (Child et al., 2004; Hakki and Geballe, 2005; Hakki et al., 2006).

OAS is an IFN-induced protein detecting dsRNA. dsRNA-bound OAS catalyzes the formation of 2'-5' linkage of ATP, creating dimerization and activation of RNase L (Clemens and Williams, 1978; Ratner et al., 1978; Zilberstein et al., 1978). Activated RNase L subsequently leads to the degradation of mRNA and rRNA (Player et al., 1998). Furthermore, OAS elicits anti-viral activity independently of RNase L in a paracrine manner, directly inhibiting viral proliferation (Kristiansen et al., 2010; Thavachelvam et al., 2014).

HCMV pUL126a, a latency-associated protein present in the nucleus and dispensable for productive viral replication (White et al., 2000), was identified to decrease OAS expression in IFN-treated and untreated cells. This prevents RNase L activation and subsequent RNA degradation (Tan et al., 2011). pTRS1 and pIRS1 are also implicated to inhibit the OAS pathway (Marshall et al., 2009). Additionally, pTRS1 is able to interact with Beclin 1 directly blocking autophagosome biogenesis (Chaumorcel et al., 2012).

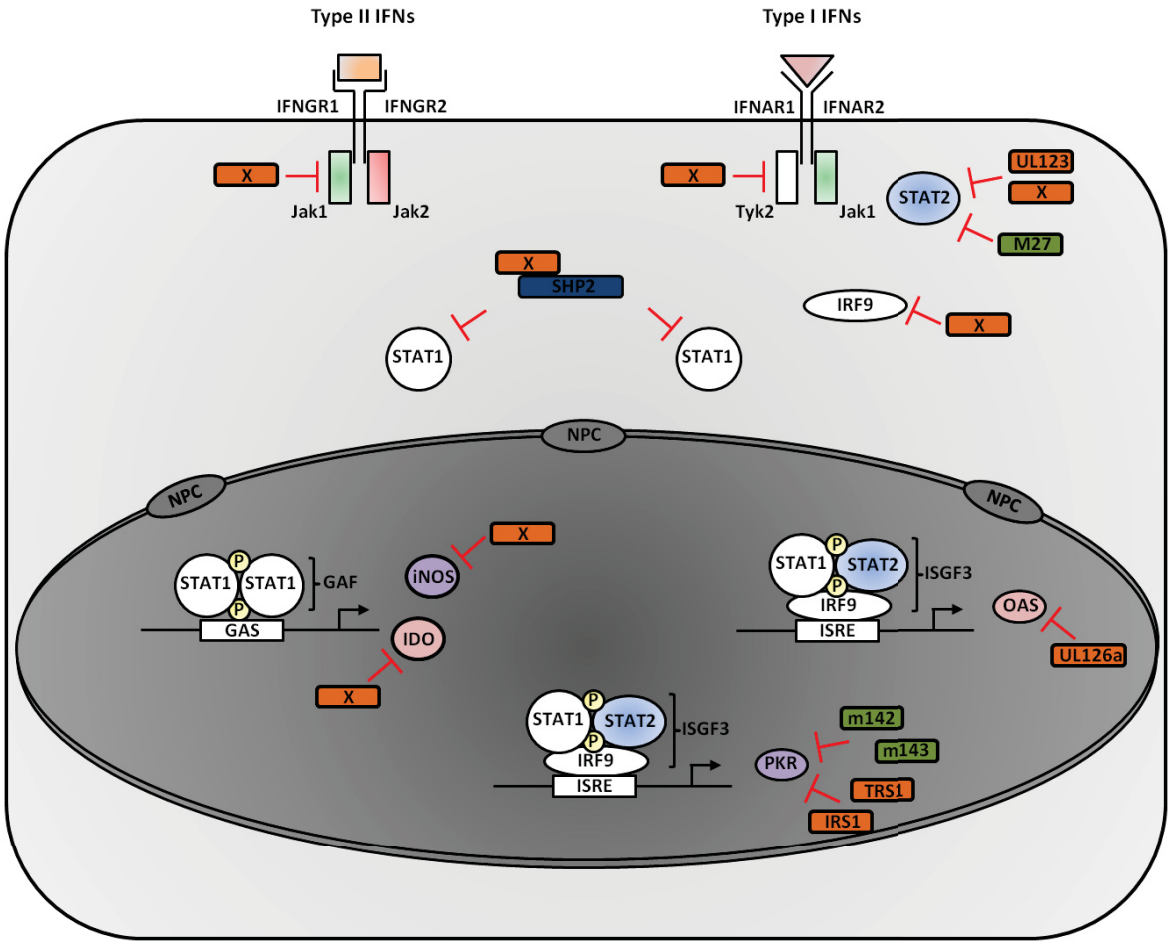

Figure 3: Multi-layered CMV immune evasion of IFN signaling. HCMV and MCMV encode multiple proteins to dampen IFN signaling. Proteins encoded by HCMV are depicted in orange, MCMV-encoded proteins in green. HCMV is able to degrade STAT2, Jak1 and IRF9. STAT1 is dephosphorylated and the protein amounts of IFNGR1, IFNAR2 and Jak2 are reduced. Furthermore, the ISGs PKR, OAS, IDO and iNOs are inhibited. The viral protein products responsible for these phenotypic changes that have not yet been identified are marked with an X.

1.3.4 HCMV-encoded ISG antagonists unidentified

Reactive nitric oxide (NO) species have been shown to have critical protective capacities against DNA viruses (Croen, 1993; Karupiah et al., 1993). iNOS is the enzyme responsible for the generation of these reactive species. HCMV is able to block the iNOS pathway, although it is not able to counteract exogenously applied NO (Bodaghi et al., 1999). In mice, iNOS-mediated antiviral activity of macrophages plays a protective role, as mice deficient for iNOS are more susceptible to a lethal infection with MCMV (Fernandez et al., 2000; Noda et al., 2001).

To limit the available amount of the essential amino acid tryptophan, IDO catalyzes the conversion of tryptophan to L-kynurenine. It has been shown that IDO is induced in HCMV infected monocytes and that it impairs virus replication in retinal pigment epithelium cells (Bodaghi et al., 1999; Furset et al., 2008), astrocytes (Suh et al., 2007) and fibroblasts (Zimmermann et al., 2014).

HCMV is able to efficiently inhibit IDO expression in different cell types, e.g. placental tissue, fibroblasts and mesenchymal stem cells (MSC) (Heseler et al., 2013; Lopez et al., 2011; Meisel et al., 2014). This is achieved by blocking transcription of IFNγ-induced IDO mRNA (Zimmermann et al., 2014). HCMV was also reported to downregulate mRNA expression of iNOS (Bodaghi et al., 1999).

1.4 MCMV-mediated immune evasion

As CMVs are very species specific, *in vivo* studies with HCMV are not possible. However, an established *in vivo* mouse model gives the opportunity to obtain insights concerning HCMV-mediated immune evasion as both viruses, HCMV and MCMV, share a certain number of gene homologies (Rawlinson et al., 1996). Both viruses have evolved similar strategies to evade the IFN response although different proteins and mechanisms are responsible for these strategies. Nevertheless, the understanding of MCMV-mediated inhibition of IFN induction and signaling enables the possibility to identify HCMV genes previously not associated with a specific function.

1.4.1 MCMV-encoded IFN antagonists assigned to a defined MCMV gene

MCMV is able to initiate proteasomal degradation of STAT2 by the viral early/late encoded protein pM27. Deletion mutants lacking *M27* reach similar viral titers in infected fibroblasts *in vitro*, however it is essential for efficient replication *in vivo*, as the delta *M27* virus mutant is highly attenuated in mice (Abenes et al., 2001; Zimmermann et al., 2005). pM27 interacts with the ubiquitin ligase complex adaptor protein DNA-damage DNA-binding protein 1 (DDB1) facilitating the poly-ubiquitination of STAT2 via Cullin-4A/B-RocA complexes. This leads to proteasomal degradation of STAT2 (Trilling et al., 2011). The STAT2 degradation is crucial for efficient viral replication as the transcription and protein expression of immuno-proteasome subunits is inhibited, disabling the cell to present peptides to T cells (Khan et al., 2004). However, it was shown that the HCMV-encoded homologue of pM27, pUL27, is not responsible for the HCMV-mediated degradation of human STAT2 as virus mutants lacking *UL27* are still able to degrade STAT2 (Le et al., 2008a).

RIP3 kinases were identified to play essential roles for necroptosis upon tumor necrosis factor (TNF) and IFN induction (Cho et al., 2009; He et al., 2009; Robinson et al., 2012; Zhang et al., 2009). The ISGF3 complex and PKR are required to sustain the RIP3 activation leading to caspase-mediated cell death (McComb et al., 2014; Thapa et al., 2013). RIP kinases together with the activation of caspase 8 regulate antibacterial immune responses (Weng et al., 2014) or suppress RNA virus replication by promoting the activation of NLRP3 inflammasomes (Wang et al., 2014a). Notably, RIP3 also plays an important role to activate necroptosis in certain *Herpes virus* infections, shown for HSV1 and MCMV infections (Upton et al., 2010; Upton et al., 2012; Wang et al., 2014b).

MCMV is very efficiently inhibiting TNFα-induced activation of NF-κB by binding of pM45 to, and thereby inhibiting, RIP1 (Mack et al., 2008). As RIP is involved in signal transduction of the cytoplasmic DNA sensor DNA-dependent activator of IRFs (DAI), pM45 inhibits the DAI-mediated

activation of NF-κB (Rebsamen et al., 2009). The DAI-mediated virus-induced necrosis is dependent on the RIP homotypic interaction motif (RHIM) complexing DAI with RIP3. pM45 suppresses this interaction by binding to the RHIM domain, thereby suppressing interaction of DAI and RIP (Upton et al., 2008; Upton et al., 2012). Additionally, M45 inhibits NFκB signaling by targeting the NFκB essential modulator (NEMO), the regulatory subunit of the IKK complex, to autophagosomes, resulting in lysosomal degradation of NEMO (Fliss et al., 2012).

MCMV expresses two 'functional homologues' of HCMV TRS1 and IRS1, pm142 and pm143. These block PKR-mediated translation shutdown. The two viral proteins are essential for virus replication but virus growth can be complemented if TRS1 is expressed from the MCMV genome (Valchanova et al., 2006). Both proteins are able to bind dsRNA and are specific inhibitors of PKR as activation of the OAS/RNase L pathway could not be detected (Budt et al., 2009).

1.4.2 MCMV-encoded IFN antagonists unidentified

Most of the proteins involved in Jak-STAT signaling, except STAT2 and STAT3, remain unaffected by an MCMV infection (Trilling et al., 2014). This is a crucial difference between HCMV- and MCMV-mediated immune evasion. However, MHC-II presentation upon IFN-γ stimulation is also inhibited by blocking the IFN-γ-induced promoter assembly thereby paralyzing macrophage responses (Heise et al., 1998a; Heise et al., 1998b; Popkin et al., 2003). In addition, productive IFN-β enhanceosome formation is inhibited as IRF-3, NF-κB and ATF/cJun are targeted (Le et al., 2008b). Furthermore, albeit an increase in phospho-STAT3 protein levels, MCMV infection leads to a decrease in STAT3 amounts and blocks STAT1/STAT3-dependent target gene expression (Trilling et al., 2014).

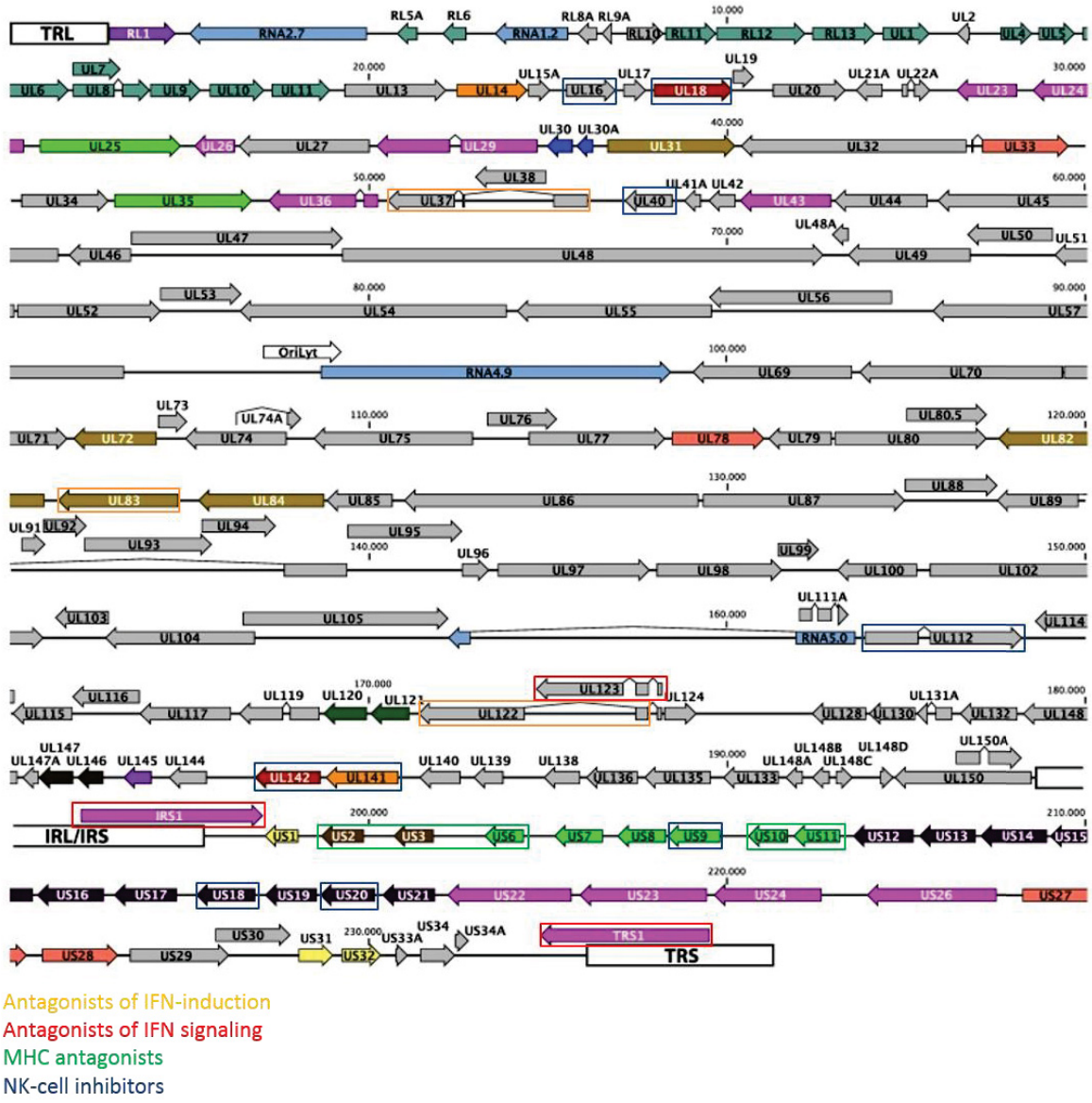

Figure 4: HCMV encodes for multiple immune evasion genes. Depicted is the HCMV genome (Sijmons et al., 2014). Additionally, genes with known antagonistic capacities were highlighted. The color was chosen according to the type of inhibition. The virus is able to translate at least ten NK cell antagonists and six MHC antagonists. Furthermore, so far six viral genes have been implicated in IFN evasion.

1.5 Cellular protein degradation

The degradation of cellular proteins for adaptation to changing conditions and the recovery of amino acids as building blocks for the translation of newly synthesized proteins is a continuous process in cells. It is highly regulated and controlled and has important implications in protein quality control of misfolded proteins (Chhangani et al., 2014; Fang et al., 2014), antigen presentation in the adaptive immune responses (Aki et al., 1994; Dahlmann et al., 2000) as well as cell cycle control (Sudakin et al., 1995). Viruses use these degradation pathways for their benefit, e.g. initiating the degradation of host restriction factors, thereby evading the cellular

immune system and enabling proper replication and egress of virus progeny. The three major cellular pathways used for protein degradation are described hereafter.

1.5.1 Proteasomal degradation

A variety of cellular proteins is degraded via the ubiquitin-proteasome system (UPS), a tightly regulated pathway that 'tags' proteins for proteolytic degradation (Mahon et al., 2014). In this system, three enzyme classes work in a stepwise fashion: An E1-ligase activates an ubiquitin-polypeptide, E2 ligases conjugate this polypeptide while E3 ligases confer the ubiquitin on protein substrate targets. This cascade is followed by protein elimination from the cell by the proteasome (Ciechanover, 2013; Hershko and Ciechanover, 1998). The type of ubiquitin lysine linkage (i.e. Lys11, Lys48 and Lys63) is controlled by a combination of E2- and E3-ubiquitin ligases, resulting in a complex network, as an expanding number of more than 600 E3-ubiquitin ligases have been identified (Deshaies and Joazeiro, 2009; Spratt et al., 2014). E3-ligases are classified into three major groups: RING, U-box and *Homologous to E6AP C-Terminus* (HECT) E3-ligases. RING and U-box E3 ligases directly transfer ubiquitin from E2-ligases to the substrate whereas HECT E3 ligases form an Ub-thioester intermediate (Kamadurai et al., 2009; Maspero et al., 2011; Metzger et al., 2014; Spratt et al., 2014). Cullin-RING E3 ligases, constituting the largest group of E3-ligases, consist of an adaptor subunit, a substrate receptor and a regulator of Cullin/RING box protein 1 (Mahon et al., 2014). Additionally, the scaffold has to be activated by the ubiquitin homologous protein NEDD8, a process called neddylation, for active scaffolding function (Lydeard et al., 2013; Soucy et al., 2009). HECT E3 ligases are comprised of a carboxy-terminal HECT domain, between two and four WW-domains and an amino-terminal C2 domain (Maspero et al., 2011; Scheffner and Kumar, 2014).

Ubiquitinated target proteins are degraded by the 26S proteasome. This large ATP-dependent proteolytic cellular machinery is composed of two subunits: the 20S core particle and a 19S regulatory particle (Ben-Nissan and Sharon, 2014; Goldberg, 2003; Schmidt and Finley, 2014; Schwartz and Ciechanover, 2009). The 20S core particle itself consists of 28 subunits, the three β-subunits $\beta1$, $\beta2$ and $\beta5$ being catalytically active proteases. The 19S regulatory particle is located at one or both ends of the 20S core unit and coordinates the degradation of the poly-ubiquitinated target protein. Proteolytic cleavage is initiated upon translocation of the protein to the interior of the 20S complex (Ben-Nissan and Sharon, 2014). Recent data show that proteins are also degraded by the 20S core particle in an ubiquitin-independent manner, especially in the case of misfolded or damaged proteins (Erales and Coffino, 2014; Pickering and Davies, 2012), and endogenous proteins with large unstructured segments (Ben-Nissan and Sharon, 2014; van der Lee et al., 2014).

1.5.2 Endosomal degradation

The endosomal system and endocytic routes remain poorly understood, as the size and dynamics of the endocytic pathway differ significantly in various cell lines due to an extreme degree of plasticity (Lucin et al., 2015). Endocytic and recycling activity are highly coordinated,

i.e. relocation of plasma membrane proteins to the intracellular site is achieved by decreasing the recycling rate (Steinman et al., 1983). Four major types of endocytosis are distinguished: Clathrin-dependent endocytosis, clathrin-independent endocytosis, macropinocytosis and endocytosis through deep tubular invagination. (McMahon and Boucrot, 2011).

Early endosomes utilize microtubules to travel towards the cell center. During this route additional vesicles are acquired and vesicle size increases (Huotari and Helenius, 2011; Jovic et al., 2010). Early endosomes that reach the cell center either mature into late endosomes or into large tubular endosomes known as the juxtanuclear recycling compartment (Huotari and Helenius, 2011; Jovic et al., 2010). Late endosomes that fuse with lysosomes finally facilitate host protein degradation (Huotari and Helenius, 2011; Lebrand et al., 2002).

1.5.3 Autophagy

The fusion of lysosomes with double-membraned structures for the efficient degradation of cellular organelles and intracellular aggregates for the sake of reusing amino acids and other building blocks was named autophagy, 'self-eating', nearly 50 years ago (Deter et al., 1967). Autophagy is a complex and tightly regulated cellular process that is directed by the core autophagy machinery mostly composed of autophagy-related proteins (Atg) (Klionsky and Schulman, 2014). Four major steps are synchronized in the cell: creation of an autophagosomal membrane, elongation of the autophagosomal membrane, engulfment of cellular organelles or aggregates and autophagosome formation and finally the fusion of the autophagosome with lysosomes (He and Klionsky, 2009). Multi-protein complexes and signaling platforms are required to coordinate the initiation of this process (Wang et al., 2015), namely the mammalian target of rapamycin (mTOR) complex, the unc-51-like kinase 1 (ULK1) complex, the Vps34 complex and the ubiquitin-like (Ubl) conjugation complex.

Cellular stress, e.g. nutrient starvation, hypoxia, damaged organelles or infection are key initiators of autophagy (Kroemer et al., 2010; Rabinowitz and White, 2010). The mTOR signaling pathway senses these environmental cues to execute the initiation of autophagy (Laplante and Sabatini, 2012; Zoncu et al., 2011) leading to the creation of the phagophore during the early stages of autophagosome formation (Hosokawa et al., 2009; Jung et al., 2009; Kim et al., 2011a). Beclin 1, a key regulatory protein, recruits additional regulators of this complex, either favoring (Fimia et al., 2007; He et al., 2013; Matsunaga et al., 2009; Takahashi et al., 2007; Zhong et al., 2009) or inhibiting (Matsunaga et al., 2009; Pattingre et al., 2005; Xia et al., 2014; Zhong et al., 2009) autophagy induction. As the last step of autophagosome formation, two Ubl conjugation systems have to be assembled (Moreau et al., 2011; Sakoh-Nakatogawa et al., 2013) followed by the conjugation of phosphatidylethanolamine (PE) to LC3 forming LC3-II (Kabeya et al., 2000). The autophagosomes will then fuse with lysosomes into autophagolysosomes degrading the engulfed intracellular materials (Kundu and Thompson, 2008; Yang and Klionsky, 2010).

1.6. Herpes simplex thymidine kinase (HSV-TK)

The virus needs the TK for successful viral replication upon reactivation in the trigeminal ganglion (Coen et al., 1989; Tenser et al., 1979). As nerve cells arrest their cell cycle once they are differentiated, the HSV-TK is needed for efficient phosphorylation of thymidine as the number of cellular thymidine kinases are gradually decreased during development (Caron and Unsworth, 1978). The nucleoside analogue Ganciclovir (GCV), a standard therapy for *Herpesviruses* (Smith et al., 1982), is also mono-phosphorylated by the HSV-TK. Only the HSV-TK is specific for GCV, whereas cellular kinases do not facilitate its mono-phosphorylation. After generation of the GCV mono-phosphate, cellular kinases proceed to convert the GCV to a 5'-triphosphate following the incorporation of the nucleoside analogue into the cellular DNA, resulting in irreparable DNA damage and cell death (Ladd et al., 2011). Therefore, the HSV-TK presents a useful tool to purposefully initiate cell death via GCV, targeting cells that express this protein (Grignet-Debrus et al., 2005; Moriuchi et al., 1998). This is used in the treatment of gliomas (Miyatake et al., 1997; Zhao et al., 2014) or to minimize the severity of graft-versus-host disease (GvHD) (Ciceri et al., 2005; Mailly et al., 2010).

1.7 Aim of the thesis

As HCMV codes for a vast number of translated proteins, the identification of specific inhibitors of known targets is quite challenging. One inherent problem with the identification of upstream regulators of protein stability is that their interaction with the respective target protein leads to the destabilization of the target - making identifications based on protein-protein interactions (e.g. Co-IP) cumbersome. This was experienced in the process of examining the mechanism of MCMV pM27 to degrade STAT2 (Trilling et al., 2011; Zimmermann et al., 2005).

This problem seems to be a more general phenomenon. A large screening approach using affinity- chromatography together with mass-spectrometry techniques was used to investigate the degradation of APOBEC3G by the HIV-1, identifying proteins that co-precipitate with the viral protein *vif*. Although complexes involved in degradation were identified, a direct interaction of the target protein APOBEC3G and the viral protein *vif* was not detected (Jager et al., 2012).

Based on the fact that cellular degradation pathways (e.g. proteasome and phagolysosome) are known to degrade proteins irrespective of the presence of fused protein domains, as previously shown for green fluorescent protein (GFP) coupled to a proteasome target protein leading to complete degradation of the whole protein including the GFP (Dantuma et al., 2000), a screen was envisaged in which a protein of interest (here Jak1 and STAT2) was fused to the thymidine kinase (TK) derived from *Herpes simplex* (HSV). TK catalyzes the phosphorylation of the pro-drug Ganciclovir (GCV), thereby turning it into a highly (geno-) toxic agent which abrogates cell proliferation and eventually leads to cell death. Thus, TK-positive cells die in presence of GCV, whereas TK-reduced cells become more and more GCV resistant. The expression of TK-fusion proteins physically tethers TK activity to the stability of the protein of interest (e.g. Jak1 and STAT2) and gives the opportunity to create a switchable and tunable system as cell death is only being initiated upon treatment with GCV. Simultaneous co-expression of the fusion protein and a viral antagonist in the cells should facilitate the degradation of the TK-fusion protein,

independent of the degradation pathway used. Analogous, degradation of the Jak1- and STAT2-TK fusion proteins should lead to a loss of the TK domain conferring resistance to GCV and a proliferative advantage of cells expressing a protein which destabilizes the fusion protein, either by targeting the TK part or the specific part (e.g. Jak1).

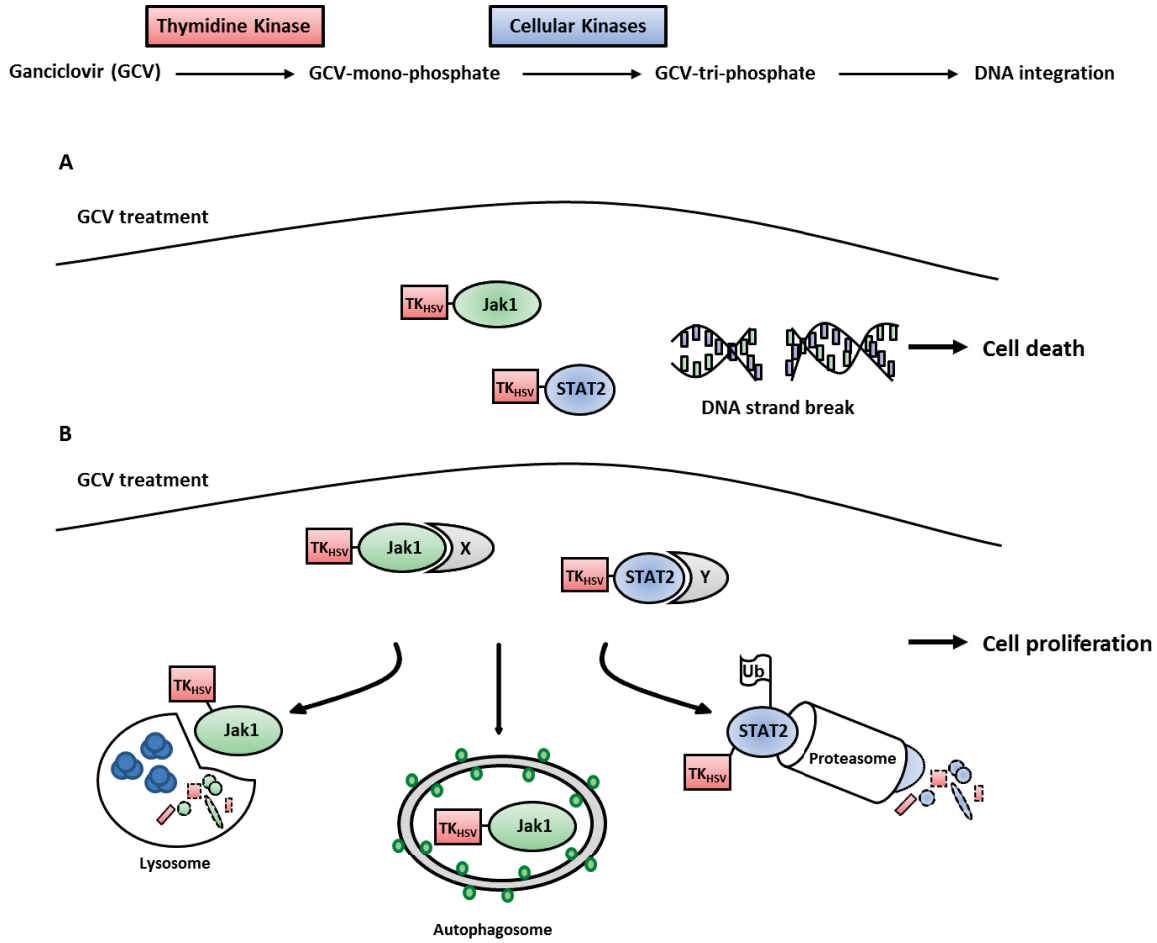

Figure 5: A novel screening strategy turning protein degradation into a survival advantage. Graphical representation of the screening approach to be developed. Viral TK initiates mono-phosphorylation of GCV subsequently leading to DNA integration and cell death. (A) Cells expressing TK-fusion proteins die upon GCV treatment. (B) Simultaneous expression of a viral, or cellular, antagonist that is able to degrade the fusion protein leads to degradation of the TK domain, resulting in a survival advantage of the cells upon GCV treatment.

Although the existence of HCMV-encoded Jak1 and STAT2 antagonists has been confirmed by several groups (Le et al., 2008a; Miller et al., 1998; Miller et al., 1999), the responsible gene products have not yet been identified. Screening systems using CMV deletion mutants have the disadvantage that redundant effects often used by CMVs might be overlooked and antagonistic gene products might not be detected. The genes eliciting a redundant antagonistic function are not deleted in the virus mutants would still be able to have the antagonistic effect. Therefore, the task was to establish, validate and apply a novel screening approach, using fusion proteins of

the HSV-TK and Jak1 or STAT2, respectively. An HCMV ORF expression library comprising 164 HCMV-encoded proteins (Salsman et al., 2008) and a novel lentiviral cDNA library of HCMV-infected cells were available to be used in the screening approach for the identification of the specific viral IFN-antagonists.

2. Results

2.1 HCMV downregulates protein amounts of Jak1 and STAT2

HCMV evades from the antiviral effects of IFN by circumventing IFN induction and signal transduction (reviewed in (Le-Trilling and Trilling, 2015; Trilling et al., 2012)), enabling viral replication despite the presence of PAMPs and/or type I or type II IFN.

HCMV strain TB40 growth was determined by classic foci titration over a period of 12 days (see Materials and Methods, Chapter 4.2.2.2), in which cell supernatants (of freeze/thawed MRC-5 cells) were taken in three-day intervals. An IFN-α treatment regime that started at the time of infection only slightly (~5-fold) reduced viral titers after 3 d p.i., and showed almost congruent growth kinetics at day 6 p.i. and later. An IFN-α pre-treatment phase of 24 h resulted in a slightly more effective, yet not significant, reduction of one order of magnitude and prolonged the anti-viral effect until day 6 p.i. However, from day 9 p.i. on, viral titers in treated and untreated cells were undistinguishable (Figure 6 A). The same pattern in terms of reliance of the effect on a phase of pre-incubation emerged upon treatment with IFN-γ. The antiviral effect of IFN-γ was stronger than the effect of IFN-α (~100-fold) but the virus titers also reached similar levels like untreated cells 9 d p.i. (Figure 6 B). The ant-viral effect of type I and type II IFN is more pronounced in other viruses (e.g. *Encephalomyocarditis virus* (EMCV) (Stebbing et al., 1983) and *Vesicular Stomatitis virus* (VSV) (Masters and Samuel, 1983), accentuating the high efficiency of HCMV to counteract the anti-viral state elicited by IFNs.

In this experimental setting, the degradation of Jak1 and STAT2 has been described previously (Le et al., 2008a; Miller et al., 1998). Consistent with previous reports from our and other groups, Jak1 and STAT2 protein amounts were reduced to barely detectable levels in HCMV-infected cells (Figure 6 C, lane 2), whereas protein amounts remain stable in MRC-5 cells infected with UV-inactivated virus (Figure 6 C, lane 3). This clearly highlights that viral gene expression is essential to initiate Jak1 and STAT2 degradation in HCMV-infected cells.

2.2 Generation of stable cell lines expressing thymidine kinase (TK) fusion constructs

Many viruses encode proteins which initiate the degradation of specific host restriction factors, e.g. proteins involved in Jak-STAT signal transduction and the IFN signaling cascade. Although the respective target proteins are often well-described, the identity of the responsible viral regulators remains often elusive (see Table 3 in the Discussion). Therefore, a novel, broadly applicable screening system was designed to identify the HCMV-encoded antagonists of Jak1 and STAT2 (Figure 5). Using plasmid expression libraries or lentiviral cDNA libraries, high throughput analysis can be performed to identify the unknown HCMV antagonists.

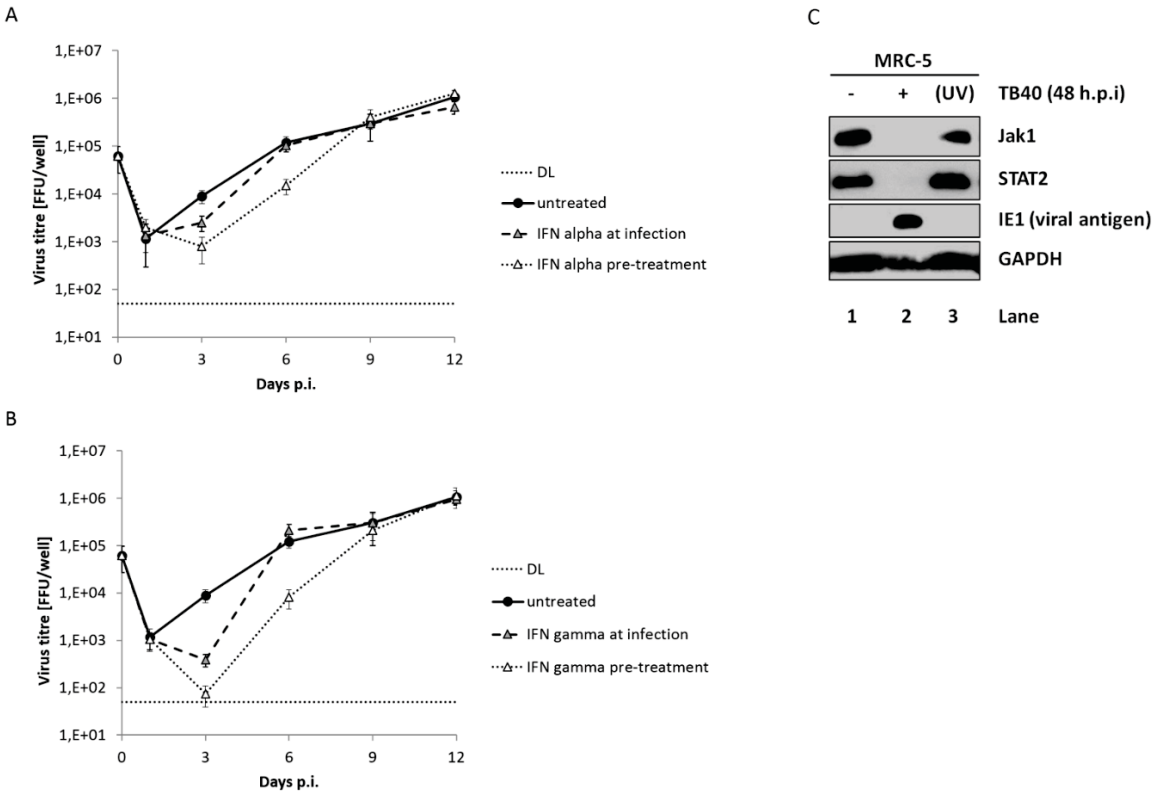

Figure 6: HCMV is not susceptible towards IFN treatment. MRC-5 fibroblasts were infected with HCMV strain TB40 (0.3 PFU/cell) and cells were frozen (-80°C) at indicated time points. To assess the effect of IFN on viral growth properties, cells were either treated with (A) IFN alpha (500 U/ml) or (B) IFN gamma (500 U/ml). Pre-treatment of cells was performed for 24 h prior to infection. Viral titers were assessed by titration as described in the M&M section. DL, detection limit; FFU, focus forming units. (C) MRC-5 fibroblasts were infected with HCMV strain TB40 (MOI 3) or UV-inactivated virus for 48 h. Cells were lysed and subjected to western blot analysis. IE1 served as control for viral gene expression. All experiments shown have been independently performed at least 2 times.

To create the novel screening system, cells expressing Jak1 or STAT2 fused to the TK had to be generated. To this end, plasmids containing a fusion of both proteins connected by a hexa-glycine linker were established. For the STAT2-TK construct, the *stat2* gene was cloned into pcDNA3.1(+) expression plasmids with a hexa-glycine linker and without a stop codon. The TK was integrated via an *Xba*I digestion. All point mutations in *stat2* or the *tk* that occurred during the PCR were repaired via site-directed mutagenesis. For the TK-Jak1 construct, the cloning was performed vice versa. For a more detailed description of the cloning process see the Materials and Method section. As a next step, cells were transfected with the fusion constructs and different cell types stably expressing the fusion constructs were selected (Figure 7 A). Next to the two forms of endogenous STAT2 (Sugiyama et al., 1996), the fusion proteins were expressed with expected size. Additionally, cells harboring Jak1 or STAT2 fused to a tandem-affinity-purification-tag (TAP-tag) were generated, to examine if potential antagonists recognize Jak1,

STAT2 or TK. In contrast to parental cells, only cells containing Jak1 or STAT2 fused to a TAP-tag showed a clear protein band when probing whole cell lysates with an antibody specific for the TAP-tag (Figure 7 B).

Figure 7: Generation of stable cell lines expressing thymidine kinase (TK) fusion constructs. (A) Indicated cell lines were transfected with the respective fusion construct harboring a neomycin or zeocin resistance cassette and cultured in presence of Geneticin (G418)/Zeocin. Surviving cell clones were expanded and whole cell lysates were subjected to western blot analysis. (B) SPA-tagged proteins were constructed and stable cell lines were generated as described in (A). All experiments shown have been independently performed at least 3 times.

2.3 TK-fusion proteins seem to be properly folded and able to reconstitute IFN signaling events

As the fusion of the TK to the target protein could potentially impair protein folding and/or location of the target protein in the cell, the two fusion proteins STAT2-TK and TK-Jak1 were tested concerning retained function, at least in fibroblasts like U6A and U4A lacking STAT2 and Jak1, respectively. Only if an efficient STAT2 phosphorylation has taken place upon IFN I stimulation, phosphorylation of STAT1 occurs (Leung et al., 1995). Hence, STAT2-deficient cells (called U6A) do not allow STAT1 phosphorylation upon IFN-α treatment unless functional STAT2 is (re-)introduced. U6A cells were transfected with the STAT2-TK fusion construct and stimulated with IFN-α. STAT2-TK became phosphorylated in presence of IFN and restored STAT1 phosphorylation (Figure 8 A). The same experimental approach was taken to assess the functionality of the TK-Jak1 construct, since IFN-I signaling cannot be initiated in absence of Jak1 (Figure 8 B). Jak1-deficient cells (U4A) were transfected with the fusion construct and treated with IFN-α. pSTAT1 could be detected in the transfected cells as seen in Figure 8 A, although to a lesser extent compared to restored STAT1 phosphorylation by STAT2-TK in U6A cells. This shows that the Jak1- and STAT2 parts of the fusion proteins must have retained proper folding to a certain degree to allow STAT1 phosphorylation despite the TK fusion.

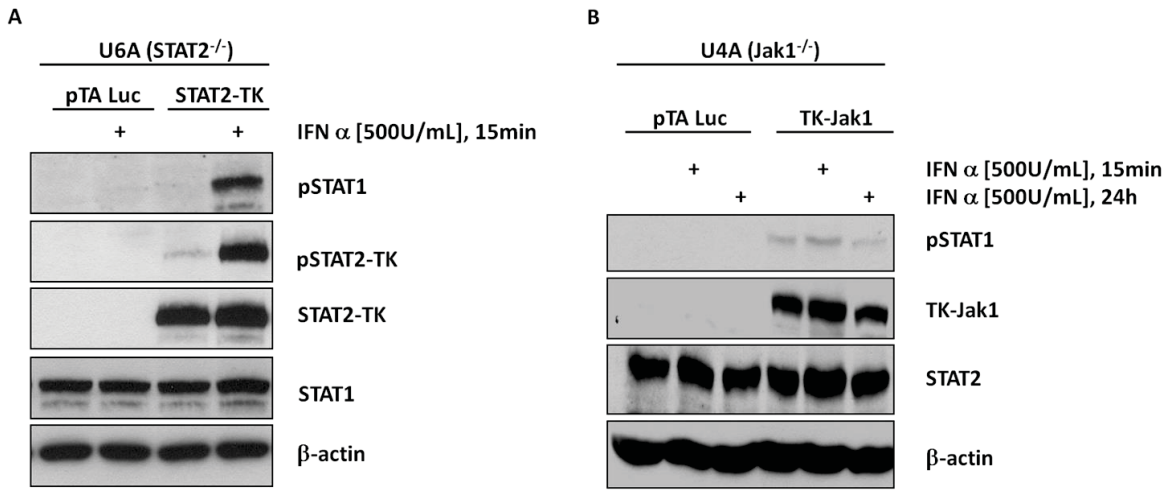

Figure 8: TK-fusion proteins are properly folded and able to reconstitute IFN signaling events. (A) U6A (STAT2$^{-/-}$) cells were transfected with control plasmid or STAT2-TK for 24 h and stimulated with IFN-α for 15 min. Whole cell lysates were subjected to western blot analysis. (B) U4A (Jak1$^{-/-}$) cells were transfected with control plasmid or TK-Jak1 for 24 h and stimulated with IFN-α for 15 min or 24 h. Whole cell lysates were subjected to western blot analysis. All experiments shown have been independently performed at least 3 times.

2.4 Expression of TK-fusion proteins results in susceptibility towards ganciclovir (GCV) treatment

To test the effectiveness of the TK fusion constructs, parental and stable cell lines expressing either STAT2-TK or TK-Jak1 and their respective parental cell lines were treated for 96 hours with different concentrations of GCV. Stable cell lines expressing the TK fusion constructs died upon GCV treatment as documented by loss of crystal violet staining, a dye which stains living cells (Figure 9 A). Highly significant differences concerning cell viability were observed even with nanomolar GCV concentrations (100 nM) leading to a dose-dependent induction of cell death.

The V protein of Parainfluenza virus 2 (PIV2V) and the mouse cytomegalovirus pM27 degrade STAT2 (Andrejeva et al., 2002; Precious et al., 2005). Assuming that our screening approach works, co-expression of these known STAT2 destabilizing proteins should lead to a measurable survival advantage of STAT2-TK expressing cell lines upon GCV treatment. The V protein was able to degrade the STAT2-TK fusion product dose-dependently in co-transfection experiments (Figure 9 B) – again suggestive for appropriate folding of the STAT2 moiety of the chimera. More importantly, STAT2-TK cells co-transfected with PIV2V or pM27 for 24 hours prior to GCV treatment led to highly significant survival advantage compared to STAT2-TK cells lacking a STAT2 degrading protein (Figure 9 C).

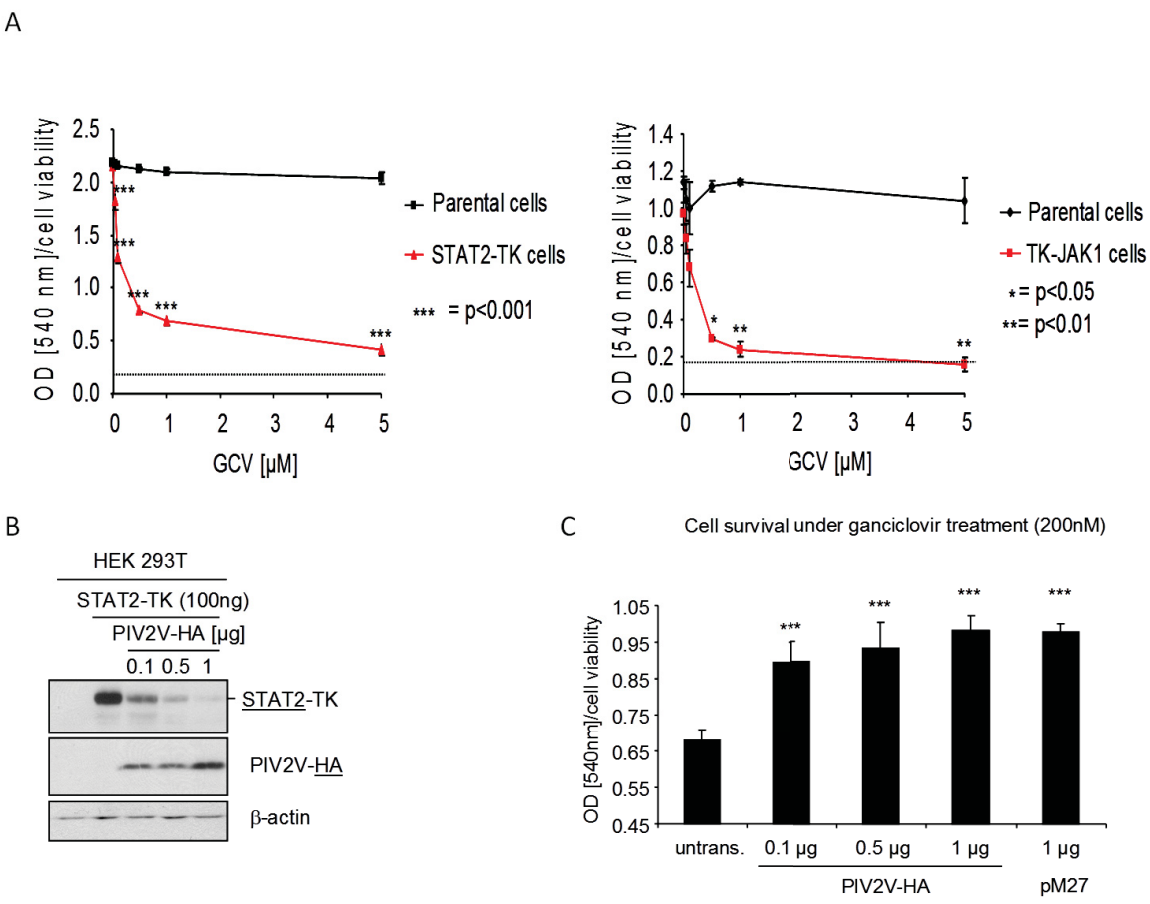

Figure 9: Co-expression of viral antagonists leads to survival advantage upon GCV treatment. (A) STAT2-TK cells, TK-Jak1 cells and their respective parental cell lines were treated for 96 hours with grading GCV concentrations (0.1 to 5 µM). Cell viability was assessed by crystal violet staining and photometric measurement at 540 nm (see Materials and Methods section). (B) 293T cells were transfected with STAT2-TK and the V protein of Parainfluenza virus type 2 (PIV). Whole cell lysates were subjected to western blotting. Underlined words specify the primary antibody. (C) STAT2-TK cells were transfected with the indicated viral protein 24 h prior to GCV treatment. Cells were treated with 200 nM GCV for 96 h and cell viability was assessed as in (A). All experiments shown have been independently performed at least 3 times.

2.5 Using a previously described collection of cloned HCMV open-reading-frames, no HCMV-encoded STAT2 antagonist was identified

To screen for a viral STAT2 antagonist, a collection containing 164 individually cloned ORFs (Salsman et al., 2012; Salsman et al., 2008) was used in the novel screening system. As for the control experiment with the PIV2V, single ORFs were co-transfected 24 hours prior to GCV treatment and cell viability was examined 96 hours after treatment. In comparison to cells transfected with the control plasmid, cells showed significant differences in cell viability under all three experimental conditions (untreated, 0.25 µM and 2.5 µM GCV), i.e. had a slight but significant survival advantage in the presence of low or high amounts of GCV (Figure 10 A) upon transfection with one out of 14 ORFs (see below). From these 14 transfected ORFs, the ones

leading to proliferative effects without the presence of GCV, as well as ORFs described as 'late' genes were excluded from further analysis because late gene expression is dispensable for HCMV-mediated STAT2 degradation (Le et al., 2008a). The three remaining potential antagonists were examined per immuno-blot analysis concerning their capacity to reduce STAT2 in co-transfection experiments (Figure 10 B). Unfortunately, none of the three ORFs (US30, UL33 and UL41) reproducibly and convincingly reduced levels of the target protein STAT2.

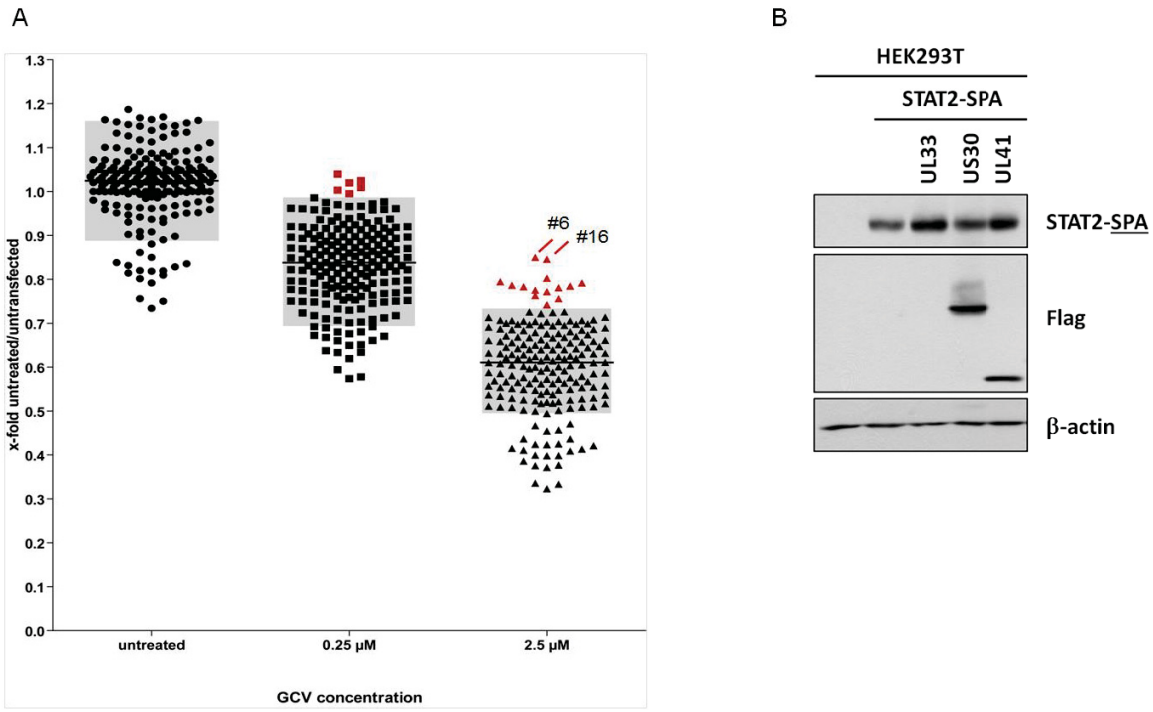

Figure 10: The novel screen does not select a gene being sufficient to reduce STAT2 amounts from a collection of individually cloned cDNAs. (A) STAT2-TK cells were transfected with plasmids encoding individual HCMV ORFs 24 h prior to start of GCV treatment (0.25 or 2.5 µM) and cell viability was measured using crystal violet (n = 6), each dot represents one ORF. Depicted are the fold differences in cell viability of each ORF in comparison to untransfected cells. Grey squares indicate the highest standard deviation measured of all ORFs tested. ORFs increasing cell proliferation significantly compared to untransfected cells are depicted in red. Complete table summarizing clones that had significant growth advantages is depicted in the Appendix. (B) 293T cells were transfected with STAT2-SPA and the indicated HCMV ORF, lysed 24 h post-transfection and subjected to western blot analysis. Depicted is a representative western blot analysis of STAT2 degrading capability of three ORFs increasing cell proliferation in (A). A complete table summarizing clones that had significant growth advantages is depicted in the Appendix (see Suppl.-Table 1).

We concluded that either the screen failed to select the HCMV-encoded STAT2 antagonist, e.g. because a hetero dimer is required to operate, the protein mediates a toxic effect or that the respective gene is not part of the cDNA collection. Please note that recent data suggest that HCMV encodes for more than 750 translation products (Stern-Ginossar et al., 2012) whereas the collection only comprises ~170 ORFs.

2.6 Global screening with lentiviral cDNAs leads to survival of cell clones expressing potential HCMV Jak1 antagonists

Contemporaneously with the above-mentioned screening approach using the HCMV ORF collection described in 2.7, a global screening approach was conducted using lentiviruses expressing eGFP and a single cDNA expressed during HCMV infection (HCMV strain AD169 and HCMV strain TB40). This lentiviral cDNA library was constructed by Marco Maywald, Heinrich-Heine University Düsseldorf. Stable TK-Jak1 cells were infected with the lentiviral cDNAs, sorted for eGFP-positive cells, as all lentiviral cDNAs additionally expressed an eGFP cassette, and expanded in medium containing Zeocin (to force the cells to retain the expression construct) and GCV for selection. Cell clones surviving these conditions were analyzed for protein expression of the TK-Jak1 construct (see Figure 11 A for a schematic overview). All cell clones surviving the screen did not, or to significantly lesser amount, express the TK-Jak1 fusion protein. Nevertheless, the surviving cells still contained the TK-Jak1 DNA, as revealed by PCR analysis of cellular DNA of the cell clones (Figure 11 B). The integrated cDNA contained in these cells was determined via PCR, using primers attaching to specific regions in the lentiviral construct flanking the cDNA, and subsequently sequenced. Of the cells infected with the HCMV AD169 cDNAs, 17 (61%) surviving cell clones revealed the integration of the HCMV gene *UL42* and of the cells infected with the HCMV TB40 cDNAs, 6 (38%) surviving cell clones the integration of a gene region comprising *UL73* and *UL74A* as potential HCMV antagonists of Jak1 (Table 1; please see Table 3 in the discussion for functions of integrated cDNAs of cellular origin).

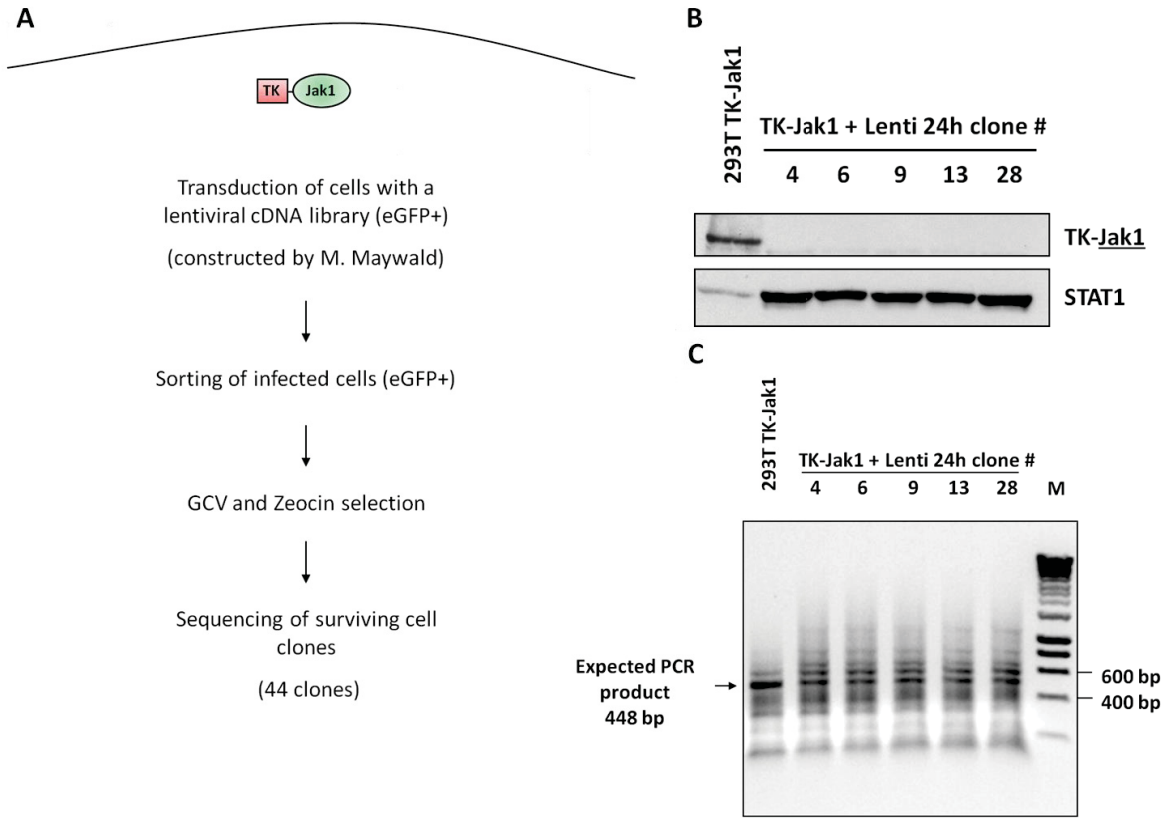

Figure 11: Global screening with lentiviral cDNAs leads to survival of cell clones expressing lower protein amounts of TK-Jak1 (A) Representation of the global screening approach using a lentiviral cDNA library. 293T TK-Jak1 cells were transduced with the lentiviral cDNA library (MOI 0.1), sorted for infected cells (eGFP+) and cultured in presence of low concentrations of GCV (0.25 µM). (B) Protein lysates of surviving cell clones were subjected to western blot analysis to determine TK-Jak1 protein amounts. (C) DNA of surviving cell clones was prepared and subjected to PCR analysis to test if TK-Jak1 DNA was retained. All experiments shown have been independently performed at least 2 times.

Table 1: Integrated lentiviral cDNA of surviving TK-Jak1 single cell clones

	Integrated cDNA	Number of surviving clones
AD169 Lentiviral cDNA (24h)	HCMV UL42	17
	SDHAF1	5
	SLC35B3	4
	ISG15 ubiquitin-like modifier	1
	Lst2	1
TB40 Lentiviral cDNA (72h)	HCMV gene region UL73-UL74A	6
	endosulphine alpha isoforrm 3	4
	CommD7	2
	HCMV TRL7	1
	PDCD5	1
	Tim8B	1
	Cop E	1

2.7 UL74A does not suffice to reduce protein amounts of Jak1

The degradative capacity of pUL74A to reduce the protein amounts of Jak1 was tested in co-transfection experiments. Therefore, *UL74A* was cloned via PCR using the DNA of surviving cell clones as template (primer pair #37 and #38) and integrated into expression plasmids. Unfortunately, the protein amounts of Jak1 were not reduced in presence of increasing amounts of pUL74A (Figure 12). Therefore, pUL74A had to be regarded as false positive screening result – see discussion for potential explanations. However, the integrated cDNA in the surviving cell clones would in principle allow the simultaneous expression of the HCMV gene *UL73*. Therefore, it is conceivable that either pUL73 or the combined expression of pUL73 and pUL74A allow for cell survival.

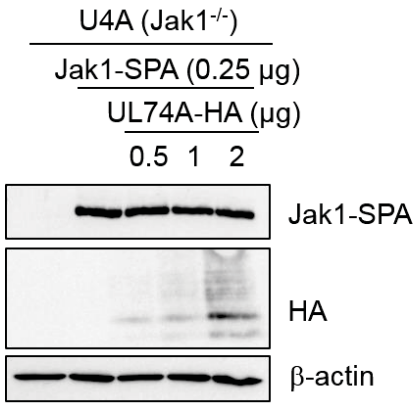

Figure 12: pUL74A alone does not convincingly reduce Jak1 protein amounts. (A) U4A (Jak1$^{-/-}$) cells were transfected with plasmids encoding Jak1-SPA and different amounts of pUL74A-HA, lysed and subjected to western blot analysis. All experiments shown have been independently performed at least 3 times.

2.8 UL42 reduces protein amounts of TK-Jak1 and Jak1 dose-dependently

To confirm the Jak1-degradative capacity of pUL42, co-transfection experiments were performed using *UL42* in conjunction with *TK-Jak1* or *Jak1*, respectively. pUL42 was indeed able to reduce protein amounts of the pTK-Jak1 and pJak1 constructs dose-dependently (Figure 13 A and B), identifying for the first time a HCMV protein that is able to reduce Jak1 protein amounts.

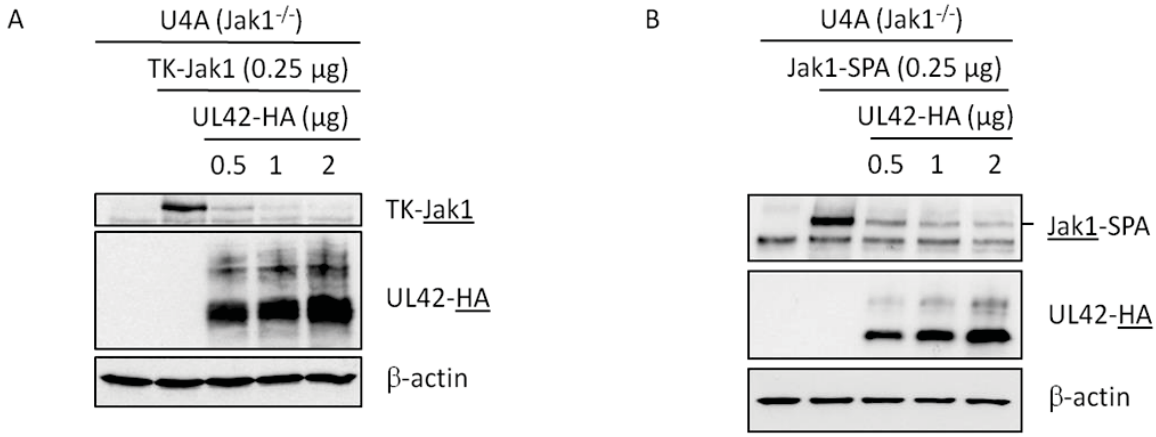

Figure 13: pUL42 reduces the protein amounts of TK-Jak1 and Jak1-SPA dose-dependently (A) U4A (Jak1$^{-/-}$) cells were transfected with plasmids encoding TK-Jak1 or (B) Jak1-SPA and different amounts of UL42-HA, lysed and subjected to western blot analysis. All experiments shown have been independently performed at least 3 times.

To test if a simultaneous expression of pUL42 and pJak1 is crucial for the antagonistic effect, transfection order was altered. The capacity of pUL42 to reduce pJak1 protein amounts was

even more pronounced (Figure 14). Even trace amounts of pUL42 were able to reduce large quantities of pJak1 (Figure 14, lane 4). When *UL42* was transfected 1 day prior to *Jak1* transfection, pJak1 even became virtually undetectable in immune blot analysis (Figure 14, lane 5).

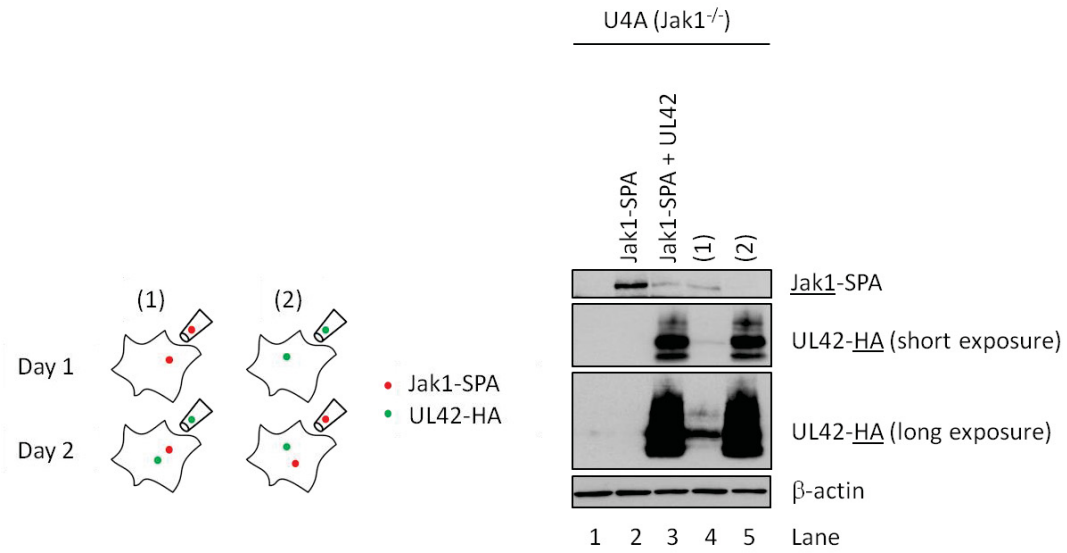

Figure 14: pUL42 reduces the protein amounts of Jak1-SPA irrespective of transfection order U4A cells were transfected with plasmids encoding either Jak1-SPA or UL42-HA, 24 h later the same cells were transfected with the other plasmid, lysed and subjected to western blot analysis. All experiments shown have been independently performed at least 3 times.

2.9 The antagonistic capacity of pUL42 does not affect untransfected bystander cells

To investigate possible effects of pUL42 on bystander cells, cells were transfected separately with a single plasmid (either expressing pJak1-SPA or pUL42) and co-cultured in different combinations the following day (Figure 15). An effect of pUL42 on the pJak1 amounts in neighboring cells did not become evident under the herein used conditions, since there was no reduction in Jak1 protein amounts when pJak1-SPA cells shared the same cell culture dish and cell culture media with pUL42-expressing cells (Figure 15, lane 5), in contrast to the above-mentioned experiments in which both proteins were expressed in the same cell (see 2.10).

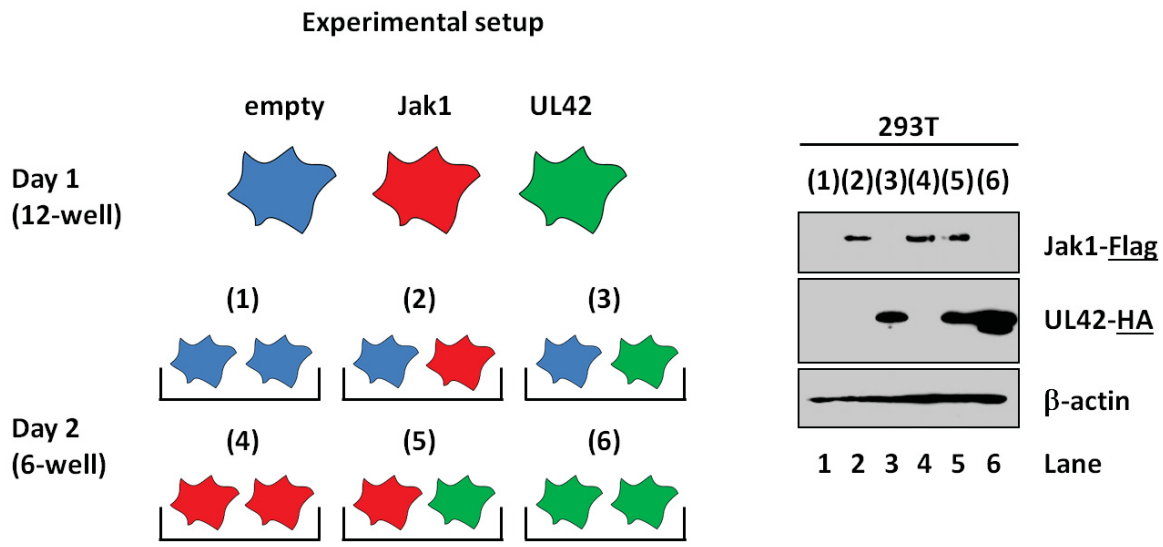

Figure 15: The antagonistic capacity of pUL42 is restricted to pUL42-expressing cells. 293T cells were transfected with control plasmid pFRTZ Tomato (allowing evaluation of transfection efficiency), *Jak1-SPA* and *UL42-HA* (1 µg), respectively. 24h post transfection cell populations were (re-)seeded together in depicted combinations. Cells were lysed 24 h after (re-)seeding and subjected to western blot analysis. All experiments shown have been independently performed at least 3 times.

2.10 HCMV pUL42 is subjected to complex N-glycosylation in the Golgi apparatus

Ectopic expression of pUL42-HA showed a smear with higher molecular weight in SDS-PAGE (see Figure 13 and 14, HA-Blot). We therefore speculated if pUL42 might be glycosylated. Bioinformatic characterization of the predicted pUL42 primary amino acid (aa) sequence revealed that the protein contains a transmembrane domain segment (TMD) and a unique N-glycosylation target site localized C-terminally to the TMD but no signal sequence - consistent with a type II transmembrane topology according to the classification of Goder and Spiess (Goder and Spiess, 2001) (Figure 16 A). To characterize the protein and subsequently get information concerning its cellular trafficking, cell lysates were treated with the two specific endoglycosidases Endo H and PNGase F (Figure 16 B) which cleave high mannose structures or all N-linked oligosaccharides, respectively. pUL42 was found to be resistant to Endo H digestion, but sensitive towards treatment with PNGase F, indicating complex glycan structures to be acquired in the ER and subsequently modified in the Golgi leading to Endo H resistance. The viral protein contains only one predicted N-glycosylation site (NxT 115-117). A mutant was constructed (pUL42 N115A) by side-directed mutagenesis disrupting the potential N-glycosylation site (NIT to AIT). Indeed, the mutated pUL42 did not show any glycosylation in whole cell lysates in comparison with the non-mutated pUL42 (Figure 16 C). Consistently, pUL42 was denominated glycoprotein (gp)UL42 from this time point on.

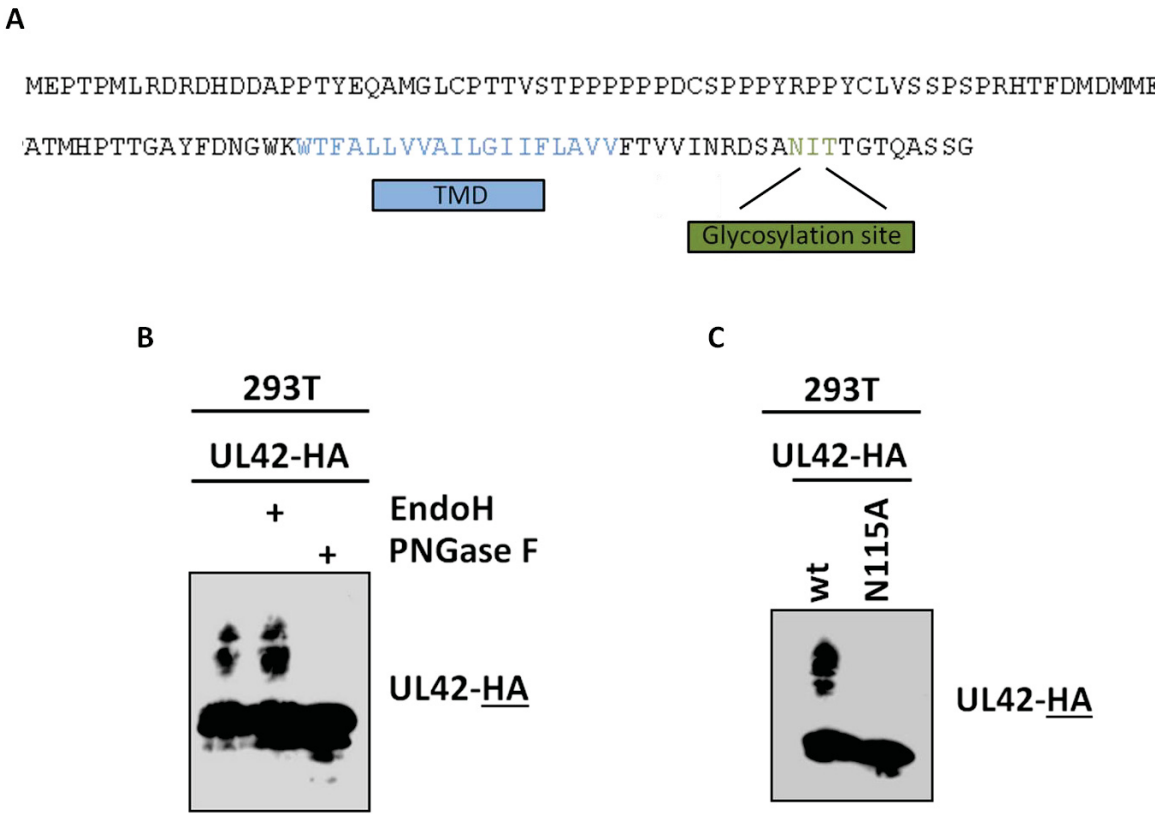

Figure 16: HCMV pUL42 is subjected to complex N-glycosylation in the Golgi apparatus. (A) Amino acid sequence of HCMV pUL42. The transmembrane domain and the N-glycosylation site are highlighted (blue and green, respectively). (B) Cells were transfected with *UL42* and whole cell lysates were prepared. Cell lysates were treated with Endo H or PNGase F for 16 h and subjected to western blotting. (C) A mutated version of pUL42 was constructed via site-directed mutagenesis (UL42 N115A). Cells were transfected with wt- or mutant-UL42 for 24 h before whole cell lysates were prepared and subjected to western blot analysis. All experiments shown have been independently performed at least 3 times.

2.11 gpUL42 is a surface protein

In-silico prediction of the amino acid sequence of gpUL42 revealed a transmembrane domain (TMD) upstream of the C-terminal N-glycosylation site. As the epitope-tag (HA) is located C-terminally of the TMD segment, cytometry analysis of transfected cells was performed to determine whether gpUL42 is located on the cell surface with the HA-tag oriented outside of the cell (Figure 17). Transfected cells were stained with antibodies against STAT2 (a strictly intracellular protein lacking TMD segments) and HA, treating half of these cells with saponin to permeabilize the cellular surface membrane. Cells treated with saponin were stained double-positive for STAT2 and HA. Interestingly, when the cellular membrane was not permeabilized, cells remained positive for gpUL42 (HA-positive) but negative for the intracellular protein STAT2, indicating that the cell integrity was retained throughout the experimental procedure and thus that gpUL42 is located on the cell surface membrane (Figure 17). A similar staining pattern became evident upon transfection of an *UL74A*-expressing plasmid, a known HCMV type II transmembrane protein (see UniProt [C8CPG5]), resulting in a comparable staining pattern.

Taken together, the experimental data are consistent with a typical type II transmembrane topology (as classified by Goder and Spiess (Goder and Spiess, 2001)).

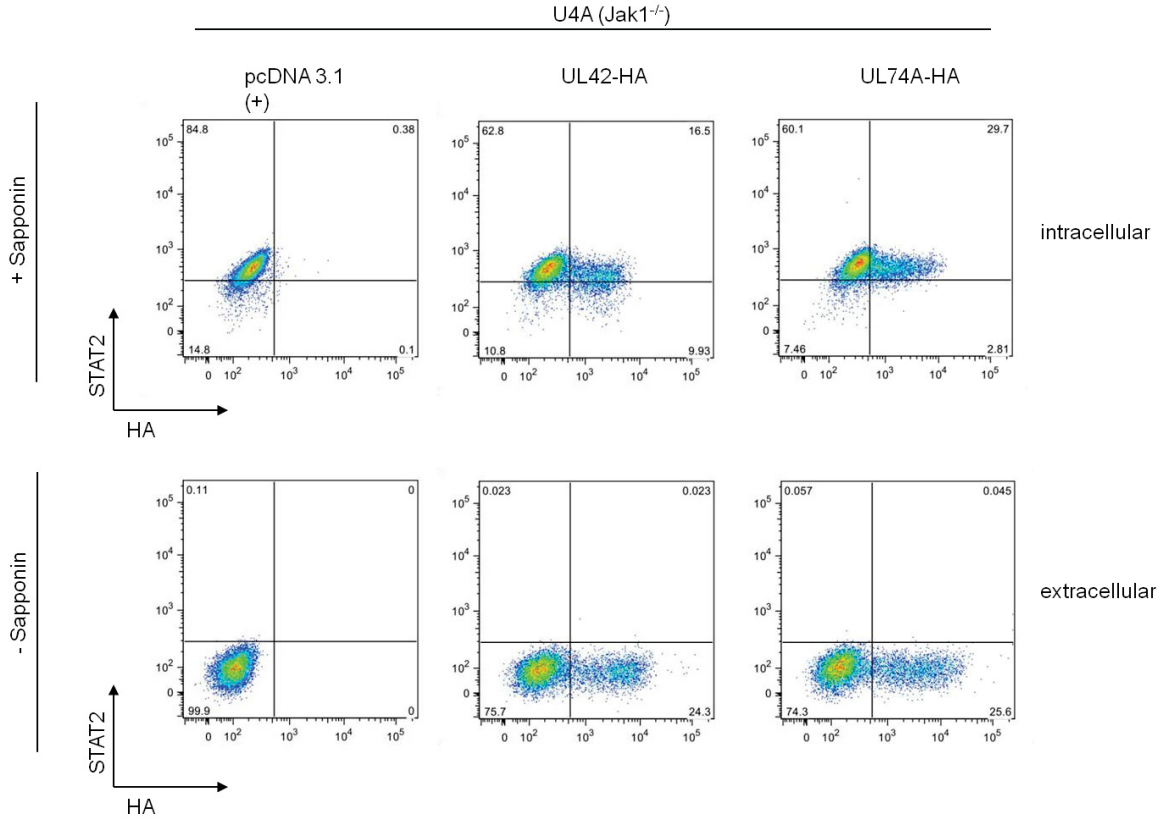

Figure 17: gpUL42 is located in the cellular surface membrane. Cells were transfected with control plasmid, gpUL42- or gpUL74A-expressing plasmids for 24 h. Half of the cells were permeabilized with saponin (upper panel) to form pores in the cellular membrane, followed by incubation with antibodies against STAT2 (a strictly intracellular protein) and HA for 1 h. After incubation with fluorochrome-labelled secondary antibodies for 1 h, cells were analyzed via cytometry (see Materials and Methods section). All experiments shown have been independently performed at least 3 times.

2.12 gpUL42 reduces Jak1 amounts post-transcriptionally

Northern blot analysis of cellular RNA was performed to determine if the antagonistic capacity of gpUL42 is operative on transcriptional or post-transcriptional levels. Co-transfection of gpUL42- together with Jak1-expressing plasmids did not decrease mRNA amounts of Jak1 (Figure 18). RNA levels of Jak1 even seem to be slightly elevated with increasing amounts of gpUL42. Thus, the antagonistic capacity of gpUL42 seems to be elicited on a step beyond transcription. Also, a reduction of the mRNA stability is rather unlikely after this experiment.

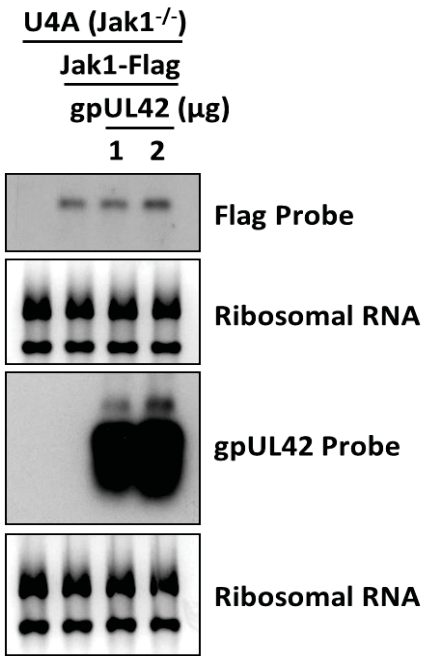

Figure 18: gpUL42 reduces Jak1 amounts post-transcriptionally. Cells were transfected with pJak1-, and different amounts of gpUL42-expressing plasmid. Cellular RNA was isolated and subjected to Northern blot analysis, detecting RNA with specific probes for the SPA-tag or gpUL42 (see Materials and Methods section for details), respectively. All experiments shown have been independently performed at least 3 times.

2.13 Translation of gpUL42 is essential for the negative effect on Jak1

As HCMV encodes also for multiple miRNAs and several abundantly expressed long non-coding RNAs (Kim et al., 2015; Stern-Ginossar et al., 2012), it had to be determined if gpUL42-mediated Jak1 protein reduction was indeed mediated by translated gpUL42. Therefore, two gpUL42 frame-shift mutants were generated, one mutant harboring a frameshift mutation after the ATG starting at position 18, the other mutant was intentionally mutated at the ATG at position 66. The *UL42* frameshift mutated within ATG 66 lost detectable protein expression. Consistent with the notion that translation of gpUL42 is a prerequisite for Jak1 reduction, Jak1 amounts remained stable despite co-transfection of this mutated *UL42* sequence, in contrast to the Jak1 protein reduction observed upon co-transfection of wt *UL42* (Figure 19, lane 3 and 5). Surprisingly, *UL42* frameshift mutation within ATG 18 did not abrogate protein expression, since a smaller gpUL42 protein form was expressed (Figure 19, lane 4), presumably initiated from a downstream ATG and thus devoid of the N-terminus. Interestingly, this truncated gpUL42 protein form was impaired in its propensity to reduce Jak1 protein amounts, suggesting the existence of functionally relevant domains within the N-terminus of gpUL42.

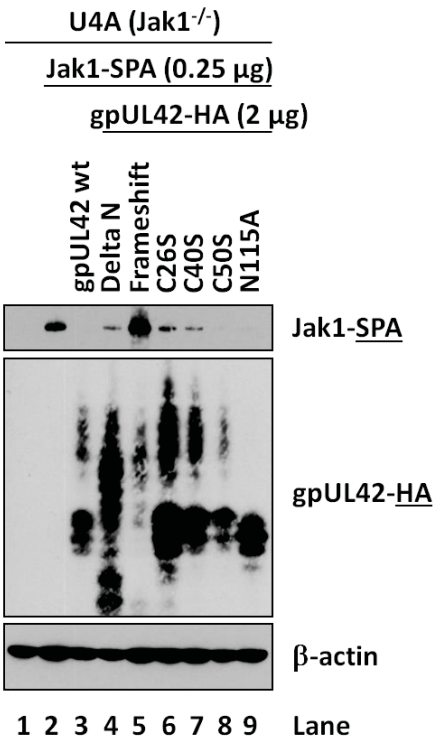

Figure 19: Jak1 protein levels are partially restored upon co-transfection of certain *UL42* mutants. Specific gpUL42 mutants (delta N [see text], frameshift, C26S, C40S, C50S and N115A) were constructed via site-directed mutagenesis (see Materials and Methods section). Cells were (co-) transfected with Jak1- and mutated gpUL42-expression plasmids for 24 h. Cells were lysed and subjected to western blot analysis. All experiments shown have been independently performed at least 3 times.

MCMV pM27-mediated degradation of STAT2 is crucially dependent on three cysteines comprising a zinc-finger motif required for pM27 interaction with DDB1 (Trilling et al., 2011). Therefore, three cysteine (C) mutants of gpUL42 were generated (gpUL42-C26S, gpUL42-C40S and gpUL42-C50S, respectively) in which the cysteines were mutated to serines (S), as serine is the closest replacement to cysteine, and their contribution to Jak1 protein reduction was assessed (Figure 18 lane 6-8). One of the cysteines was dispensable for Jak1 reduction (C50) as the gpUL42-C50S mutant was still able to efficiently reduce Jak1 protein amounts. However, the two other cysteines expressed in gpUL42 (C26 and C40) seem to contribute to Jak1 protein reduction, as a partial rescue of Jak1 protein levels was observed. The cysteine mutated gpUL42 proteins were similarly expressed compared to the protein levels of non-mutated gpUL42.

The N-glycosylation does not seem to have an effect on the antagonistic capacity of gpUL42 towards Jak1 (Figure 19 lane 9). The protein amount of Jak1 was still reduced when co-transfecting Jak1 together with the N-glycosylation-incompetent gpUL42. This effect was equal to non-mutated gpUL42.

2.14 The conserved tyrosine Y45 is important for Jak1 protein reduction

Throughout different HCMV strains gpUL42 is highly conserved with only minor sequence differences in the C-terminus (Figure 20). Interestingly, a conserved proline-rich PPPY-protein motif was revealed aligning the homologous gpUL42 proteins of different simian CMVs (Figure 21).

```
pUL42_refseq        MEPTPMLRDRDHDDAPPTYEQAMGLCPTTVSTPPPPPPDCSPPPYRPPYCLVSS
pUL42_Han1          MEPTPMLRDRDHDDAPPTYEQAMGLCPTTVSTPPPPPPDCSPPPYRPPYCLVSS
pUL42_TB40e         MEPTPMLRDRDHDDAPPTYEQAMGLCPTTVSTPPPPPPDCSPPPYRPPYCLVSS
pUL42_PAV21         MEPTPMLRDRDHDDAPPTYEQAMGLCPTTVSTPPPPPPDCSPPPYRPPYCLVSS
pUL42_Han13         MEPTPMLRERDHDDAPPTYEQAMGLCPTTVSTPPPPPPDCSPPPYRPPYCLVSS
pUL42_VR1814        MEPTPMLRERDHDDAPPTYEQAMGLCPTTVSTPPPPPPDCSPPPYRPPYCLVSS
pUL42_JP            MEPTPMLRDRDHDDAPPTYEQAMGLCPTTVSTPPPPPPDCSPPPYRPPYCLVSS
pUL42_BE_28_2010    MEPTPMLRDRDHDDAPPTYEQAMGLCPTTVSTPPPPPPDCSPPPYRPPYCLVSS
                    ********:**********************************************

pUL42_refseq        PSPRHTFDMDMMEMPATMHPTTGAYFDNGWKWTFALLVVAILGIIFLAVVFTVV
pUL42_Han1          PSPRHTFDMDMMEMPATMHPTTGAYFDNGWKWTFALLVVAILGIIFLAVVFTVV
pUL42_TB40e         PSPRHTFDMDMMEMPATMHPTTGAYFDNGWKWTFALLVVAILGIIFLAVVFTVV
pUL42_PAV21         PSPRHTFDMDMMEMPATMHPTTGAYFDNGWKWTFALLVVAILGIIFLAVVFTVV
pUL42_Han13         PSPRHTFDMDMMEMPATMHPTTGAYFDNGWKWTFALLVVAILGIIFLAVVFTVV
pUL42_VR1814        PSPRHTFDMDMMEMPATMHPTTGAYFDNGWKWTFALLVVAILGIIFLAVVFTVV
pUL42_JP            PSPRHTFDMDMMEMPATMHPTTGAYFDNGWKWTFALLVVAILGIIFLAVVFTVV
pUL42_BE_28_2010    PSPRHTFDMDMMEMPATMHPTTGAYFDNGWKWTFALLVVAILGIIFLAVVFTVV
                    ************************************************************

pUL42_refseq        INRDSANITTGT
pUL42_Han1          INRDNTNITTGTQASTSG
pUL42_TB40e         INRDSANTTTGVSSSSG
pUL42_PAV21         INRDSANTTTGVSSASSG
pUL42_Han13         INRDSANTTTGVSSASSG
pUL42_VR1814        INRDSANTTTGVSSSSG
pUL42_JP            INRDSSNTTTGRQST
pUL42_BE_28_2010    INRDNSTATGTSSSSG
                    ****.:. *
```

Figure 20: Alignment of gpUL42 sequences of different HCMV strains. Protein sequences of gpUL42 were aligned using Clustal Omega web tool. Asterisks (*) indicate fully conserved residues, colons (:) indicate groups with strongly similar properties, periods (.) indicate groups with weakly similar properties.

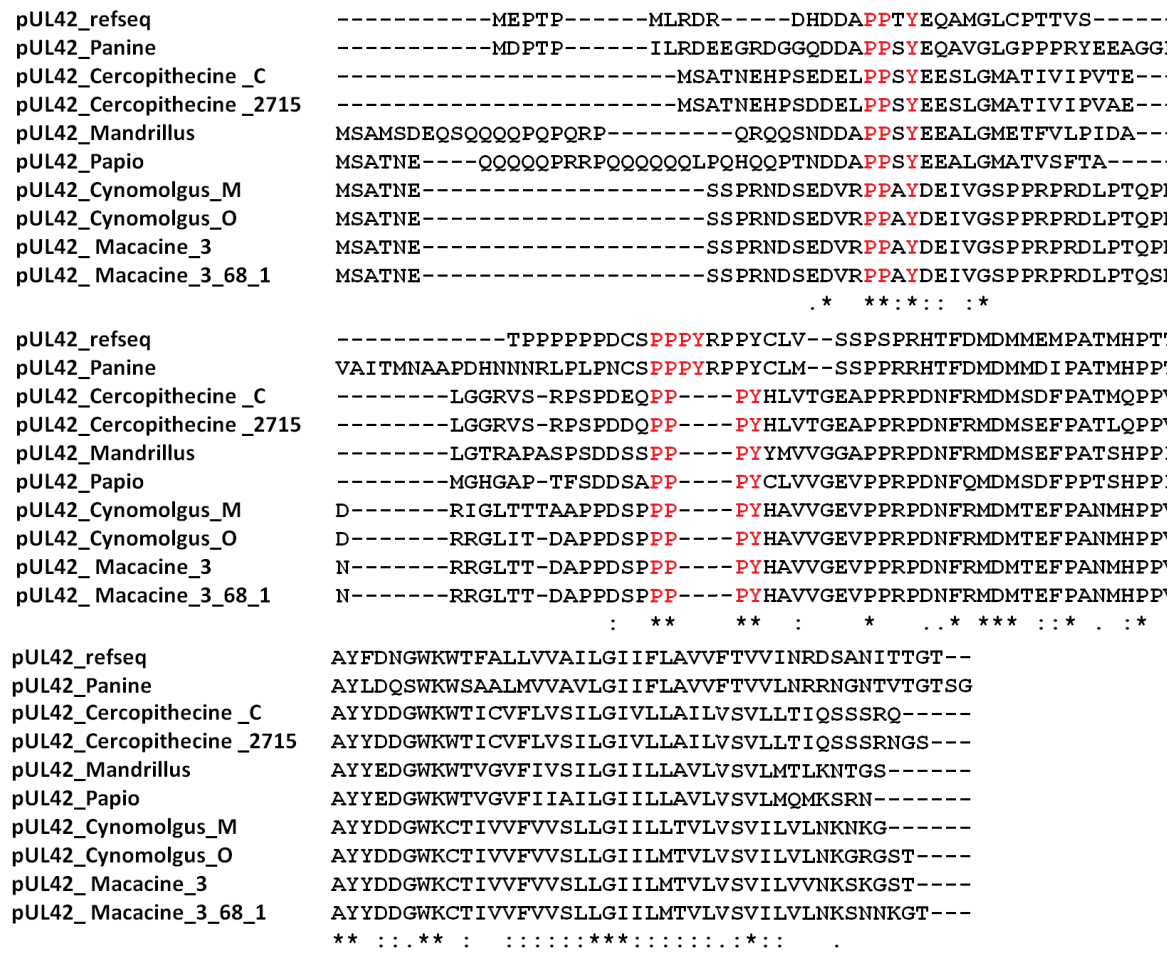

Figure 21: Alignment of gpUL42 sequences of different simian CMVs. Protein sequences of gpUL42 were aligned using Clustal Omega web tool. The two conserved PPXY motifs are highlighted in red. Asterisks (*) indicate fully conserved residues, colons (:) indicate groups with strongly similar properties, periods (.) indicate groups with weakly similar properties.

To test if the conserved stretch is the consequence of purifying selection operating on a functionally relevant part of the protein, the codon for tyrosine (Y) at position 45 of this motif was mutated into a codon for alanine (A) to assess the importance of this domain for the reduction of Jak1. Interestingly, the single Y→A mutation in this PPPY motif (gpUL42-Y45A) rescued Jak1 protein amounts in co-transfection experiments (Figure 22), i. e. protein amounts of Jak1 were comparable as in the cells only transfected with Jak1-expressing plasmid. This was not due to a difference in gpUL42 protein amount, as both gpUL42 proteins were similarly expressed.

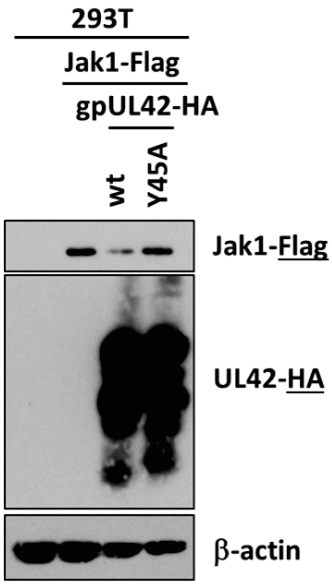

Figure 22: The conserved tyrosine Y45 is important for Jak1 protein reduction. Cells were transfected with *Jak1* and wt-*UL42* or *UL42*-Y45A mutant for 24 h. Cell lysates were prepared and subjected to western blot analysis. All experiments shown have been independently performed at least 3 times.

2.15 No indication for gpUL42-dependent Jak1 protein reduction via autophagy

In an attempt to probe into the mechanism by which gpUL42 reduces the protein amounts of Jak1, different drugs were used to inhibit major cellular degradation pathways, namely autophagy (3-methyl adenine [3-MA] and Wortmannin), endosomal-lysosomal degradation (Bafilomycin A) or proteasomal degradation (MG-132). Both drugs (3-MA and Wortmannin) efficiently decreased the protein amounts of phosphorylated Akt – which is a well-known consequence of inhibited autophagy (Noguchi et al., 2014) – indicating that the pharmacological inhibition was efficient (Figure 23 A and B). gpUL42 decreased Jak1 amounts despite the presence of 3-MA treatment (Figure 23 A). A similar pattern arose when cells were treated with Wortmannin (Figure 23 B). Treatment with Wortmannin did also not lead to a restoration of Jak1 protein in presence of gpUL42. Hence, autophagy-mediated degradation of Jak1 by gpUL42 has to be considered unlikely.

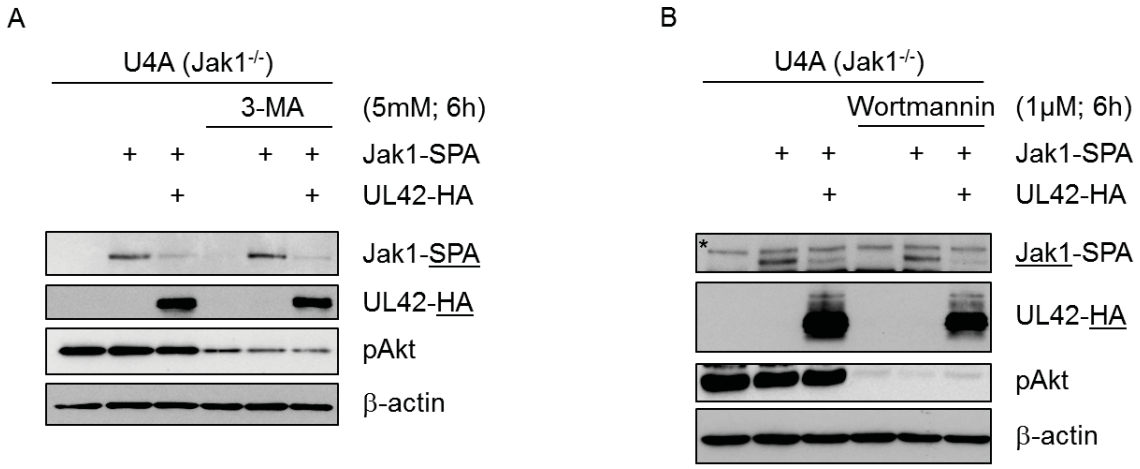

Figure 23: gpUL42-dependent Jak1 protein reduction occurs despite inhibited autophagy. (A) Cells were transfected with pJak1- and gpUL42-expressing plasmids overnight and treated with 3-MA (5 mM) for 6 h. Whole cell lysates were prepared and subjected to western blot analysis. (B) Cells were prepared as in (A) but treated with Wortmannin (1 μM) instead of 3-MA. Western blot analysis was performed with cell lysates. The asterisk indicates an unspecific band detected by the Jak1 antibody. All experiments shown have been independently performed at least 3 times.

2.16 The proteasome controls Jak1 amounts in uninfected cells

Two other important pathways involved in protein degradation are the proteasomal and the endosomal pathway. Use of the proteasomal inhibitor MG-132 increases the protein amounts of transfected Jak1 irrespective of gpUL42 expression (Figure 24 A), consistent with the notion that Jak1 turnover is dependent on the proteasomal degradation of Jak1 by SOCS proteins (Boyle et al., 2009). This effect was found to be specific for Jak1 as the STAT2 protein amounts were if at all decreased upon MG-132 treatment (Figure 24 A). The strong effect of MG-132 on Jak1 amounts in cells in absence of HCMV infection or gpUL42 compromises an unambiguous interpretation of the results concerning the contribution of the proteasome to gpUL42-mediated Jak1 protein reduction. Treatment of cells with MG-132 did not lead to an increase of Jak1 protein amounts in the presence of gpUL42 which was more pronounced than the increase in untreated or control-treated cells. The increase of Jak1 amounts in MG-132-treated cells was again observed (Figure 24 B, lane 5). Interestingly, the amounts of gpUL42 seemed to slightly decrease, when the cells were treated with MG-132 in comparison with untreated cells.

Treating the cells with the endosomal inhibitor Bafilomycin A1 also led to an increase in Jak1 protein amounts (Figure 24 C, lane 5). Jak1 amounts in the gpUL42 co-transfected cells (Figure 24 C, lane 6) were also slightly increased however it is hard to distinguish if this observed increase is larger than in lane 5. In contrast to the treatment with MG-132, Bafilomycin A1 led also to an increase in gpUL42 protein amounts. The observed increases in protein amounts of Jak1 upon treatment with proteasomal or endosomal inhibitors impedes a convincing interpretation of an observed recovery of Jak1, especially in the Bafilomycin A1 treated cells (Figure 24 C, lane 6). However, there is evidence that tyrosine-phosphorylated Jak1 can be found in endosomal compartments shortly after interferon stimulation, together with the interferon receptor chains (Payelle-Brogard and Pellegrini, 2010).

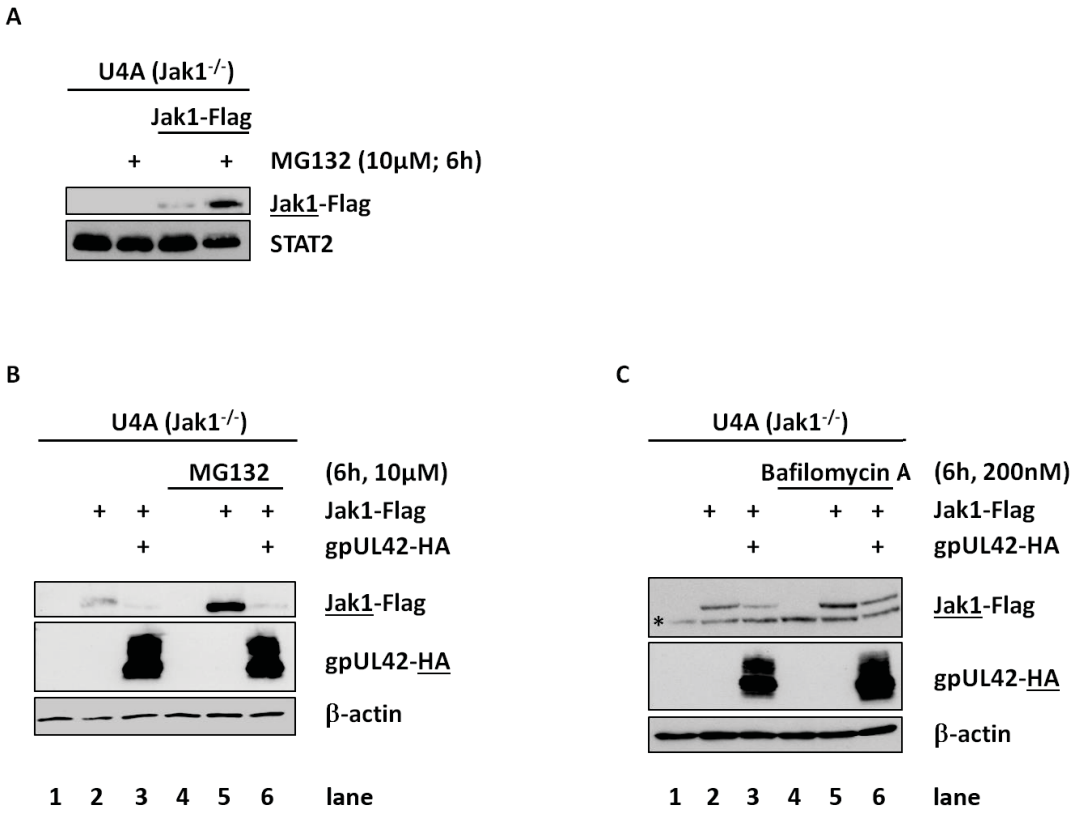

Figure 24: The proteasome controls Jak1 amounts irrespective of gpUL42 expression. (A) Cells were transfected with Jak1 overnight and treated with MG-132 for 6 h. Whole cell lysates were prepared and subjected to western blot analysis. (B) Cells were transfected with Jak1- and gpUL42-expressing plasmids overnight and treated with MG-132 for 6 h. Whole cell lysates were prepared and subjected to western blot analysis. (C) Cells were transfected as in (B) but treated with Bafilomycin A instead of MG-132. Western blot analysis was performed with the cell lysates. The asterisk indicates an unspecific band recognized by the Jak1 antibody. All experiments shown have been independently performed at least 3 times.

2.17 Overexpression of gpUL42 affects several co-transfected proteins

Since HCMV is able to degrade various proteins during infection (Hwang and Kalejta, 2007; Le et al., 2008a; Miller et al., 1998), the capability of gpUL42 to antagonize other proteins was investigated (Figure 25). gpUL42 was able to reduce protein amounts of death-domain associated protein (Daxx) (Figure 25 B) in co-transfection experiments. Daxx is located both in the nucleus as well as in the cytosol. Also, protein amounts of pUS6, a HCMV-encoded ER-resident MHC class I antagonist (Ahn et al., 1997; Hengel et al., 1997; Jun et al., 2000), were reduced upon co-transfection of gpUL42 (Figure 25 C). In co-transfection experiments, gpUL42 was also able to reduce protein amounts of STAT2 (Figure 25 A), however, this might constitute an artificial effect based on artificial (over-) expression since HCMV mutants lacking gpUL42 are still able to reduce STAT2 amounts as wt-virus (see below).

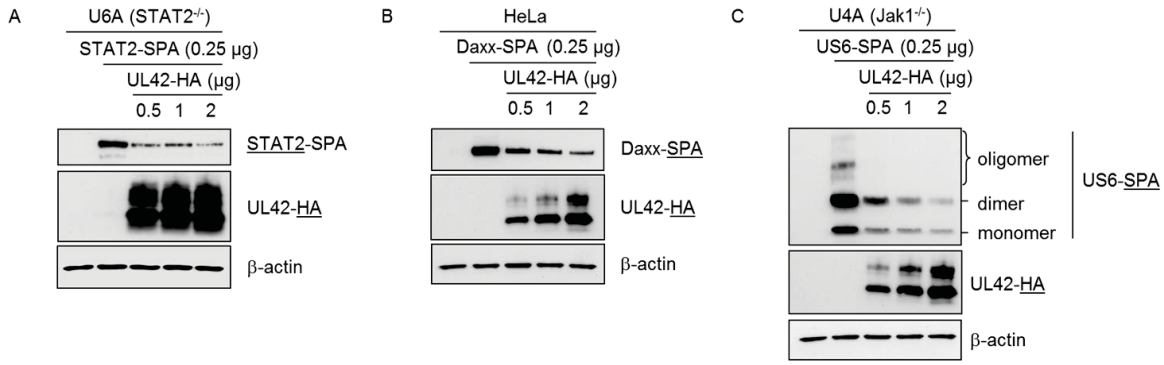

Figure 25: Co-transfection of gpUL42-expressing plasmids reduces protein amounts of various other co-transfected proteins. Indicated cell lines were co-transfected with (A) STAT2-, (B) hDaxx- or (C) pUS6-expression plasmids and indicated amounts of gpUL42-expression plasmids for 24 h. Whole cell lysates were prepared and subjected to western blotting. All experiments shown have been independently performed at least 3 times.

2.18 HCMV mutants lacking the ability to express gpUL42 exhibit reduced and protracted Jak1 reduction

As the co-transfection of gpUL42 and Jak1 leads to reduction of Jak1 protein amounts, an HCMV double gene knockout lacking the genes *UL42* and *UL43* was tested for its ability to reduce Jak1 protein amounts during infection. Upon infection with wt-HCMV, Jak1 protein amounts were strongly reduced 24 h p.i. as compared to uninfected MRC-5 cells (Figure 26 A, please compare lanes 1 and 2). The double knockout virus lacking the capacity to express gpUL42 (and pUL43) failed to reduce Jak1 amounts as efficient as wt-HCMV (Figure 26 A, lane 3). However, at 48 h p.i., Jak1 protein amounts were also reduced upon infection with Δ*UL42-UL43*-HCMV suggesting that further Jak1 down-regulating functions of HCMV must exist. HCMV-mediated STAT2 degradation is known to occur later than Jak1 degradation (Le et al., 2008a), therefore only a partial reduction of STAT2 protein can be observed after 48 h p.i., however the STAT2 protein levels are comparable in the wt- and Δ*UL42-UL43* virus (Figure 26 A, please compare lanes 5 and 6). The viral protein pp72 was expressed similarly in both viruses.

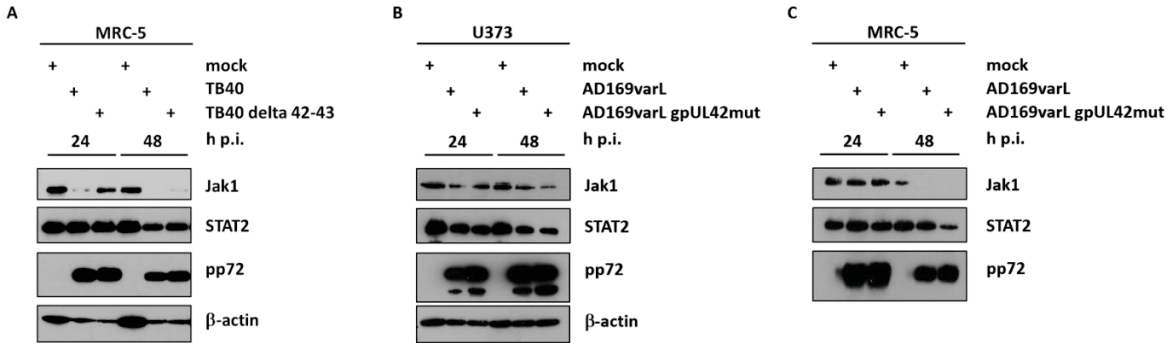

Figure 26: Jak1 degradation is less effective in HCMV delta *UL42-UL43*. MRC-5 fibroblasts and U373 cells were infected with (A) wt-HCMV$_{TB40}$ or ΔUL42-43-HCMV$_{TB40}$ or (B) and (C) HCMV$_{AD169varL}$ wt or HCMV$_{AD169varL}$ gpUL42mut (generated by Dr. Vu Thuy Khanh Le-Trilling) for the indicated time points. Whole cell lysates were prepared and subjected to western blot analysis. All experiments shown have been independently performed at least 3 times.

2.19 gpUL42mut-HCMV$_{AD169varL}$ has comparable growth characteristics as wild-type HCMV in absence of IFN

Based on background of strain AD169 *varL* HCMV (called BAC2, described in (Le et al., 2011)), Dr. Vu Thuy Khanh Le-Trilling generated an HCMV mutant harboring a frame-shift mutation of the gene *UL42* (gpUL42mut-HCMV$_{AD169varL}$). To assess if the gpUL42mut-HCMV$_{AD169varL}$ virus exhibits growth differences in comparison with wt-HCMV$_{AD169varL}$ virus, foci titration assay with both viruses was performed. gpUL42mut-HCMV$_{AD169varL}$ virus showed similar growth kinetics as the wt-HCMV$_{AD169varL}$ virus (Figure 27); gpUL42mut-HCMV$_{AD169varL}$ virus reached even slightly higher titers after 6 and 9 d p.i. than the wt-HCMV$_{AD169varL}$ virus.

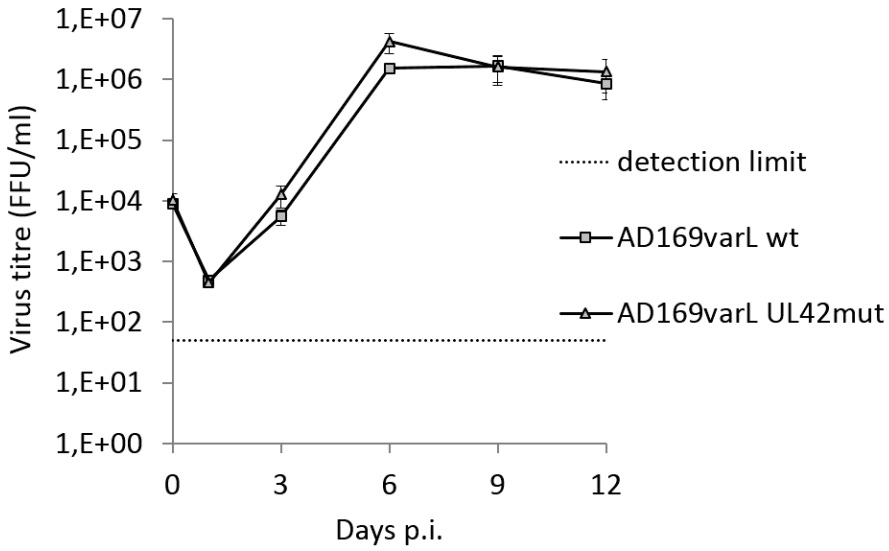

Figure 27: gpUL42mut-HCMV$_{AD169varL}$ has comparable growth characteristics as wt-HCMV$_{AD169varL}$. MRC-5 fibroblasts were infected with gpUL42mut- or wt-HCMV$_{AD169varL}$ (MOI 0.3) and cells were frozen at -80°C at the indicated time points. Viral titers were assessed by foci titration assay on MRC-5 cells followed by AEC staining after 96 h of infection. DL, detection limit; FFU, focus forming units. All experiments shown have been independently performed at least 2 times.

2.20 gpUL42mut-HCMV$_{AD169varL}$ and wt-HCMV$_{AD169varL}$ are susceptible towards IFN-γ pre-treatment

The effect of IFN on viral growth could be elucidated upon pre-treatment of MRC-5 fibroblast with IFNs (Figure 28). Pre-treatment of cells with IFN-α lead to only slightly decreased viral titers (~ 1 x $\log_{10}$) until day 9 p.i. in both viruses (Figure 28 A). From this day on, both viruses reached similar viral titers comparable to titers in untreated cells. However, IFN-γ pre-treatment decreased viral titers of both gpUL42mut- HCMV$_{AD169varL}$ and wt-HCMV$_{AD169varL}$ up to 12 days p.i (Figure 28 B). Viral titers were reduced for up to 2.5 x $\log_{10}$. The decrease in viral growth was comparable between gpUL42mut- HCMV$_{AD169varL}$ and wt-HCMV$_{AD169varL}$.

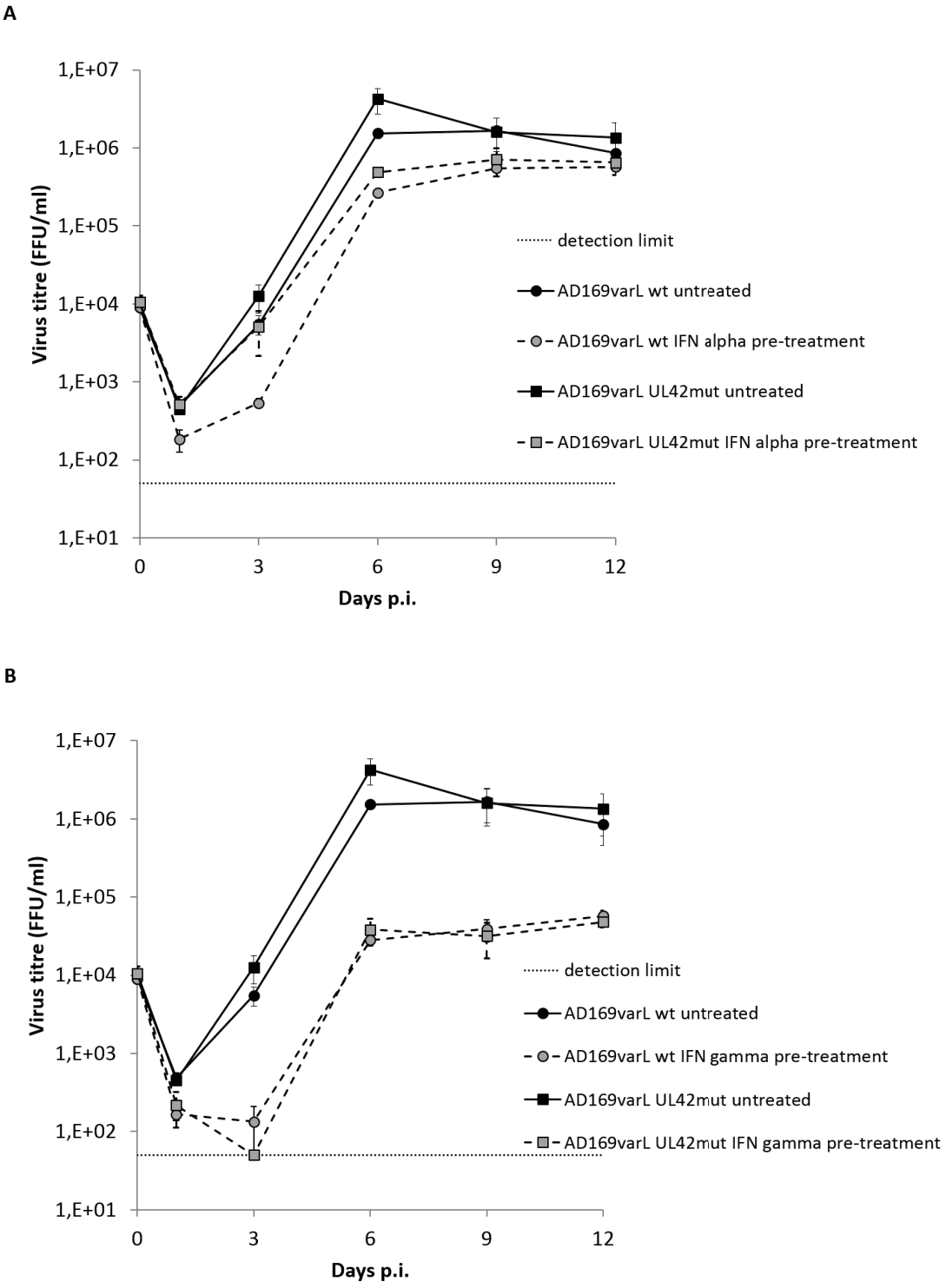

Figure 28: gpUL42mut- and wt-HCMV$_{AD169varL}$ are susceptible towards IFN-γ pre-treatment. MRC-5 fibroblasts were infected with gpUL42mut- or wt-HCMV$_{AD169varL}$ (MOI 0.3) and cells were frozen at -80°C at the indicated time points. To assess the effect of IFN on viral growth properties, cells were pre-treated with (A) IFN-α or (B) IFN-γ (500 U/ml) 24h prior to infection. Viral titers were assessed by foci titration assay on MRC-5 cells followed by AEC staining after 96 h of infection. DL, detection limit; FFU, focus forming units. All experiments shown have been independently performed at least 2 times.

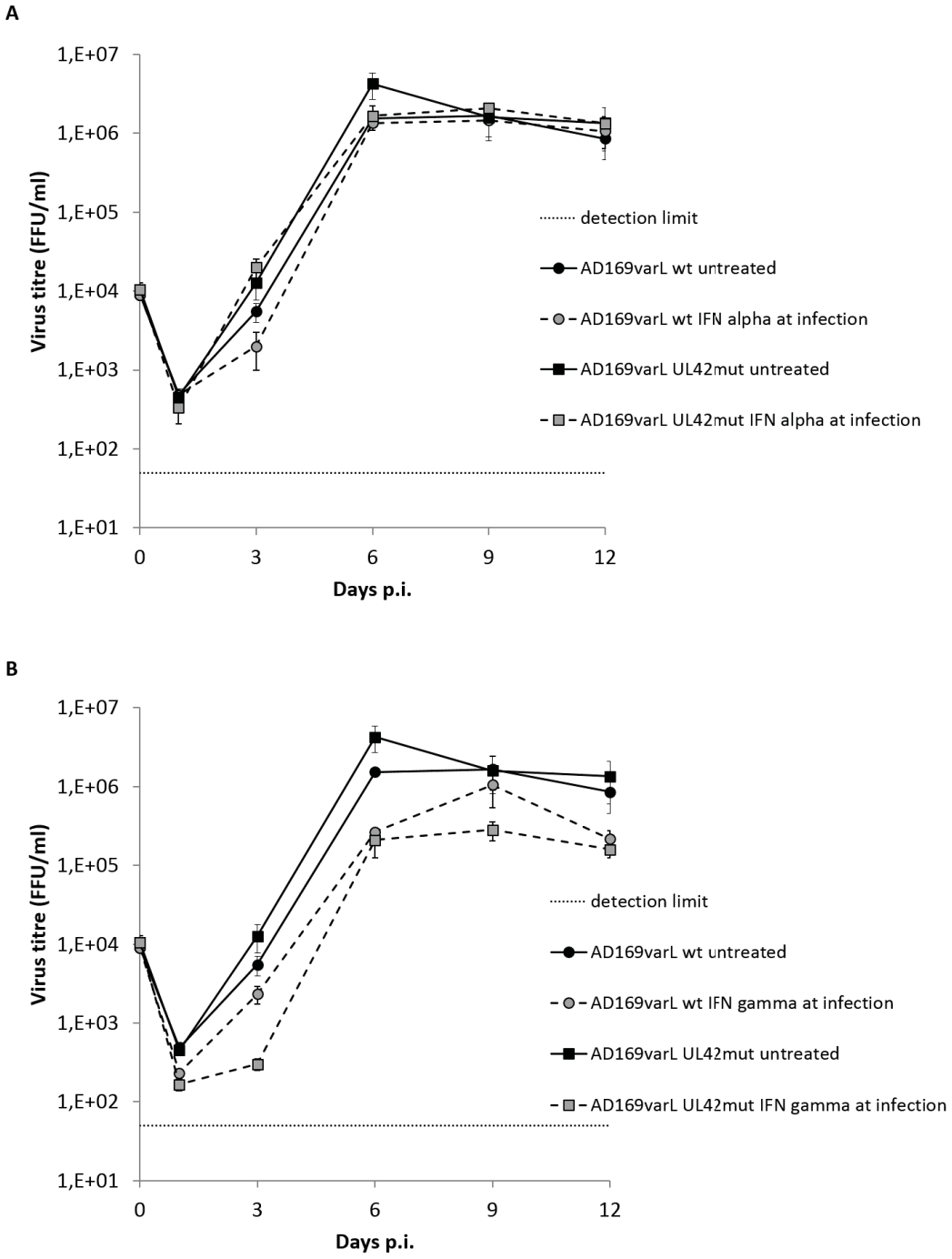

Figure 29: In the first 3 days of infection, gpUL42mut-HCMV$_{AD169varL}$ is slightly more susceptible towards IFN-γ treatment at the time of infection. MRC-5 fibroblasts were infected with gpUL42mut- or wt-HCMV$_{AD169varL}$ (MOI 0.3) and cells were frozen at -80°C at the indicated time points. To assess the effect of IFN on viral growth properties, cells were treated with (A) IFN-α or (B) IFN-γ (500 U/ml) at the time of infection. Viral titers were assessed by foci titration assay on MRC-5 cells followed by AEC staining after 96 h of infection. DL, detection limit; FFU, focus forming units. All experiments shown have been independently performed at least 2 times.

2.21 gpUL42mut-HCMV$_{AD169varL}$ is more susceptible towards IFN-γ treatment in the first 3 days than wt-HCMV$_{AD169varL}$

Increased protein amounts of Jak1 in gpUL42mut-HCMV$_{AD169varL}$ (Figure 26) could elicit an increased anti-viral effect upon IFN treatment. Therefore, viral replication of gpUL42mut- and wt-HCMV$_{AD169varL}$ upon IFN treatment was investigated. IFN treatment of MRC-5 cells at the time of infection did not elicit an inhibition of viral growth in either of the two viruses over the complete replication kinetics of 12 days (Figure 29). Treatment of MRC-5 cells with IFN-α at the time of infection lead to identical viral titers compared to untreated cells in gpUL42mut- or wt-HCMV$_{AD169varL}$-infected cells (Figure 29 A). Treatment of cells with IFN-γ was slightly more efficient in reducing the viral load, however only led to a 10-fold reduction of virus progeny (Figure 29 B). Notably, there was a difference in viral titers comparing the two different viruses in the first 3 days of viral growth. gpUL42mut-HCMV$_{AD169varL}$ was reduced in viral titers by about 42-fold (in comparison to untreated gpUL42mut-HCMV$_{AD169varL}$) whereas wt-HCMV$_{AD169varL}$ titers were only reduced 2.5-fold. At later time points, the growth kinetics of both viruses was identical.

3. Discussion

3.1 HCMV gpUL42 was identified as a Jak1 antagonist using a novel screening system

Many viruses, including HCMV, use viral proteins to initiate the degradation of host restriction factors to evade cellular interferon signaling (see Table 2). HCMV is relatively IFN resistant as it is able to degrade Jak1, STAT2 and IRF9 (Le et al., 2008a; Miller et al., 1998; Miller et al., 1999), proteins crucial for an effective IFN signal transduction. The viral genes responsible for the degradation of Jak1 and STAT2 have not been identified so far, therefore a novel screening system was developed turning the degradation of a target protein, i.e. Jak1 or STAT2, into a survival advantage. This was achieved by fusion of the HSV-TK to our target protein leading to cell death upon treatment with GCV. Only cells expressing proteins leading to the degradation of the target protein were able to survive under GCV treatment as the HSV-TK was degraded simultaneously. Transduction of these cells with a lentiviral cDNA library generated from HCMV-infected cells and subsequent selection with GCV identified HCMV gpUL42 as a viral protein antagonizing Jak1. gpUL42 is a type II transmembrane protein located at the cell surface (see Figure 17), subjected to complex N-glycosylation supposedly in the Golgi apparatus (see Figure 16). Furthermore, it contains two conserved PPXY motifs that seem to play important roles in Jak1 protein reduction (see Figure 19 and 22). On the other hand, it is evident that gpUL42 protein amounts also increase upon Bafilomycin A treatment, but not upon MG132 treatment (Figure 24 C), indicating that gpUL42 might be also rather dependent on the endosome than on the proteasome.

So far, the exact molecular function of gpUL42 had not been elucidated. However, functional profiling by mutational analysis, either by a random transposon or directed mutagenesis revealed that HCMV *UL42* is not essential for viral replication (Dunn et al., 2003; Yu et al., 2003). Furthermore, two independent groups could show that the first published genome sequence of HCMV was based on the analysis of a mutant virus which lacked 929 base pairs affecting the gene region *UL42-UL43* (Dargan et al., 1997; Mocarski et al., 1997). Both groups showed that HCMV mutants lacking this region grew to similar titres, with even slightly higher titres at the beginning of infection, in comparison with other pUL42-encoding HCMVs. These findings are in accordance with the growth kinetics we observed with the newly constructed gpUL42mut-$HCMV_{AD169varL}$ (Figure 26). Above mentioned mutation gives rise to a truncated pUL42 protein which still comprises the 76 carboxyterminal amino acids (Dargan et al., 1997; Mocarski et al., 1997) (see alignment in Figure 30, in the discussion). Interestingly, the truncated protein that could be translated from the mutated *UL42* gene lacks both conserved PPXY motifs that were shown – in this work – to have important functions for the Jak1 reduction. Importantly, the Jak1-degradation experiments by Miller *et al* used the HCMV strain Towne (Miller et al., 1998) which is known to harbour an intact *UL42-UL43* gene region (Dargan et al., 1997; Mocarski et al., 1997).

3.2 Importance of Jak1 and IFN signaling in anti-viral activity

Detection of viral nucleic acids by PRRs leads to the expression of IFN-β and subsequent Jak-STAT signaling in a para- and autocrine manner. This will lead to the expression of specific ISGs and the presentation of peptides by MHC molecules which are presented at the cell surface to T-cells. Activation of cytotoxic T-cells will lead to the clearance of the virus and virus-infected cells and to the survival of the infected individual. On the one hand, a *Herpesvirus* infection e.g. with HCMV is mostly asymptomatic in immune-competent individuals. On the other hand, *Herpesviruses* remain latently in their hosts throughout the hosts life-span. This shows the balanced interplay between efficient activation of the immune system to control further spread of the virus and an efficient immune evasion by the virus to remain latently in already infected cells.

```
pUL42_refseq          --------------------MEPTPMLRDRDH------DDAPPTYEQAMGLCPTT---
pUL42_AD169_varS      RNARWRTRWLSSWLTRPSAALSPPTLARTCRSWFVASAARCCVSAGQTVGSSPTATCY
                                        :.*  : *            .  :  *::* .**:

pUL42_refseq          VSTPPP-PPPDCSPPPYRPPYCLVSSPSPRHTFDMDMMEMPATMHPTTGAYFDNGWKW
pUL42_AD169_varS      VTLDISILKVWRRPSCAATSSCLVSSPSPRHTFDMDMMEMPATMHPTTGAYFDNGWKW
                      *:           *       ************************************

pUL42_refseq          ALLVVAILGIIFLAVVFTVVINRDSANITTGT-----
pUL42_AD169_varS      ALLVVAILGIIFLAVVFTVVINRDSANITTGTQASSG
                      *********************************
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Figure 30: Alignment of gpUL42 sequences of AD169varS and 'repaired' AD169. Protein sequences of gpUL42 were aligned using Clustal Omega web tool. Both virus strains share 76 AA at the carboxyl terminus. Asterisks (*) indicate fully conserved residues, colons (:) indicate groups with strongly similar properties, periods (.) indicate groups with weakly similar properties.

Miller *et al.* showed in their landmark paper that HCMV inhibits IFN-γ-induced MHC II presentation. MHC II molecules are constitutively expressed on antigen presenting cells like B cells, dendritic cells and macrophages to be recognized by $CD4^+$ T cells (reviewed in Fooksman, 2014). In fibroblasts, MHC II transcription is initiated mainly via IFN-γ by the transcription of the transcription of class II transactivator (CIITA) (Steimle et al., 1994). At least for MCMV, it was demonstrated that there is an important role for $CD4^+$ T cells in the control of MCMV infection (Jonjic et al., 1990; Lucin et al., 1992). The inhibitory mechanism of HCMV was found to act upstream of CIITA and IRF-1 leading to the conclusion that Jak-STAT signaling is actively antagonized by HCMV. Consistently, STAT1 phosphorylation could not be initiated in HCMV-infected cells. Finally, they could show that the inhibition of MHC II presentation is due to degradation of Jak1, the kinase which is essential for IFN signaling (Miller et al., 1998). There are no described phenotypes of Jak1 deficiencies in humans in accordance with the early postnatal lethality of Jak1 KO mice which have deficits in lymphopoiesis and of neurological origin (Rodig et al., 1998). Additionally, some gynecologic cancer cells evade tumor antigen recognition by frame-shift and truncation mutations in Jak1 (Ren et al., 2013). The HCMV evasion strategy represents a highly efficient way to evade all IFN-mediated anti-viral effects since Jak1 is essential for type I, II and III signal transduction (see Figure 1). In addition to Jak1 degradation, Le

et al. showed that STAT2 is also degraded during HCMV infection adding a further level of complexity to HCMV-mediated evasion of Jak-STAT signaling (Le et al., 2008a). Recently, a large proteomic approach confirmed that Jak1 and STAT2 protein amounts are reduced in HCMV infected cells and uncovered additional targets of HCMV (Weekes et al., 2014).

Table 2: Overview of viral proteins degrading cellular proteins of the Jak-STAT-signaling pathway

Pathogen	Target protein	Relevant protein	
MCMV	STAT2	pM27	Zimmermann A *et al.* J Exp Med. 2005 Trilling M *et al.* PLoS Pathog. 2011
PIV2	STAT2	V protein	Parisien JP *et al.* Virology 2001 Andrejeva J *et al.* J Virol. 2002
Dengue Virus	STAT2	NS5	Ashour J *et al.* J Virol. 2009
SV5	STAT1	V protein	Andrejeva J *et al.* J Virol. 2002 Ulane CM and Horvath CM. Virology 2002
KSHV	IRF7	RTA	Yu Y *et al.* Immunity 2005
HCMV	Jak1	UL42 + unknown	Miller DM *et al*. J Exp Med. 1998 This work
HCMV	STAT2	unknown	Le VTK, Trilling M *et al*. J Gen Virol. 2008
HCMV	p48/IRF9	unknown	Miller DM *et al*. J Immunol. 1999
HSV-1	STAT2	unknown	Chee AV and Roizman B. J Virol. 2004
HSV-1	Jak1	UL41 + unknown	Chee AV and Roizman B. J Virol. 2004
HSV-1	IFNAR	unknown	Chee AV and Roizman B. J Virol. 2004
hMPV	Jak1	unknown	Ren J *et al.* PLoS One. 2011
hMPV	Tyk2	unknown	Ren J *et al.* PLoS One. 2011
HCV	STAT1/STAT3	unknown	Stevenson NJ *et al.* FEBS Lett. 2013

Despite the fact that HCMV-mediated Jak1 degradation was described more than 15 years ago (Miller et al., 1998), to our knowledge the causative virus-encoded gene has remained elusive. The herein newly established screening approach yielded gpUL42 as a potential candidate which contributes to the reduction of Jak1 protein amounts in HCMV-infected cells. Furthermore, the success implies that the general screening procedure might be applicable to identify other viral proteins being responsible for the degradation of other target proteins (see below -> section 3.10).

3.3 Redundancy in HCMV-mediated Jak1 protein reduction

Among the human pathogenic viruses, HCMV has the largest and most complex genome resulting in the expression of >750 translation products (Stern-Ginossar et al., 2012). For MCMV it has been shown that large parts of the viral genome are not essential for viral replication *in vitro* (Cicin-Sain et al., 2007) indicating that these non-essential proteins regulate the interaction with the host. Consistently, HCMV can afford to devote multiple gene products to the inhibition of one particular aspect of the immune system. It was shown that HCMV encodes numerous antagonists which target MHC presentation (Ahn et al., 1997; Furman et al., 2002; Hengel et al., 1997; Jones and Sun, 1997; Jones et al., 1996; Kim et al., 2011b; Wiertz et al., 1996) or NK cell activation (Arnon et al., 2005; Fielding et al., 2014; Hassan-Walker et al., 1998; Stern-Ginossar et al., 2007; Tomasec et al., 2000; Tomasec et al., 2005; Welte et al., 2003; Wills et al., 2005). Additionally, the virus targets several proteins of the Jak-STAT signaling pathway (e.g. Jak1, STAT2 and IRF9) to inhibit transcription of ISGs and to block the initiation of an ISG-dependent anti-viral state. Although the Jak1 protein reduction was significantly reduced in the two mutant viruses (ΔUL42-43-HCMV$_{TB40}$ and gpUL42mut-HCMV$_{BAC2}$) after 24 h. p.i., both viruses were able to reduce Jak1 as efficient as wt-virus after 48 h (Figure 26). This leads to the conclusion that at least one additional Jak1 antagonist apart from gpUL42 is encoded by HCMV.

A possible candidate might be one (or both) of the detected ORFs (UL73 and/or UL74A) identified by the second TB40-mediated cDNA library used in the screening approach (Table 1). *UL73* was successfully cloned into expression plasmids but no pUL73 protein expression was established in transfection experiments and consecutive western blot analysis. UL74A alone did not reduce Jak1 protein amounts in co-transfection experiments (see Figure 12). However, that does not necessarily exclude a contribution of this protein in Jak1 protein reduction as pUL73 and pUL74A might be needed as a hetero-dimeric protein complex for functional reconstitution in gain-of-function experiments. Co-expression of both proteins might be necessary as the cells surviving the screening integrated a cDNA from which both proteins could be transcribed (see Figure 31). This was a fundamental difference to the cDNA of gpUL42 integrated in cells which survived the screen upon transduction with the AD169-derived cDNA library, where only the single gene could be expressed. Interestingly, pUL73 and pUL74A show similarities to gpUL42. All three proteins have potential N-glycosylation sites, a trans-membrane domain and are expressed on the cell surface.

It was excluded that late genes play a role in HCMV-mediated Jak1 degradation because late gene inhibition by the drugs PAA or GCV do not abrogate/interfere with Jak1 degradation (Miller et al., 1998). As Jak1 protein amounts are also reduced after 48 h p.i. in cells infected with viruses lacking gpUL42 protein expression (ΔUL42-43-HCMV$_{TB40}$ and gpUL42mut-HCMV$_{BAC2}$) (see Figure 26), there seems to be a contribution of at least one more viral protein or virally-encoded miRNA. Therefore, it would be interesting to see if the on-going Jak1 protein reduction is present in cells infected with ΔUL42-43-HCMV$_{TB40}$ or gpUL42mut-HCMV$_{BAC2}$ that were treated with late gene inhibitors. A possible contribution of late genes in Jak1 protein reduction could have been overlooked by Miller *et al.* as gpUL42 might still be expressed in this experimental setup leading to a sustained Jak1 protein reduction even in the presence of PAA or GCV. Also, the creation of a mutant BAC2 gpUL42-HA would be important to impose the gpUL42 expression kinetics during viral replication with the reduction of Jak1 protein amounts.

Alignment of the AA sequence of pUL42 of different HCMV strains and different CMVs of other species revealed high conservation in two PPXY motifs (see Figure 20 -22). Hence, the redundant viral protein could harbour a PPXY motif if the mode of Jak1 reduction is similar. Interestingly, two viral proteins with known function (pUL55 and pUL150) and one viral protein so far uncharacterized (pUS29) have a PPXY motif. The viral protein pUL55 (envelope protein gB) is needed for attachment and entry of the virus (Wille et al., 2013). The second viral protein with a PPXY motif is pUL150. The gene region of *UL128-UL150* was shown to have a role in entry into epithelial and endothelial cells (Ryckman et al., 2006). The functionally uncharacterized viral protein is a membrane protein (see UniProt [D2K3X8]) and a target of the virally encoded miRNA-US33 (Shen et al., 2014). pUL42 and pUS29 share a homologous PDXXPPPY motif. This motif is also shared by 9/10 simian CMVs as the D at position 39 is shared by 9/10 simian CMVs implicating an importance of the PD motif N-terminal to the PPPY motif. A possible contribution of pUS29 towards pJak1 reduction could be easily investigated by cloning of a tagged pUS29 followed by co-transfection experiments with pJak1.

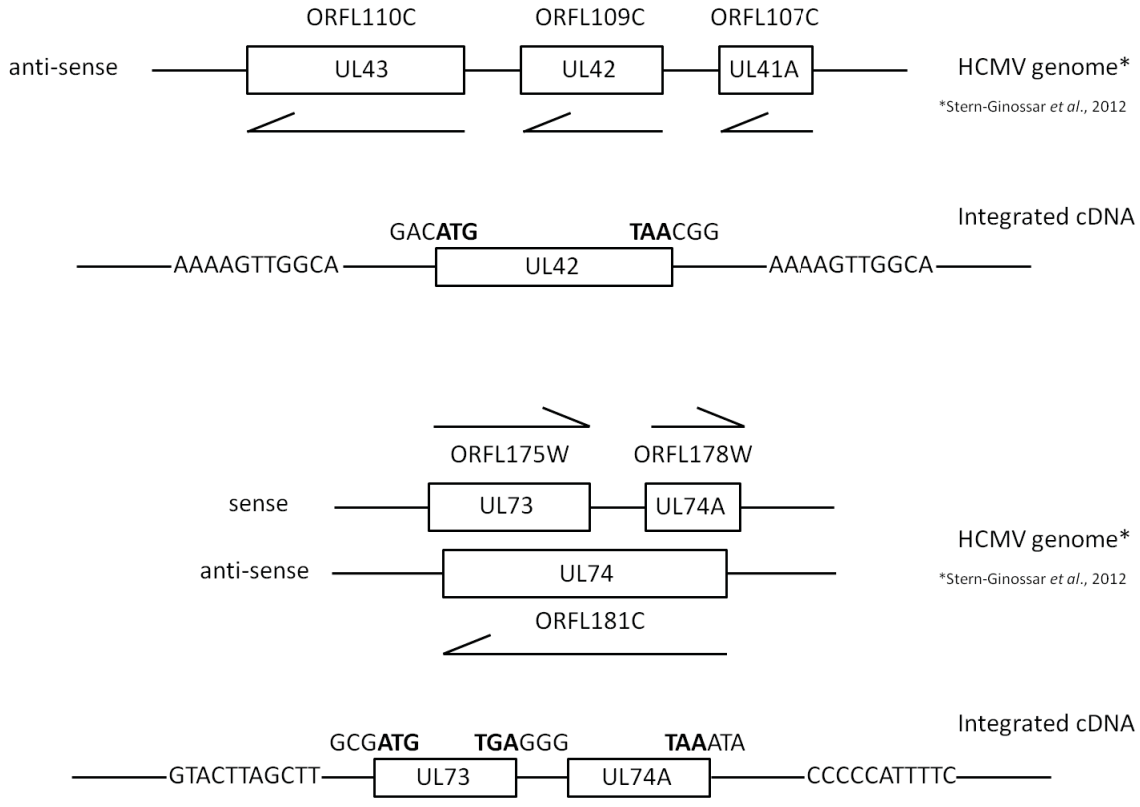

Figure 31: Overview of integrated lentiviral cDNAs in the surviving cell clones. Depicted is a graphical representation of the HCMV genome and the possible proteins expressed by the integrated lentiviral cDNAs. Cells surviving the global screening approach transduced with the AD169 lentiviral cDNA are only able to express one possible protein product. However, cells surviving the global screening approach transduced with the TB40 lentiviral cDNA could, in principle, express two different proteins.

3.4 Cellular cDNAs apparently leading to a selection advantage in TK-Jak1 cells

In addition to surviving cell clones containing lentiviruses which harbor genes coding for HCMV-encoded proteins (e.g. *UL42* and *UL73*/*UL74A*), a number of cell clones survived the screening approach which harbored cDNAs from cellular origin. Obviously, screening approaches always bear the risk of false positives surviving the screening. This became evident in the first screening approach using the single transfection of specific expression plasmids (Figure 10). Although the growth advantage was rather modest when transfected with specific HCMV ORFs, further validation with WB showed no reducing capabilities of these proteins on STAT2 protein levels.

However, some of the integrated cDNAs in cells surviving the screening approach with the lentiviral cDNA library might be theoretically linked either to Jak1, TK or GCV. It is tempting to speculate that some of the cellular proteins might induce Jak1 degradation, inhibit TK activity or interfere with GCV toxicity explaining their survival advantage during the selection process.

Table 3: Integrated lentiviral cDNAs of cellular origin in surviving TK-Jak1 single cell clones

Integrated cDNA library	Protein function	Selected reference
succinate dehydrogenase assembly factor 1 (SDHAF1)	Mitochondrial electron transport chain Maturation of the iron-sulfur subunit of succinate dehydrogenase	Ghezzi D *et al.* Nat Genet. 2009 Na U *et al.* Cell Metab. 2014
solute carrier organic anion transporter family member 1B3 (SLCO1B3 or Lst2)	Organic anion transporter in the liver	van de Steeg E *et al.* J Clin Invest. 2012
Endosulphine alpha (ENSA)	Regulator of insulin secretion Inhibitor of protein phosphatase 2A	Bataille D *et al.* Cell Mol Life Sci. 1999 Virsolvy A *et al.* Br J Pharmacol. 2002 Mochida S FEBS J. 2014 Labandera AM *et al.* Biochem Biophys Res Commun. 2015
Mitochondrial import inner membrane translocase subunit Tim8 B (Tim8B)	Chaperone involved in the import of mitochondrial carrier proteins	Paschen SA *et al.* EMBO J. 2000
Coatomer subunit epsilon (CopE)	Recycling of proteins from the Golgi to the ER	Shima DT *et al.* Curr Biol. 1999
Pro-apoptotic programmed cell death protein 5 (PDCD5)	Apoptosis	Liu H *et al.* Biochem Biophys Res Commun. 1999
3'-phospho adenosine 5'-phosphosulfate transporter 2 (SCL35B3)	Transport of 3' phosphorylated adenosine 5'-phosphosulfates into the Golgi	Kamiyama S et al. J Biol Chem. 2006
ISG15	Anti-viral activity against various infections	Morales DJ and Lenschow DJ J Mol Biol. 2013
COMM domain-containing protein 7 (COMMD7)	Regulation of cullin-RING E3 ubiquitin-ligase complexes	Mao X et al. J Biol Chem. 2011

4 out of 44 cell clones which survived the GCV selection in cells expressing TK-Jak1 harboured a cDNA encoding 3'-phospho adenosine 5'-phosphosulfate transporter 2 (SCL35B3). This transporter is involved in the sulfation of glycans and mediates the transport of 3' phosphorylated adenosine 5'-phosphosulfates from the cytosol into the Golgi (Kamiyama et al.,

2006). Interestingly, it was shown that over-expression of 3'-phospho adenosine 5'-phosphosulfate transporters supports cancer growth and plays a role in colorectal cancer cell proliferation (Kamiyama et al., 2011). Therefore, one potential explanation for a selective advantage might be the induction of a proliferative phenotype and enhanced growth capabilities. GCV is a purine base analogue. Therefore, it is conceivable that the phospho-purine transporter SCL35B3 cross-recognizes GCV as substrate and either sequesters it in the Golgi apparatus or transports it even out of the cell, thereby alleviating its toxicity to a certain extent.

Another gene seemingly conferring a selection advantage during the screening procedure was ISG15. ISG15 is an IFN-inducible gene with extensive homology to ubiquitin (Ub). Like Ub, ISG15 can be covalently linked to numerous target proteins, a process which is called ISGylation and which mediates anti-viral activity against various infections (Morales and Lenschow, 2013). It was shown in co-immuno-precipitation experiments that ISG15 modifies Jak1 (Malakhov et al., 2003) and interacts with HECT-ubiquitin ligases of the Nedd4-like ubiquitin ligase family (Malakhova and Zhang, 2008; Okumura et al., 2008). Binding and ISGylation of Jak1 by ISG15 might lead to the degradation of the TK-Jak1 construct resulting in the survival of the cell clone upon GCV treatment.

COMM domain-containing protein 7 (COMMD7) is a family member of the Copper metabolism MURR1 domain-containing (COMMD) protein family. These proteins are involved in the regulation of cullin-RING E3 ubiquitin-ligase complexes, promoting the ubiquitination of substrates (Mao et al., 2011).

Lentiviral vectors will inevitably integrate into the cellular genome. This is a fundamental difference between the single screen approach using the expression library of HCMV ORFs and the lentiviral cDNA library in the global screening approach. Transduction will lead to insertion mutagenesis by which genes are inactivated by the retroviral genome insertion but also neighboring genes could be activated, e.g. by long-terminal repeats. Also, complex effects on different genes or complete transcription programs could be elicited by miRNAs. All of these scenarios might lead to survival of cell clones upon GCV selection as host genes could be influenced which are involved in Jak1 stability. Noteworthy, the integration site selection is not random for retroviruses. HIV-1 integrates preferably within active genes and the *Moloney murine leukemia virus* favors strong enhancers and active gene promoter regions (Kvaratskhelia et al., 2014). To identify the exact chromosomal integration sites of the lentiviral vectors, combination of restriction endonuclease digest and several rounds of PCR amplification followed by nucleotide sequencing could be used as this method is established for genomic DNA of transgenic animals (Bryda et al., 2006).

To test if ISG15 or COMMD7 play a direct role in Jak1 protein degradation, co-transfection and *in-vitro* binding assays could be performed. The two proteins should behave similarly to gpUL42 in their ability to decrease Jak1 protein amounts if both proteins will be directed against Jak1 (as seen in Figure 13). As COMMD7 is not directly linked to the interferon system or Jak1, another approach could be feasible. Jak1 turnover is dependent on SOCS1 (Vuong et al., 2004) and SOCS proteins are part of cullin RING E3 ubiquitin ligases. It would be interesting to see if Jak1 turnover is dependent on COMMD7. This could be achieved in comparative studies of recombinant COMMD7 in SOCS-deficient cells.

3.5 Cellular degradation mechanism exploited by gpUL42

Miller *et al.* showed in their landmark paper that HCMV is able to degrade Jak1 via the proteasome. However, identifying the underlying molecular mechanism is very challenging as the cellular turnover of Jak1 protein levels is dependent on proteasomal degradation mechanisms mediated by SOCS proteins, leading to an increase of Jak1 protein levels upon inhibition of this pathway (Figure 24 A). This effect was also already observed in the initial paper of Miller *et al*. discovering the HCMV-mediated Jak1 degradation – but not taken into account.

Jak1 turnover rates are tightly regulated by the proteasome (Vuong et al., 2004). Hence, Jak1 protein levels increase if cells are treated with proteasome inhibitors like MG-132 (see Figure 24 A). This effect is also seen in co-transfection experiments of gpUL42 and Jak1 (see Figure 24 B). Therefore, it is crucial to differentiate between cellular and truly viral-mediated degradation of Jak1. A specific tyrosine within Jak2 (Y1007) was shown to be essential for SOCS-mediated proteasomal degradation (Ungureanu et al., 2002). The use of a stable Jak1 harbouring a mutation at this tyrosine could be used to discriminate between 'turnover-dependent' and 'gpUL42-mediated' protein degradation. One could also establish a 'refined screen' using a TK-Jak1 construct with a mutated Y1007 within the Jak1 sequence to distinguish between these two forms of protein degradation in the viral infection context.

3.6 Possible co-factors involved in gpUL42-mediated Jak1 protein reduction

Northern blot analysis showed that gpUL42 reduces Jak1 protein amounts post-transcriptionally (see Figure 17). Our newly developed gain-of-function screening approach enriched *UL42*-expressing cells in agreement with the notion that gpUL42 is sufficient to reduce Jak1 protein amounts (see Figure 13). We could also show contribution of gpUL42 to reduce Jak1 protein amounts in the context of permissive HCMV infection (see Figure 23).

Bioinformatic analysis of the amino acid sequence of gpUL42 did not reveal any known proteolytic domains or domains known to be involved in protein ubiquitination. The known classes of Ub-ligases have characteristic domains (e.g. RING, U-box or HECT (Hibbert et al., 2009; Metzger et al., 2012)). Hence, gpUL42 would be either the first identified member of a novel class of Ub-ligases or not an Ub-ligase. Potential Ub-ligase activity of gpUL42 could be tested by *in vitro* assays under physiological conditions, adding recombinant Ub, E1- and E2-Ub-ligases produced in *E.coli* which do not have a canonical Ub-system, ATP and Jak1.

The binding of co-factors to facilitate the ubiquitination and degradation of target proteins, especially host restriction factors, is very well described for MCMV. The viral protein pM27 uses the interaction with DDB1 to recruit a cullin ubiquitin ligase into proximity of STAT2 thereby inducing its (poly-) ubiquitination and subsequent proteasomal degradation (Trilling et al., 2011). By analogy, gpUL42 could also constitute a viral adaptor to a so far unknown cullin ubiquitin ligase. For the two HCMV proteins US2 and US11 it is described that they are able to 'catch' MHC I molecules by binding to RING-type E3 ubiquitin ligases and transport the MHC I molecules to be degraded in proteasome-dependent pathways (Halenius et al., 2015). gpUL42 harbors two conserved PPXY motifs (AA 16-19 and AA 42-45). The mutation of the conserved Y45 leads to the

abrogation of Jak1 protein reduction in co-transfection experiments (Figure 21). In addition, the N-terminal mutant of gpUL42 lacking the first 22 AA and thereby the first conserved PPXY motif (AA 16-19) has an impairment in Jak1 protein reduction (Figure 18). Therefore, it is tempting to speculate that this motif is crucial for the gpUL42-mediated effect.

PPXY motifs constitute one of the best characterized protein motifs known to confer interaction with a conserved tryptophan-rich motif, the so called WW domain (Sudol et al., 1995). WW domains are found in several unrelated protein families, inter alia in HECT ubiquitin ligases of the Nedd4-like ubiquitin ligases (Otte et al., 2003). The Nedd4-like ubiquitin ligase family comprises nine members in Homo sapiens: Nedd4, Nedd4L, ITCH, SMURF1, SMURF2, WWP1, WWP2, NEDL1 and NEDL2. From these, seven members are associated either with virus-protein interaction or ubiquitination and degradation of anti-viral factors, e.g. proteins of the IFN signaling cascade:

WWP1 and WWP2 are involved in the budding of retroviruses such as human *T-cell leukemia virus type 1* and *koala retrovirus* (Heidecker et al., 2007; Martin-Serrano et al., 2005; Shimode et al., 2013) or virus internalization of *Adenoviruses* (Galinier et al., 2002). SMURF2 was described to be involved in *Papilloma virus* intracellular trafficking thereby decreasing infection (Dabydeen and Meneses, 2011). Nedd4L is implicated in the release of HIV-1 (Chung et al., 2008; Mercenne et al., 2015) and *Enterovirus 71* (Kuo et al., 2015). Nedd4 regulates the egress of Ebola virus and Marburg virus particles (Urata and Yasuda, 2010; Yasuda et al., 2003), as well as the release of *Lassa Fever virus*, *vesicular stomatitis virus* and *rabies virus* particles (Han et al., 2014). ITCH is essential for *Influenza A virus* release from endosomes during its viral life cycle (Su et al., 2013).

Both SMURF1 and SMURF2 are negatively regulating IFN-signaling by facilitating the ubiquitination of the adaptor protein MAVS (Pan et al., 2014; Wang et al., 2012) or STAT1 (Yuan et al., 2012). Additionally, there is a direct link between *Hepatitis C virus*-mediated TGF-β signaling interference and SMURF2 (Verga-Gerard et al., 2013). ITCH is used by a variety of different viruses to interfere with receptor and cell signaling. HIV-1 Vif binds to both ITCH and Nedd4 (Dussart et al., 2004). HIV-1 Nef down-regulates chemokine receptor CXCR4 via recruitment of either ITCH or Nedd4 (Chandrasekaran et al., 2014) and *SARS-Corona virus* triggers the ITCH-mediated degradation of MAVS by the viral ORF-9b (Shi et al., 2014). Interestingly, ITCH was also implicated in the association with herpesviral proteins. The *Epstein Barr virus* (EBV) protein latent membrane protein 2A forms complexes with ITCH in EBV-infected B-cells (Winberg et al., 2000). Latent membrane protein 2A was furthermore described to modulate IFN type I and type II signaling as it facilitates the degradation of IFN receptors in epithelial cells (Shah et al., 2009).

To address the involvement of Nedd4-like ubiquitin ligases in the gpUL42-mediated Jak1 protein reduction, it would be interesting to specifically inhibit Nedd4 ubiquitin ligase activity. Recently, a drug screen has revealed a number of compounds specifically inhibiting the interaction of Nedd4 and virus-like particles of several RNA-viruses (Ebola and Lassa fever, vesicular stomatitis and rabies viruses). These viruses were not able to bud from cells treated with the compounds, as the binding interface of the PPXY-motif containing viral proteins and Nedd4 ubiquitin ligases was impaired (Han et al., 2014). As the budding of a variety of different virus families was inhibited, it is very likely that the tested compounds block the Nedd4-binding interface to the

viral proteins. Therefore, it would be interesting to see if the compounds will block the gpUL42-mediated Jak1 protein reduction, without inhibiting cellular degradation pathways. Astonishingly, a proteomic analysis of HCMV-infected cells revealed that Nedd4-ubiquitin ligases are regulated during viral infection (Dominik A. Megger and Mirko Trilling, unpublished data). Here, Nedd4 protein levels were down-regulated during infection, whereas ITCH protein levels were significantly up-regulated. This phenotype might indicate a function of these ubiquitin ligases for virus replication. If these regulations have a direct implication in host/interferon evasion will have to be further elucidated.

This could be easily achieved with plasmid derived gpUL42, however the generation of a mutant BAC harboring an epitope-tagged gpUL42 would be essential as this approach represents the actual infection. Here, the possibility to identify additional viral interaction partners is given which is not the case in the transfection based system. However, previous studies concerning CMV mediated protein degradation and the identification of involved binding partners (Trilling et al., 2011) showed that these approaches are not trivial even if the involved degradation pathway is known.

Interaction studies of gpUL42 could be assessed by immune-precipitation experiments. Instead of visualization of specific binding partners e.g. Jak1 with SDS gel electrophoresis or general detection methods as silver or Coomassie staining, the complete sample could be analyzed by complex proteome analytical methods. For this approach, HPLC followed by mass spectrometric detection would be ideal to identify and characterize the 'complete' interactome of the protein which was precipitated. Using these methods, novel binding partners of gpUL42 e.g. involved Ub-ligases, could be identified.

3.7 Expanded 'antagonistic' capacity of HCMV gpUL42 in co-transfection experiments

To ascertain the function and mode of action of proteins involved in the degradation of other proteins is quite challenging. This is especially the case when working with virally encoded proteins used for the evasion of host restriction factors. There is a clear difference between transfection related effects and phenotypes observed during actual infection. The viral ubiquitin ligase ICP0 of HSV-1 was described to inhibit and degrade the cellular DNA sensor IFI16 (Diner et al., 2015; Johnson et al., 2013; Orzalli et al., 2012). However, a different approach showed that ICP0 is neither sufficient nor necessary for degradation of IFI16 but rather that IFI16 stability is regulated by cellular factors in response to HSV-1 infection (Cuchet-Lourenco et al., 2013).

Besides Jak1, gpUL42 elicited a seemingly 'antagonistic' capacity towards different proteins, i.e. STAT2, hDaxx and US6, in co-transfection experiments (Figure 25). However, STAT2 amounts of human fibroblasts infected with ΔUL42-43-HCMV$_{TB40}$ and gpUL42mut-HCMV$_{BAC2}$ remained on a similar level to mock- and HCMV wt-infected cells (Figure 26). The reduction of the STAT2, hDaxx and US6 protein amounts in Figure 23 therefore rather demonstrates a co-transfection (or overexpression) dependent effect than a gpUL42 specific effect.

Another plausible explanation why gpUL42 is able to reduce protein amounts of several other proteins could be the potential binding of HECT ubiquitin ligases via the PPXY motif. A recent

publication showed that the Nedd4 ubiquitin ligase ITCH is involved in the cytosolic protein quality control and co-localizes with bona fide misfolded proteins (Chhangani et al., 2014). Over-expression of proteins by transfection of cells most likely leads to misfolded proteins in the cell. A possible interaction of gpUL42 and ITCH could bring the ubiquitin ligase into proximity of these over-expressed proteins leading to their subsequent degradation. This process would then be independent of the target co-transfected with gpUL42. However, this is purely speculative as a direct interaction of ITCH and gpUL42 was not investigated.

3.8 Comparative analysis of MCMV-expressed gpUL42 homologues

Although both HCMV and MCMV have evolved several mechanisms to degrade proteins of the Jak-STAT signaling pathway, MCMV does not seem to degrade Jak1 in the course of infection as STAT1-phosphorylation is still present in MCMV-infected cells upon IFN-γ treatment (Trilling et al., 2014). MCMV expresses m42 an approximately 18 kDa type II transmembrane protein harboring a PPXY motif. Furthermore, it was shown that m42 is able to facilitate the proteasomal degradation of CD45 (Thiel N et al, Abstract 31, 9th Mini-Herpesvirus Workshop). Hence, although the two proteins are not homologous, both seem to be involved in degradation/reduction of proteins.

3.9 HCMV mutants lacking IFN antagonists as live attenuated vaccine viruses?

The development of a CMV vaccine is far more challenging due to the broad ability of CMV to evade the immune system, especially by inhibition of the interferon signaling, MHC presentation and NK-cell activation. The presence and re-infection with different strains occurring in sero-positive individuals highlights the difficulty to fully protect from HCMV infection (Pati et al., 2013; Ross et al., 2011). Multiple candidate CMV vaccines have been developed and used in clinical evaluation (reviewed in (Rieder and Steininger, 2014)). However, only two of these developed vaccines justified phase II clinical studies in healthy individuals (Griffiths et al., 2011; Kharfan-Dabaja et al., 2012). Both vaccines, lowering the rate of CMV viremia, will be further investigated in phase III studies (Griffiths et al., 2011; Kharfan-Dabaja and Nishihori, 2015).

HCMV vaccine candidates are very safe but are mostly inefficient. The use of single mutant CMVs, lacking genes with immune-modulatory functions, as vaccines, seems to be rather difficult as T cells mediate protection by targeting multiple CMV antigens (Malouli et al., 2014). However, viruses that have no or impaired IFN evasion might induce a more potent stimulation of T cells. The MCMV ΔM27 mutant lacking the ability to degrade STAT2 is highly susceptible towards IFN-γ and attenuated *in vivo* (Zimmermann et al., 2005) therefore one could postulate that a vaccine virus lacking *M27* would be quite safe at least in healthy individuals. The vaccines could still present a problem in immune-compromised patients as SCID mice infected with a mutant virus lacking *M27* succumb to the infection after about 40 days (Abenes et al., 2001).

Nevertheless, it would be interesting if there are possible differences in JAK-STAT signaling (especially phosphorylation of STAT molecules and transcription of ISGs) and MHC presentation

of gpUL42mut-HCMV$_{BAC2}$ upon IFN-γ treatment. As HCMV is able to completely block the presentation of MHC molecules on the cell surface upon IFN-γ treatment (Miller et al., 1998), MHC surface presentation should be examined in cells infected with the gpUL42 mutant virus. However, the possibility of redundant Jak1 antagonism in HCMV (see 3.5) and similar growth kinetics of gpUL42mut- or wt-HCMV$_{BAC2}$ even upon IFN-γ treatment after 6 days of infection (see Figure 28 and 29) make it rather unlikely that a virus lacking only *UL42* expression would be attenuated *in vivo*.

On the other hand, the use of CMV as vaccine vector background is well adapted. Cytomegalovirus-based vectors to vaccinate rhesus monkeys against *Simian immunodeficiency virus* (SIV) have been an approach showing partial protection against viral infection (Barouch and Picker, 2014). As rhesus CMV (RhCMV) also persists in the host, the vaccine is able to elicit a high frequency of circulating and tissue-resident effector memory T-cells in contrast to adeno-based vaccine vectors. Approximately 50 % of vaccinated monkeys were able to control SIV challenges early after exposure when vaccinated with these RhCMV-based vectors (Hansen et al., 2011; Hansen et al., 2013; Hansen et al., 2009).

3.10 General applicability of the herein established screening system

As we were able to identify gpUL42 with the developed screening system using a lentiviral cDNA library of HCMV-infected cells, it should be possible to identify Jak1 and STAT2 antagonists encoded by other viruses, e.g. hMPV, using lentiviral cDNAs that were generated from virus-infected cells. The broadly applicable nature of the screening system renders the possibility to identify the antagonistic protein of a given target protein that would be linked to the HSV-TK. The only criterion to be met by the unknown antagonistic protein is the complete degradation of the target protein fused to the HSV-TK by the cellular machinery, e.g. proteasomal or lysosomal, together with the TK. Furthermore, as endogenously encoded proteins were also integrated in cells surviving the screening system, the approach is not restricted to the identification of pathogen-derived proteins but also to help understanding cellular regulators of the specific target proteins e.g. quality control of misfolded proteins, regulatory molecules of transcription factors or proteins involved in cell cycle control.

Cellular protein degradation is a tightly regulated process involving more than 600 different E3-ubiquitin ligases (Li et al., 2008). To date, many proteins are regulated in the cell but the identity of these regulators is poorly understood. To identify and characterize the specific ubiquitin ligases involved in the degradation of one defined protein would be of great importance for the development of therapeutics in cancer, neurodegenerative and immunological disorders and viral infections (Landre et al., 2014). To date, global ubiquitination events and ubiquitinated substrates of specific E3 Ub-ligases are analyzed by Tandem Ubiquitin Binding Entities (TUBEs) followed by MS analysis (Lopitz-Otsoa et al., 2012; Yoshida et al., 2015). Here, the exogenous overexpression of E3 Ub-ligases allowed for the detection of the substrate and Ub-ligase activity (Yoshida et al., 2015). However, these methods are very challenging and are only specific for identified E3 Ub-ligases.

Our newly developed screening system could allow the identification of the 'degradome' of a specific substrate. Instead of using a lentiviral cDNA of infected cells that are comprised of virally encoded cDNAs, the use and generation of a defined cDNA library only encoding for identified ubiquitin ligases would be needed. Furthermore, these cDNAs could be DNA-barcoded to follow enrichment via qPCR or Next Generation Sequencing. Cells that have integrated and over-express the ubiquitin ligases involved in the degradation of the target protein should have a survival advantage upon GCV treatment. This approach could be crucial for the identification of Ub-substrate interactions in neurodegenerative diseases that accumulate protease resistant aggregated proteins e.g. Huntington's diseases, Alzheimer's disease, Parkinson's disease and Amyotrophic Lateral Sclerosis (reviewed in Ciechanover and Kwon, 2015). Current therapies to improve degradation of these aggregated proteins focus on the induction of degradation pathway e.g. the induction of autophagy by rapamycin, however the success of the drug is bivalent. Rapamycin treatment is beneficial in mouse models of Alzheimer's disease, Huntington's disease or Parkinson's disease with mutant α-synuclein but worsen the phenotype in mouse models of Amyotrophic Lateral Sclerosis or Parkinson's disease with mutant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Ciechanover and Kwon, 2015). Identification of specific Ub-ligases involved in the degradation of these aggregated proteins and targeted therapy could vastly improve disease outcome and reduce off-target effects mediated by general autophagy inducers.

Identification and understanding of virally encoded IFN antagonists could help in the development of pharmacological inhibitors that directly interfere with the antagonist. As many viruses use the ubiquitin proteasome system for the degradation of proteins, inhibition of this pathway might affect viruses, especially HCMV, on multiple levels. Proteasomal activity is needed by HCMV on multiple levels as proteasome inhibitors like MG-132 also block viral DNA replication (Kaspari et al., 2008). These inhibitors are widely used as anti-cancer drugs (e.g. bortezomib or carfilzomib (Crawford et al., 2014; Richardson et al., 2006)), however they elicit strong side effects. A major problem of bortezomib treatment is the inhibition of cell proliferation. T cells are not expanded further and latent and chronic viruses as *Herpesviruses* and *Hepatitis B virus* are reactivated (Hussain et al., 2014; Tong et al., 2007) leading to additional disease burden in already critically ill patients. Therefore, the focus will lie on the targeted inhibition of defined E3 ubiquitin ligases as drug targets (reviewed in (Bielskiene et al., 2015)). The identification and further characterization of gpUL42 as a Jak1 antagonist could be used to test the efficacy of these pharmacological inhibitors as wt-virus can be directly compared with the gpUL42 deletion mutant.

Furthermore, Jak inhibitors or Jakinibs are therapeutic options in auto-immune disease or in specific cancers (reviewed in (Kontzias et al., 2012)). However, some cancers are resistant to ATP-competitive inhibitors i.e. INCB018424 that is currently used in the clinics as they harbor Jak1 and Jak2 mutations (Hornakova et al., 2011). This is crucial in malignant diseases with mutated Jaks harboring constitutive active kinase domains e.g. Jak2 V617F present in various myeloproliferative diseases such as polycythaemia vera, chronic myelomonocytic leukemia and acute myeloid leukemia (Baxter et al., 2005; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). Somatic Jak1 mutations are a problem in adult and childhood acute lymphoblastic leukemia (Flex et al., 2008; Jeong et al., 2008; Mullighan et al., 2009). Identification of virally

encoded Jak inhibitors like gpUL42 and the understanding of their exploitation of the cellular machinery to degrade these kinases could help to develop new targets for an effective Jak inhibition.

Linking the HSV-TK to cellular proteins and therefore turning the degradation of a target protein into a cellular growth advantage represents a novel approach for the identification of virus and cell-encoded restriction factors. Future development of the screening could prove an important research tool to understand and identify cellular processes involved in protein degradation on a general level.

4. Materials and Methods

4.1 Materials

4.1.1 Equipment

-80 °C Freezer:	Sanyo, Ultra low temperature freezer
-20 °C Freezer:	Liebherr Medline
	Liebherr Comfort UG1211
Blotting apparatus:	PerfectBlue™,Semi-Dry'-Elektroblotter, peqlab
Cell culture bench:	HERA safe, Thermo Scientific
Centrifuges:	5415 D, Eppendorf
	5417 R, Eppendorf
	5424, Eppendorf
	5427 R, Eppendorf
	Allegra X-15R, Beckman Coulter
Electrophoresis chamber:	PerfectBlue™ Gelsysteme Mini und Midi, peqlab
	PerfectBlue™, vertikale Doppelsysteme
	Twin S, L und ExW S, peqlab
ELISA reader	TECAN Sunrise
FACS sorter:	MoFlo XDP, Beckman-Coulter
Flow cytometer:	FACSCanto II, BD
Fridges:	Liebherr
	UK 1720, Liebherr
Gel documentation:	GeneGenius Gel Imaging System, Syngene
	Science Imaging Advanced Fluorescence Imager, INTAS Science Imaging
Homogeniser:	Dounce Tissue Grinders, Wheaton
Hybridisation oven:	OV 3, Biometra

Incubators:	Binder
	Heraeus, Thermo Scientific
	Max Q6000, Thermo Scientific
	Incubat, Melag
Light source microscopy:	EL6000, Leica
Magnet fish:	MR Hei-Standard, Heidolph
Microwave:	Siemens
Microscopes:	CKX41, Olympus
	Primovert, Zeiss
NanoDrop:	NanoDrop2000c, Thermo Scientific
Nitrogen Tanks:	Biosafe MD, Cryotherm
pH-Measurement:	Lab 850, Schott Instruments
Photometer:	Bio Photometer plus, Eppendorf
Power supply:	Electrophoresis power supply EV202, peqlab
	Mini Power Pack PS300T, Biometra
Scales:	440-47N, Kern
	EMB 1000-2, Kern
Thermoblocks:	Thermomixer Comfort, Eppendorf
	ThermoStat plus, Eppendorf
Thermocycler:	T Professional Trio, Biometra
Transilluminator:	MWG-Biotech
Vortex:	IKA® Vortex Genius 3, Sigma-Aldrich
	VortexerTM, Heathrow Scientific
Waterbath:	GFL
WB developing:	Cawomat 2000 IR, BEMA

4.1.2 Chemicals

Acetic acid (96 %)	Roth
Acrylamid/Bisacrylamid (30 %)	Roth
Agarose	Biozym
3-amino ethylcarbacol (AEC)	Sigma
Ammoniumpersulfate (APS)	Roth
Ampicillin (Amp)	AppliChem
AmpliTaq DNA Polymerase	Applied Biosystems
Bacitracin	Sigma
β-Mercaptoethanol	Roth
Boric acid	Roth
Bromphenol blue	Merck
Calcium chloride	Roth
Calf Intestine Phosphatase (CIP)	New England Biolabs
Color Protein Standard Broad Range	New England Biolabs
Complete Protease Inhibitors	Roche
Coomassie Blue G-250	LKB
Crystal Violet	Roth
Desoxyribonukleosidtriphosphate (dNTPs)	New England Biolabs
DIG-Blocking-Reagenz	Roche
DIG-Easy Hyb	Roche
Dimethyl sulfoxid (DMSO)	Roth
Dithiothreitol (DTT)	Roth
Dulbeccos Modified Eagle Medium (DMEM)	Gibco
Endoglycosidases Endo H and PNGase F	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Roth
Ethanol	Roth

Fetal calf serum (FCS)	Biochrom AG
Fuji medical X-Ray Film	Fujifilm
Ganciclovir	Sigma
GelRed	Biotium
GeneRuler DNA Ladder Mix	Thermo Scientific
Geneticin (G418)	Roth
Glycerol	Roth
Glycine	Roth
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Roth
Hydrochloric acid	Roth
Hydrogenperoxide (H_2O_2)	Roth
HyperfilmeTM ECL	GE-Healthcare
Interferon α (human)	PBL
Interferon γ (human)	PBL
Isopropanol	Roth
Leupeptin	Roth
Luc-Lysis buffer	Roche
Lysogeny broth (LB)-Medium	Roth
Lysogeny broth (LB)-Agar	Roth
Magnesium chloride	Roth
Maleic acid	Roth
Methanol	Roth
MG-132	Tocris Bioscience
N,N-dimethylformamide	Roth
Nitrocellulose Blotting Membran 0,2/0,45 µm	GE-Healthcare
NP40/ Igepal	Sigma
Nylon membrane, positively charged	Roche

Paraformaldehyde (PFA)	Roth
Penicillin/Streptomycin (P/S)	Gibco
Pepstatin	Roth
Phenylmethanesulfonylfluoride (PMSF)	Roth
Phosphate buffered saline (PBS)	Gibco
Polyethylenimine (PEI)	Sigma
Ponceau S	Roth
Potassiumacetate	Roth
Potassiumchloride	Roth
Proteinase K	Roche
Restrictions endonucleases	New England Biolabs
RNase	AppliChem
Saccharose	Roth
Saran-Foil	Sarogold
Serva DNA Stain Clear G	Serva
Skim milk powder	Sucofin
Sodium azide	Roth
Sodium chloride	Roth
Sodiumdodecylsulfate (SDS)	Roth
Sodiumhydroxide	Roth
Sodiumorthovanadat (Na3VO4)	AppliChem
Sorbitol	Roth
Superfect	Qiagen
T4 DNA-Ligase	New England Biolabs
TEMED	Roth
Tricin	Roth
Trinatriumcitratdihydrat	Roth

Tris	Roth
Triton X 100	Roth
Trypsin	Gibco
Trypsin/EDTA	Gibco
Tween 20	Roth
Whatman 3MM Chr Paper	Whatman
Zeocin (Zeo)	Invitrogen

4.1.3 Buffers and solutions

10x Alkaline Phosphatase (AP)-buffer:	1 M NaCl 1 M Tris-HCl, pH 9,5
Bradford-Reagent:	8,5% (v/v) Phosphoric acid 4,75% (v/v) Ethanol 100 mg/l Coomassie Blue G-250
Buffer P1:	50 mM Tris/HCl, pH 8,0 10 mM EDTA 100 µg/ml RNase A
Buffer P2:	200 mM NaOH 1 % SDS
Buffer P3:	3 M Potassiummacetat, pH 5,5
Denaturation buffer:	0,5 M NaOH 1,5 M NaCl

FACS-Buffer:	10x PBS; pH 7,4
	3% FCS
10x Laemmli-Electrophoresis buffer:	250 mM Tris
	1,92 M Glycin
	1 % (w/v) SDS
LB (Lysogeny Broth)-Medium:	1 % (w/v) Bacto-Trypton
	5,5 % (w/v) Bacto-Yeastextract
	1 % (w/v) NaCl
LB-Agar:	LB-Medium
	1,5% (w/v) Agar
Maleic acid buffer:	0,15 M NaCl
	0,1 M Maleic acid, pH 7,5
Neutralisation buffer:	3 M NaCl
	0,5 M Tris/HCl, pH 7,5
Ponceau:	0,1% (w/v) Ponceau-S
	5% (v/v) Acetic acid
Proteinase K-Puffer (4x); pH 8,0:	2% (w/v) SDS
	40 mM Tris
	20 mM EDTA

RIPA+-Lysisbuffer	50 mM Tris/HCl; pH 7,5
	150 mM NaCl
	1% (v/v) NP-40/Igepal
	0,1% (v/v) SDS
	1% (v/v) Na-Deoxycholat
	0,2 nM PMSF
	1 mM DTT
	1 µg/ml Leupeptin
	1 µg/ml Pepstatin
	50 mM NaF
	0,1 mM Na-Vanadat
	Complete Protease Inhibitors
10x Semidry-Blotting-buffer:	48 mM Tris
	28 mM Glycin
	20 % (v/v) Methanol
20x Sodiumchloride Sodiumcitrate (SSC)	1,5 M NaCl
	0,15 M Trinatriumcitratdihydrat
Sorbitol-Cushion:	20% (w/v) Sorbitol
	50 mM Tris; pH 7,4
	1 mM MgCl2
	100 µg/ml Bacitracin

6x TBE sample buffer:	10 % (v/v) Glycerol
	6x TBE
	Bromphenolblue
10x TBST:	100 mM Tris/HCl, pH 8,0
	1,5 M NaCl
	5 % (v/v) Tween-20
Wash-Buffer 1 (SB):	2x SSC
	0,1% SDS
Wash-Buffer 2 (SB):	0,1x SSC
	0,1% SDS
5x Westernblot sample buffer:	0,25 M Tris/HCl, pH 6,8
	25 % (v/v) Glycerol
	20 % (w/v) SDS
	0,5 % (v/v) β-Mercaptoethanol
	Bromphenol blue

4.1.4 Commercial kits

All commercial kits were used according to manufacturer's protocol.

AmpliTaq DNA Polymerase, SixPaq with GeneAmp	Roche
Phusion® High-Fidelity DNA Polymerase	NEB
NucleoSpin® Gel Extraction Kit	Macherey-Nagel
Plasmid Midi Kit	Qiagen
QuikChange II XL Site-Directed Mutagenesis Kit	Agilent Technologies

4.1.5 Primers

All primers were synthesized by MWG Biotech.

Table 4: Primers for PCR

Nr	Name	Sequence 5'-3'
1	hSTAT2 fo	CGCGGCCGCATGGCGCAGTGGGAAATGCTGC
2	hSTAT2 re	GTCTAGATCCTCCTCCTCCTCCTCCGAAGTCAGAAGGCATCAAGGGTC
3	TK fo #1	GTCTAGAATGGCTTCGTACCCCGGCCATC
4	TK re #1	GTCTAGATCAGTTAGCCTCCCCCATCTCC
5	TK fo #2	CGCGGCCGCATGGCTTCGTACCCCGGCCATC
6	TK re #2	GTCTAGATCCTCCTCCTCCTCCTCCGTTAGCCTCCCCCATCTCCCG
7	hJak1 fo	GCCTCTAGAATGCAGTATCTAAATATAAAAGAGG
8	hJak1 re	CTCTAGATTATTTTAAAAGTGCTTCAAATCCT
9	UL42-HA fo	ATCGTCAAGCTTATGGAGCCCACGCCGATGCTC
10	UL42-HA re	GACGATGAATTCTTACGCGTAATCTGGAACATCGTATGGGTACCCCGATGATGCTTGCGT
11	UL42deltaN-HA fo	ATGCTCCGCGACCGGTGATCACGACGACGCGC
12	UL42deltaN-HA re	GCGCGTCGTCGTGATCACCGGTCGCGGAGCAT
13	UL42FS-HA fo	CCTACGAGCAGGCCACTGGGTCTGTGCCCGAC
14	UL42FS-HA re	GTCGGGCACAGACCCAGTGGCCTGCTCGTAGG
15	UL42C26S-HA fo	CAGGCCATGGGTCTGTCCCCGACGACGGTTTC
16	UL42C26S-HA re	GAAACCGTCGTCGGGGACAGACCCATGGCCTG
17	UL42C40S-HA fo	CCGCCACCACCCGACTCCAGCCCACCGCCCTA
18	UL42C40S-HA re	TAGGGCGGTGGGCTGGAGTCGGGTGGTGGCGG
19	UL42C50S-HA fo	TATCGACCCCCGTACTCCCTGGTTAGTTCGCC
20	UL42C50S-HA re	GGCGAACTAACCAGGGAGTACGGGGGTCGATA
21	UL42N115A-HA fo	AACCGGGACAGTGCCGCTATAACAACGGGGAC
22	UL42N115A-HA re	GTCCCCGTTGTTATAGCGGCACTGTCCCGGTT
23	UL42Y45A-HA fo	TGCAGCCCACCGCCCGCTCGACCCCCGTACTG
24	UL42Y45A-HA re	CAGTACGGGGGTCGAGCGGGCGGTGGGCTGCA
25	PIV2V-HA fo	ATCGTCAGATCTATGGCCGAGGAACCAACATAC

26	PIV2V-HA re	AGGAATTCTCACGCGTAATCTGGAACATCGTATGGGTAATGATCTCCTTCACATTCTCC
27	STAT2-SPA fo	CCTCGAGATGGCGCAGTGGGAAATGCTGC
28	STAT2-SPA re	GGCGGCCGCGAAGTCAGAAGGCATCAAGGGTC
29	Jak1-SPA fo	ATCGTCCTCGAGATGCAGTATCTAAATATAAAA
30	Jak1-SPA re	ATTGCTCATATGTTTTAAAAGTGCTTCAAATCC
31	NB-SPA fo	TTTAAGAAAATCTCATCCTCCGGGG
32	NB-SPA re	CTTGTCATCGTCATCCTTGTAGTCGA
33	NB-UL42-HA fo	ATCGTCAAGCTTAAAAGTTGGCAGGTATTTGTG
34	NB-UL42-HA re	CGGCGAACTAACCAGGCAGTACGGGGGTCGATA
35	m13	GTAAAACGACGGCCAGTG
36	CMVfwd	CGCAAATGGGCGGTAGGCGTG

4.1.6 Plasmids

pcDNA3.1(+)	(Invitrogen)
pIRES eGFP	(Dr. Vu Thuy Khanh Le-Trilling, Uniklinik Essen)
pIRES-pM27-Flag	(Prof. Dr. Mirko Trilling, Uniklinik Essen)
PMZ3F vector	(Zeghouf et al., 2004)

Table 5: Plasmids

Name	Primer Pair
pcDNA3.1(+):STAT2	1 and 2
pcDNA3.1(+):STAT2-TK	3 and 4
pcDNA3.1(+):TK	5 and 6
pcDNA3.1(+):TK-Jak1	7 and 8
pcDNA3.1(+):UL42-HA	9 and 10
pcDNA3.1(+):UL42-DeltaN-HA	11 and 12
pcDNA3.1(+):UL42-FS-HA	13 and 14
pcDNA3.1(+):UL42-C26S-HA	15 and 16

pcDNA3.1(+):UL42-C40S-HA	17 and 18
pcDNA3.1(+):UL42-C50S-HA	19 and 20
pcDNA3.1(+):UL42-N115A-HA	21 and 22
pcDNA3.1(+):UL42-Y45A-HA	23 and 24
pIRES:eGFP-PIV2V-HA	25 and 26
PMZ3F:STAT2-SPA	27 and 28
PMZ3F:Jak1-SPA	29 and 30

4.1.7 Antibodies

mAb anti-ß-actin:	Sigma [#A1978]
rabbit polyclonal anti-HA:	Sigma [#H6908]
pSTAT1:	Cell Signaling [#7649]
STAT1:	Santa Cruz [#sc-346]
pSTAT2:	MERCK [#07-224]
STAT2:	Santa Cruz [#sc-476]
Jak1:	BD [#610232]
IE1-pp72:	MERCK [#MAB810R]
pp65 (CH12):	Abcam [#ab53489]
GAPDH:	Santa Cruz [#sc-25778]
Flag:	Sigma [#3165-1mg]

4.1.8 Bacteria

XL1-Blue:	*F´::Tn10 proA+B+ lacIq Δ(lacZ)M15/recA1 endA1 gyrA96 (Nalr) thi hsdR17 (rK- mK+) glnV44 relA1 lac*
DM1 (Invitrogen):	*F- dam-13::Tn9(CmR) dcm- mcrB hsdR-M+ gal1 gal2 ara- lac- thr-leu- tonR tsxR Su0*

4.1.9 Human cell lines

4.1.9.1 Commercially available cell lines

MRC-5	Human foetal lung fibroblasts, ATCC CCL-171
HeLa	Human cervix carcinoma-cell line, ATCC CCL-2
HEK293T	Human foetal kidney cells, ATCC CRL-3216
U373	Human astrocytoma-cell line, ATCC HTB-17
2fTGH	Human sarcoma-cell line
U4A (Jak1-deficient 2fTGH)	(McKendry et al., 1991)
U6A (STAT2-deficient 2fTGH)	(McKendry et al., 1991)

4.1.9.2 Transgenic cell lines

HeLa STAT2-TK (G418 resistant)

$TK^{-/-}$ STAT2-TK (G418 resistant)

U373 STAT2-TK (G418 resistant)

U373 STAT2-SPA (G418 resistant)

HEK293T TK-Jak1 (zeocin resistant)

U373 Jak1-SPA (G418 resistant)

4.1.10 Viruses

HCMV-TB40/E	(Sinzger et al., 1999)
HCMV-TB40/E Δ42-43	(PD Dr. Albert Zimmermann, HHU, Düsseldorf)
HCMV BAC2	(Dr. Vu Thuy Khanh Le-Trilling, UK Essen)
HCMV BAC2 UL42mut	(Dr. Vu Thuy Khanh Le-Trilling, UK Essen)

4.2 Methods

4.2.1 Cell culture

All infectious and non-infectious cell culture work was spatially separated. The cells were incubated at 37°C, 80% humidity and a CO_2-concentration of 5%.

The used D-MEM culture medium was decanted and the adherent cells were washed with pre-warmed (37°C) PBS. Afterwards, the cells were detached from the flask with trypsin (0.5% in PBS; both from Invitrogen). The cells were then re-suspended in fresh, pre-warmed 37°C D-MEM culture medium and transferred into a new culture flask, mostly by a factor of 1/5 or 1/8.

Selection antibiotics (Geneticin or Zeocin) were supplemented to the cell culture medium of the generated transgenic cell lines.

4.2.1.1 Cryo-conservation of human cells

Human cells were frozen in the logarithmic phase of their growth and with a confluence of at least 75%. Adherent cells were washed and detached from the cell culture flask as described previously. Then, cells were re-suspended in fresh, pre-warmed 37°C D-MEM and centrifuged (70g, 23°C for 10 min; Beckman-Coulter). Cell pellets were re-suspended in a freezing medium (50% FCS, 40% D-MEM and 10% DMSO) and transferred into cryotubes (Nunc). Cells were then slowly (-1°C/min) frozen in cryo-freezing containers (Qualifreeze, Qualilab) at -80°C before storing them in liquid nitrogen.

4.2.1.2 Transfection of human cells

The different cell types were transfected with Superfect reagent (Qiagen) or Polyethylenimine (PEI) [1 mg/ml stock solution; 3µg PEI per 1µg DNA]. All transfections were performed according to the manufacturer's protocol.

4.2.1.3 Production of transgenic cell lines

Different human cell lines (HeLa, $TK^{-/-}$, U373 and HEK293T) were transfected with the plasmid of interest (e.g. STAT2-TK). On the next day the cells were washed and detached from the wells as described previously. The cells were re-suspended in fresh D-MEM supplemented with a specific selection antibiotic (G418 or zeocin, respectively). All cells were transferred to a hybridoma dish and incubated for several days. The cells were regularly washed with pre-warmed PBS and D-MEM supplemented with the selection antibiotic. The cells were then washed and detached

from the hybridoma dish and transferred into a 96-well-plate. This procedure was repeated until the cells could be grown in a 25 cm^2 flask and were ready to be tested for the expression of the target gene.

For all generated cells a non-transfected control was prepared to ensure the effect of the selection antibiotic.

4.2.1.4 Testing the transgenic cell line ganciclovir (GCV) susceptibility

Transgenic cell lines expressing the herpesviral thymidine kinase are not able to survive when GCV is added to the culture medium. To assess the minimal lethal dose of GCV, 10^4 parental cells or transgenic cells were seeded into a 12-well- plate, treated with different concentrations (0 to 5 µM) of GCV and incubated for 96 h. Afterwards, the medium was aspirated and the cells were stained with Crystal Violet (0.05% (w/v) in 20% (v/v) ethanol) and washed with pre-warmed 37°C PBS. The cells were then washed with 200 µl 100% methanol to extract the Crystal Violet, from which 100 µl were transferred into a 96-well ELISA plate (Nunc) to be analysed by an ELISA reader (TECAN Sunrise) at 540 nm.

For the identification of STAT2-TK degrading proteins, cells were transfected with PIV2V or pM27, respectively, 1 day prior to the treatment with GCV.

4.2.1.5 Single cell screen of HCMV ORF collection

To find an unknown HCMV antagonist, the transgenic cells were transfected in duplicates ON with one plasmid expression ORF (Salsman et al., 2008) or with a non-coding control plasmid. Afterwards, the cells were washed and detached from the wells and re-suspended in 10 ml fresh D-MEM. 1 ml of the cells was transferred in 1 24-well (9 wells per transfection in total) and incubated in triplicate either with 0 µM, 0.25 µM or 2.5 µM GCV for 96 hours. Afterwards the cell viability was assessed as described previously (4.2.1.4).

4.2.1.6 Global screen of lentiviral cDNA library

Production of lentiviral particles, transduction of HEK293T TK-Jak1 and sorting of infected cells was performed by Dr. Marco Maywald, Heinrich-Heine University Düsseldorf. Hek293T cells were co-transfected with lentiviral vectors and helper plasmids coding for VSV-G and gag/pol (pcz-VSV-G; pCD/NL-BH). After over-night incubation, cell culture medium was changed and cells were incubated for a further night (37°C, 5% CO_2). 2 ml of filtered supernatant (0.45 µm filters, Sarstedt) was added to 1.5 ml cell culture medium and protamine sulfate (10 µg/ml) and TK-Jak1 cells were infected. After two days of incubation, sorting of cells was performed with the MoFlo XDP (Beckman-Coulter) at the Core Flow Cytometry Facility at the Institute of Transplantation Diagnostics and Cell Therapeutics, HHU Düsseldorf. Cells were treated as described previously

(without permeabilization), filtered (Celtrics 50µm, Sysmex Pardec) and transferred to sterile FACS tubes (BD). Sorted cells were then incubated with cell culture medium (DMEM supplemented with 10% (v/v) FCS, streptomycin, penicillin and 2 mM glutamine + zeocin [1 mg/ml] and GCV [250nM])

4.2.1.7 Cytometry

Cells were incubated in 0.5% (w/v) trypsin (Invitrogen) in PBS and taken up in FACS buffer (10 x PBS + 3% FCS). After washing with FACS buffer (1500 rpm, 3 min, 4°C (Eppendorf, 5810R)), half of the cells were fixed with 3% (w/v) paraformaldehyde (10 min at RT) and permeabilized with saponin (0.05% in FACS buffer) for 10 min at 4°C. Cells were incubated with specific antibodies (anti-STAT2, L-20, Santa Cruz; anti-HA, H3663, Sigma-Aldrich) for 30 min at 4°C. After additional incubation (APC-conjugated goat anti-mouse Ig, BD; biotinylated donkey anti-rabbit IgG, Jackson ImmunoResearch; Streptavidin-FITC, BioLegend) and washing steps, cells were taken up in 200-300 µl FACS buffer and measured by FACSCanto II (BD).

4.2.2 Virological methods

4.2.2.1 Preparation of a HCMV virus stock

For the purification of a HCMV virus stock, 20 175 cm^2 cell culture flasks MRC-5 cells were infected with HCMV (~ MOI 0.05). After 9 days, MRC-5 cells were harvested and centrifuged for 15 min at 5000 g at 10°C (J2-21 centrifuge). Afterwards, the cell supernatant was centrifuged for 3 h at 20000 g at 10°C (J2-21 centrifuge). The virus pellet was re-suspended in 7 ml D-MEM, dounced and placed on a 20 % sorbitol cushion. The sorbitol cushion was centrifuged for 1 h at 60000 g at 10°C (OptimaL-K70 Beckman Ultracentrifuge), re-suspended in 1-2 ml cold PBS (depending on the size of the pellet), dounced and transferred into 20 µl aliquots, which were stored at -80°C.

4.2.2.2 Virus titration of a HCMV virus stock

The virus stock used for titration was frozen at -80°C and slowly thawed at 4°C just before the titration. The titration is performed on MRC-5 cells on a 96-well-plate with a pre-diluted virus stock (2 µl virus stock solution in 198 µl D-MEM) and a subsequent serial dilution of 1/10 (20 µl virus solution in 180 µl D-MEM). The infection was enhanced by centrifugation (see 4.2.2.3 - HCMV infection). After 3 days, HCMV plaques were stained. Therefore, cells were fixed with 100 µl ice-cold methanol (-20°C) per well and incubated over night at -20°C. On the next day, the methanol was removed and the cells were dried under the bench. After washing cells with PBS,

cells were incubated for 1 h at RT with 50 µl primary antibody (1/200 [v/v] in 1% [w/v] skim-milk TBST; Clone CCH2, Dako, directed against HCMV-antigen p52). After incubation, cells were washed 3 times with PBS and incubated for 1 h at RT with secondary, peroxidase-linked anti-mouse antibody (1/200 [v/v] in 1% [w/v] skim-milk TBST; Sigma). Peroxidase-activity was detected with 3-Amino-Ethylcarbacol (AEC, Sigma), resulting in a red precipitate in infected cells. After washing 3 times with PBS, 50 µl substrate (1/4 AEC tablet in 1 ml N,N-dimethylformamide, 4 ml 0.1 M Na-Acetate [pH 5.5] and 10 µl 30% [v/v] H_2O_2) per well was given to the cells and incubated for 30 minutes at RT. The reaction was stopped by washing with PBS. The titration was analysed via the light microscopy.

4.2.2.3 Infection of cells with HCMV

All infections with HCMV were enhanced via centrifugal enhancement. Two-times centrifugation at 23°C at 800g for 15 min (Beckman-Coulter Allegra) of the cell culture plates increases the infectivity of the virus 10- to 100-times (Osborn and Walker, 1968).

4.2.3 Molecular biological methods

Restriction of plasmids and PCR products by restriction enzymes (NEB), agarose gel electrophoresis, ligation of plasmids and isolation of DNA were performed according to Sambrook *et al.* (1989), Sambrook and Russell (2001) or via kits according to the manufacturer's protocol.

4.2.3.1 Cloning of thymidine kinase fusion proteins

For the generation of the STAT2-TK fusion protein, MRC-5 RNA was used as a template in an RT-PCR to clone the human STAT2 (primer pair #1 and #2). The PCR product was cloned into a pcDNA3.1(+) (Invitrogen) via *Not*I – *Xba*I digestion. As a template for the HSV1 TK, DNA of HSV1 infected HeLa cells was used (primer pair #3 and #4). The PCR product was inserted after an *Xba*I digest into the pcDNA3.1(+):STAT2 vector.

For the production of the TK-Jak1 fusion constructs, DNA of HSV1 infected HeLa cells was used as a template in a PCR (primer pair #5 and #6). The PCR product was inserted into pcDNA3.1(+) via a *Not*I – *Xba*I digestion. The human Jak1 was cloned by an RT-PCR, using MRC-5 RNA as a template (primer pair #7 and #8). The PCR product was inserted via an XbaI digest into the pcDNA3.1(+):TK vector.

Prior to the second *Xba*I digest, the pcDNA3.1(+) plasmids were transformed in dam-methylation incompetent *dm1-E. coli*, to remove methylation from the *Xba*I restriction sites.

Protocol for RT-PCR (Qiagen):

Template	0.5 µl	50°C	30 min	
5 x reaction buffer	4.0 µl	95°C	15 min	
Primer 1	1.2 µl	94°C	30 s	
Primer 2	1.2 µl	55°C	30 s	
dNTPs	0.8 µl	72°C	45 s	30 cycles
Enzyme mix	0.8 µl	72°C	10 min	
ddH20	11.5 µl			
Total	20 µl			

Protocol for PCR (Phusion polymerase):

Template	0.5 µl	98°C	10 min	
5 x reaction buffer	4.0 µl	98°C	30 s	
Primer 1	0.8 µl	55°C	30 s	
Primer 2	0.8 µl	72°C	1 min	38 cycles (1000 bp per min)
dNTPs	1.0 µl	72°C	10 min	
Polymerase	0.2 µl			
ddH20	12.7 µl			
Total	20 µl			

4.2.3.2 Cloning of Tandem-Affinity-Purification (SPA) constructs

For the production of STAT2 and Jak1 constructs, that can be used for immuno-precipitation or affinity purification techniques, PMZ3F vectors (Zeghouf et al., 2004) were kindly provided by the lab of Jack Greenblatt of the University of Toronto.

The vectors pcDNA3.1(+):STAT2 and pcDNA3.1(+):TK-Jak1 were used as template in a PCR (primer pairs #9 and #10 or primer pairs #11 and #12, respectively). The PCR was performed as described previously. The PCR products were inserted into the PMZ3F vectors by *Xho*I and *Not*I or *Xho*I and *Nde*I restriction digest, respectively. Functional protein expression was examined by Western Blot analysis with a mouse anti-Flag antibody (Sigma).

4.2.3.3 Production of chemically competent E. coli ($CaCl_2$ method)

To express the created fusion proteins, chemically competent *E. coli XL1 Blue* were made chemically competent. Therefore, 100 ml LB medium were incubated with 3 ml of overnight culture at 37°C and grown to an optical density of 0.5. Bacteria were centrifuged for 10 min at 4°C at 4000 rpm (Allegra X-15R, Beckman Coulter) and placed on ice. The supernatant was decanted and the bacteria were re-suspended in 50 ml 0.1 M $MgCl_2$. Afterwards the bacteria were centrifuged for 10 min at 4°C at 4000 rpm and the supernatant was decanted. The pellet was re-suspended in 50 ml 0.05 M $CaCl_2$ and centrifuged again. After centrifugation the pellet was re-suspended in 2 ml 0.05 M CaCl with 15% (v/v) Glycerol and frozen in 50 µl aliquots at -70°C.

4.2.3.4 Site-directed mutagenesis

To remove or introduce point mutations from/into plasmid DNA, Quikchange® mutagenesis (Agilent) was performed according to the manufacturer's protocol. After the PCR reaction, DNA was precipitated with 50 µl isopropanol and washed with 70% (v/v) ethanol. Afterwards, digestion with *Dpn*I (NEB) was performed prior to transformation into *E .coli* XL1 Blue. Point mutations were verified by DNA sequencing.

4.2.3.5 RNA methods (Isolation of cell RNA and Northern Blot)

Cells were permeabilized using QIAShredder-columns (Qiagen). The RNA was isolated using the RNeasy Mini Kit (Qiagen). All methods were performed according to the manufacturer's protocol. All samples were treated exclusively with filter pipette tips and RNase-free buffers to prevent degradation of RNA.

For Northern Blot analysis, cellular RNA was separated and detected with DIG-Easy-labelled probes on a nylon membrane (Hybond NX, Amersham). For each condition, ~ 6 x 10^6 cells were used (6-well-plate). After isolation, the RNA was separated in an agarose gel, containing 10% (v/v) 10 x MEN and 5% (v/v) formaldehyde at 400 mA, 60 V for 2 hours, using running buffer containing 10% (v/v) 10x MEN. RNA sample buffer was added to the RNA and the probes were denaturized for 10 min at 70°C prior to gel loading. Comparable RNA amounts were afterwards controlled by UV exposure and comparison of the 28S and 18S rRNA bands. The agarose gel was then incubated in 20 x SSC during Northern Blot assembly. RNA is blotted on the nylon

membrane through capillary force therefore paper towels and 3 sheets of dry Whatman paper are place in a reservoir. One sheet of Whatman paper, equilibrated with 20 x SSC, is placed on top, followed by the nylon membrane, equilibrated in 20 x SSC. Then, 3 sheets of Whatman paper, equilibrated with 20 x SSC, are connected with Whatman paper to two reservoirs containing 20 x SSC buffer and the system is closed. Blotting was performed ON.

After blotting, the RNA is cross-linked to the nylon membrane and washed 2 times with H_2O. Then, the nylon membrane is hybridised with DIG-Easy for 2 hours at 55°C, followed by incubation with a specific DIG-labelled probe ON at 55°C. The specific probe was produced by PCR using DIG-labelled dNTPs (primer pairs #33 and #34). After incubation, the nylon membrane is washed 2 times with stringent wash #1 followed by 2 times incubation for 20 min at 68°C with stringent wash #2. After short washing with H_2O, the nylon membrane is incubated for 30 min with 1 x blocking solution in maleic acid buffer. Then, the nylon membrane is incubated with anti-digoxigenin antibody (1:20000) in blocking solution for 30 min and washed 3 times for 10 min with maleic acid buffer. For detection, the nylon membrane is dried using Whatman paper, incubated with CDP Star (Sigma) for 5 min, dried again and detected using photo films (GE Healthcare).

4.2.4 Protein biochemical methods

4.2.4.1 Preparation of whole cell lysates

Adherent human cells were detached from the cell culture well with a cell scraper (Renner) and centrifuged for 3 min at 4°C at 3000 rpm (Eppendorf 5417R). The supernatant was aspirated and the cell pellets were washed 3 times with cold (4°C) PBS and centrifuged for 1 min at 4°C at 3000 rpm (Eppendorf 5417R). After the last washing step, the pellet was re-suspended in 100 µl ice-cold $RIPA^+$ buffer and stored at -20°C. After thawing, the samples were centrifuged for 25 min at 4°C at 13000 rpm (Eppendorf 5417R) and the pellet was removed. Cell pellets were stored one ice throughout the complete experimental procedure. The supernatant was used as whole cell protein lysate. Usage of phosphatase inhibitors NaF and Na-Vanadate enabled the detection of phosphorylated proteins.

4.2.4.2 Bradford assay

To normalize protein amounts for analysis, whole cell lysate supernatants were photometrically measured using Bradford reagent. To do so, 3.5 µl of the supernatant were given to 996.5 µl Bradford reagent; all samples were measured in the linear range (OD values of 0.1 – 0.7). Optical density was measured at 595 nm wavelength in a spectrophotometer (UV mini 1240, Shimadzu). Lysates were equalized (in respect to the lowest protein amount) with their respective buffer.

Equal protein loading was confirmed with Ponceau red staining and β-actin protein amounts on the membrane.

4.2.4.3 SDS-PolyAcrylamide-Gel-Electrophoresis (SDS-PAGE)

With this method, proteins can be separated in an electric field by the pores in a polyacrylamide gel by their respective molecular weight. All proteins obtain a negative charge through the decoration with SDS (Laemmli, 1970).

For the separation of the proteins two different gel types (separation and stacking gel) were prepared. The following table illustrates the pipetting scheme for the two gel types:

All protein lysates were denaturized for 5 minutes at 95°C with SDS sample buffer containing β-mercapto-ethanol. 15 – 50 µl of the sample were applied to the polyacrylamide gel, separating the proteins at 20 mA, 400 V for 90 min in a small gel or at 12 mA, 150 V ON in a medium gel, respectively.

		2 small gels				2 medium gels			
		separation			stacking	separation			stacking
		8%	10%	12%		8%	10%	12%	
30% PAA	[ml]	3,2	4	4,8	1	25,6	32	38,4	6
2M Tris/HCl (pH 8.8)	[ml]	2,5	2,5	2,5		20	20	20	
0.5M Tris/HCl (pH 6.8)	[ml]				0,8				4,8
20% SDS	[µl]	60	60	60	30	480	480	480	180
60% Saccharose	[ml]				1,4				8,4
dH_2O	[ml]	6,1	5,3	4,5	2,8	48,8	42,4	36	16,8
TEMED	[µl]	24	24	24	8	192	192	192	48
10% APS	[µl]	144	144	144	80	1000	1000	1000	240

4.2.4.4 Western Blot

To investigate properties (e.g. degradation or up-regulation) of specific proteins, the separated proteins in the polyacrylamide gel can be transferred onto a nitro-cellulose membrane.

To do so, three sheets of Whatman paper are soaked with 1 x Western Blot buffer and placed onto the Western blotter (Biometra). A sheet of nitro-cellulose membrane is soaked with Western Blot buffer and placed on top of the Whatman paper. The gel is then placed onto the

nitro-cellulose membrane, followed by another layer of three sheets soaked Whatman paper. The proteins are blotted at 1000 mA, 14 V for 65 min. Complete transfer of the proteins is checked by proper transfer of the protein marker (Spectra BR, Thermo Scientific) and Ponceau staining. The nitro-cellulose membrane is shortly washed in 1 x TBST and then incubated in 5% (w/v) skim milk TBST for 1 h. Afterwards a specific primary antibody directed against the protein of interest, e.g. STAT2, is given onto the membrane (according to the manufacturer's dilution). After incubation for 1 h with primary antibody, the membrane is washed 3 times with 1 x TBST and incubated for 1 h with peroxidase-linked antibody (anti-mouse 1:10000, anti-rabbit 1: 5000). As a last step, the membrane is washed 3 – 4 times with 1 x TBST.

Detection of the proteins is achieved using ECL solution (GE Healthcare). Solution A and B are mixed in the ratio 1:1 and the nitro-cellulose membrane is incubated for 3 – 5 min. After drying the membrane with Whatman paper, the proteins bands can be visualized using film (GE Healthcare).

4.2.4.5 Deglycosylation

Cells were transfected with pcDNA3.1(+)UL42-HA and whole cell lysates were prepared. Cell lysates were treated with EndoH or PNGase F (Roche) according to standard methods and subjected to western blotting.

5. References

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Appendix

Suppl.-Table 1: Overview of HCMV ORFs eliciting a slight growth advantage upon GCV treatment

GCV concentration	HCMV ORF	gene class
0.25 µM	TRL14	L
	US22	E
	US28	E
	US30	E
	UL3	L
	UL33	E-L
	UL41	L
2.5 µM	TRL9	L
	TRL14	L
	US22	E
	US23	E
	US30	E
	UL3	L
	UL12	n.a.
	UL33	E-L
	UL41	L
	UL56	E
	UL77	E
	UL99	L
	UL127	n.a.

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Diese Arbeit wurde weder in der vorgelegten noch in einer ähnlichen Form bei einer anderen Institution eingereicht.

Düsseldorf den 05.12.2016

(Sebastian Howe)