Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Vegetal

# Study of a conserved herpesvirus gene inducer of cell cycle arrest and IL-8

**Diogo Bernardes Dias**

Dissertação

Mestrado em Microbiologia Aplicada

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Orientadores: Professora Maria Filomena Caeiro
Doutora Rute Nascimento

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# Index

**Acknowledgements** ..... i

**Abbreviations** ..... iii

**Resumo** ..... iv

**Abstract** ..... vii

**Introduction** ..... 1

**Objectives** ..... 11

**Materials and Methods** ..... 12

**Results** ..... 18

**Discussion** ..... 25

**Concluding Remarks and Future Perspectives** ..... 29

**References** ..... 30

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# Abbreviations

| | |
|---|---|
| ATM | Ataxia telangiectasia mutated |
| IFN | Interferon |
| DAPI | 4’,6-diamidino-2-phenylindole |
| DDR | DNA Damage Response |
| ELISA | Enzyme-Linked immunosorbent assay |
| G2M | G2 phase/ Mitosis cell cycle arrest |
| IL-8 | Interleukin-8 |
| HA | Haemaglutinin peptide |
| HCMV | Human cytomegalovirus |
| HSV-1 | Herpes simplex virus 1 |
| MHV-68 | Murine herpesvirus strain 68 |
| NEMO | NF-kB essential modulator |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| p21 | Cyclin-dependent kinase inhibitor 1 |
| p53 | Tumor supressor p53 |
| PAMP | Pathogen associated molecular patterns |
| PBS | Phosphate buffer saline |
| PVDF | Polyvinylidene difluoride |
| SDS-PAGE | Sodium dodecyl sulfate polyacrulamide gel |
| SUMO | Small ubiquitin-like modifier |
| TNF-α | Tumor necrosis factor alpha |

## Resumo

O gene UL76 do citomegalovírus humano (HCMV) pertence a uma família de genes homólogos conservados em todos os herpesvírus, envolvidos na evasão aos mecanismos de defesa do hospedeiro, denominada família de genes UL24. Esta família é alvo de estudo devido ao facto de permanecer a única família de genes homólogos ao qual ainda não foi associada nenhuma função principal. No entanto, a sua conservação e simultânea falta de homologia com genes celulares indicam de que se trata de uma família com um papel determinante no ciclo de vida dos herpesvírus. O trabalho que tem sido feito em relação a esta família visa elucidar a comunidade científica quanto aos mecanismos empregues pelos herpesvírus para escapar à detecção pelo sistema imune do hospedeiro e estabelecer uma latência que se pode prolongar durante grande parte da vida desse mesmo hospedeiro. É esta capacidade que torna os herpesvírus nunm dos grupos de patogéneos mais prevalentes e eficientes da Humanidade.

No que toca ao UL76, descobertas anteriores reportam que as principais funções associadas a este gene em particular incluem a capacidade de por si só ser capaz de induzir uma paragem no ciclo celular na transição entre a fase G2 e mitose (G2M), bem como um papel crucial na indução e manutenção da expressão da interleucina-8 (IL-8). Do ponto de vista do vírus, ambas estas acções são benéficas para o seu ciclo replicativo de vida. A paragem no ciclo celular permite à célula manter um microambiente membranar estruturado favorável à replicação viral, microambiente este que seria totalmente perdido se à célula fosse permitido entrar na fase mitótica. Enquanto esta última função beneficia a replicação do vírus, a indução de IL-8 actua ao nível da sua propagação já que esta interleucina específica é responsável pela quimioatracção de neutrófilos, os quais são infectados pelo vírus e utilizados na sua disseminação pelo hospedeiro. Enquanto a capacidade para induzir paragem no ciclo celular se encontra conservada em todos os genes da família UL24, a indução de IL-8 não se encontra propagada pelos homólogos desta mesma família. Contudo, ambas estas acções derivam da habilidade anteriormente demonstrada que o UL76 do HCMV tem de induzir danos no DNA, que levam a uma activação e resposta adequada por parte da via de *DNA Damage Response*. A activação da via previamente referida é dependente de ATM, uma cinase de serina/treonina que é activada por quebras em dupla cadeia no DNA, responsável pela transdução de um sinal que resulta na fosforilação de várias proteínas alvo que levam a várias respostas incluindo reparação do DNA e paragem no ciclo celular. A ATM inicia então uma via de sinalização em

cascata que resulta na activação de p53, um regulador do ciclo celular responsável pela manutenção da integridade genómica. Este factor controla uma proteína denominada p21, que por sua vez regula várias etapas nas transições do ciclo celular. Após activação de p53 e consequente activação de p21, este último é translocado para o núcleo onde participa então na transcrição de genes responsáveis pela resposta adequada a danos no DNA, culminando estes eventos numa paragem G2M no ciclo celular. Para além disso, os dados no DNA causados pelo UL76 podem ainda ser reconhecidos pela via de sinalização NF-kB, a qual levará à indução da expressão de IL-8. NF-kB é um complexo proteico formado por factores de transcrição cuja via de sinalização participa em vários mecanismos de resposta a stress, como por exemplo respostas imunitárias e inflamatórias. Na ausência de estímulo este complexo proteico encontra-se inactivo pelas proteínas inibitórias IkB. Após estimulação ocorre fosforilação do complexo IKK e da proteína regulatória NEMO, que formam um complexo responsável pela posterior fosforilação das proteínas IkB que se tornam assim alvo de degradação. Deste modo o complexo NF-kB torna-se activo e desloca-se para o núcleo a fim de induzir a expressão de genes alvo. O UL76 actua através da via genotóxia do NF-kB, que resulta num sinal que leva à translocação de NEMO para o núcleo, onde é modificada e complexada com a ATM. Este complexo é exportado para o citoplasma e activa o complexo IKK que fosforila então a proteína inibitória IkB, libertando o complexo NF-kB que se dirige assim para o núcleo levando assim à indução da expressão de IL-8.

Neste trabalho o meu papel foi o de determinar quais dos domínios do UL76 são responsáveis pelas funções previamente referidas, nomeadamente a indução de paragem celular em G2M e de IL-8, as principais funções associadas a este determinado gene. Estudos recentes detalham a presença de cinco domínios conservados em todos os herpesvírus localizados na região N-terminal de UL76, enquanto a região C-terminal demonstra ser altamente variável. Deste modo é possível verificar que o existe uma região N-terminal conservada e uma região C-terminal não-conservada, o que à partida se ajusta ao facto do UL76 possuir duas funções principais. Funções essas que são a capacidade de induzir paragem no ciclo celular na transição G2M, capacidade partilhada por todos os homólogos da família UL24, e a indução da expressão de IL-8, específica para o UL76 do cítomegalovírus humano. O meu trabalho começou então por utilizar enzimas de restrição de modo a obter duas metades do gene UL76, que codificam para a região N-terminal e para a região C-terminal da proteína. Ambos os mutantes de delecção foram clonados em vectores de expressão que foram posteriormente alvos de ensaios de expressão,

nomeadamente *western blots* e ensaios de imunofluorescência de modo a averiguar se ambas as proteínas deletérias eram expressas e qual a sua localização celular. Após confirmação de expressão tanto da região N-terminal como da região C-terminal o próximo passo prendia-se com a determinação de qual das regiões, se alguma, era responsável pela indução de IL-8. De modo a estudar esta função recorreu-se a ensaios de luciferase para averiguar a actividade transcripcional do gene da luciferase sob o controlo do promotor de IL-8 e a ELISA para confirmar a presença da interleucina-8 em células transfectadas com os domínios previamente obtidos do UL76. Em ambos os ensaios foi possível concluir que a indução da expressão de IL-8 está apenas a cargo da região C-terminal não-conservada e não da região N-terminal conservada, o que tem lógica uma vez que esta função apenas está presente neste homólogo pertencente ao citomegalovírus humano.

Finalmente, ambos os mutantes foram sub-clonados em vectores de expressão para produção em lentivírus, de modo a serem alvo de ensaios de ciclo celular para determinar se algum dos mutantes, ou até mesmo ambos, eram de facto capazes de induzir paragem de ciclo celular em G2. A análise do ciclo celular através de FACS leva à observação que a capacidade de induzir paragem em G2M no ciclo celular se encontra presente apenas na região N-terminal conservada e não na região C-terminal não-conservada. Mais uma vez, esta descoberta segue a lógica correcta uma vez que a indução de paragem do ciclo celular é uma característica presente em todos os homólogos da família de genes UL24 dos herpesvírus.

Através dos resultados obtidos é possível concluir que neste trabalho se identificam as regiões onde as duas principais funções de UL76 se encontram codificadas, uma vez que os resultados demonstram claramente que a região N-terminal conservada é responsável pela paragem no ciclo celular em G2M, uma função conservada em todos os homólogos de UL24, enquanto a indução de IL-8 se encontra restrita na região variável C-terminal. Está então explicado desta maneira o porquê desta função particular se encontrar apenas no homólogo pertencente ao citomegalovírus humano.

## Abstract

The UL76 gene of the human cytomegalovirus belongs to the UL24 gene family, conserved in all herpesvirus. Previous work has demonstrated that UL76 induces a cell cycle arrest at G2/M, perhaps favouring virus replication, and also induces expression of IL-8, a cytokine known to enhance virus propagation. While the former function is conserved in all genes of the UL24 family, only the HCMV homologue UL76 induces expression of IL-8. Paradoxically, both the induction of cell cycle arrest and expression of IL-8 result from activation of the DNA Damage Response (DDR) pathway. Activation of the DDR is dependent on activation of ATM and the resulting signaling cascade that ends with the activation of p53, the translocation of p21 to the nucleus and the consequent transcription of target genes associated with the DDR, resulting in a cell cycle arrest. In addition, DNA damage by UL76 can also activate the NF-kB pathway, through the translocation of the NEMO-ATM complex, leading to activation of NF-kB and then transcription of IL-8.The obvious question that arises, and is addressed in this work, is whether both the cell cycle arrest and induction of IL-8 are controlled by the same or different domains of UL76.

Recent studies reveal the presence of five conserved sequences localized in the N-terminal region of UL76, while the C-terminal region is highly variable within the UL24 family. Therefore, deletion mutants of the UL76 gene corresponding to the N-terminal and the C-terminal regions were constructed and cloned into expression vectors which were then the subject of expression and functional assays. Western blots and immunofluorescence assays confirmed that the predicted products were in fact expressed in transfected cells. The entire UL76 gene as well as the N-terminal and C-terminal regions deletion mutants were then tested for their impact on G2/M progression (cell cycle analysis through FACS inspection of propidium iodide labeled cells) and IL-8 expression (luciferase reporter assays and ELISAs for secreted IL-8).

The results clearly show that the conserved N-terminal region is responsible for the cell cycle arrest at G2, an observation consistent with conservation of the N-terminal region homologous domains and the finding that all homologues of the UL24 family induced G2/M cell cycle arrest. In contrast, induction of IL-8 is restricted to the C-terminal variable region, thus explaining why this particular function is not conserved amongst all genes of the UL24 family.

# Introduction

## 1) Herpesvirus

Herpesviruses consist of a large group of ubiquitous double-stranded DNA viruses which pose serious healthcare threats due to their involvement in a wide range of medical and veterinary pathologies [(1)]. Although most infections are asymptomatic, this particular group of viruses can be responsible for several diseases, particularly in immunocompromised hosts [(2)]. Herpesviruses achieve this by means of a primary infection in epithelial cells which will later result in an establishment of persistence to be maintained during a period of latency. This ability to remain in a latent phase for so long is one of the major characteristics of herpesviruses and constitutes a serious concern regarding this particular family of viruses since they become essentially invisible to the host immune system. Lack of detection by the immune defenses and failure to eradicate the pathogen can result in a lifelong persistent infection. Afterwards, pathological conditions will certainly arise sooner or later as a consequence of viral reactivation [(1)]. A particular feature of the herpesviruses is their large number of host evasion genes, which have evolved in order to manipulate host cell biology and immunity to ensure successful virus propagation [(2)].

Another hallmark of this group that separates them from all the other known viruses is their distinct virion morphology which is comprised by the core, the capsid, the tegument and the envelope to form a spherical shape. The core is made up of linear double-stranded DNA within an icosahedral protein capsid, which in turn is surrounded by a protein matrix called the tegument. Finally, these structures are then encased in a lipid envelope composed of several glycoproteins [(3)]. All herpesviruses possess a life cycle consisting of two phases, a lytic phase during which the virus replicates and a latent phase where it remains dormant.

After establishment of the lytic phase, as well as in viral reactivation after latency, most genes are expressed in a cascade manner with the immediate-early genes being first, since their expression is not dependent on viral protein synthesis. Later on follows the expression of the early genes, encoding for enzymes involved in DNA replication and then expression of the late genes. The latter rely on viral DNA synthesis for their expression and encode the proteins needed for virion assembly [(4)]. Replication of herpesviruses occurs in the nucleus where the newly replicated viral genome is packaged into the assembled capsid. After packaging of the genome, the aforementioned nucleocapsid is translocated to the cytoplasm through changes in

nuclear architecture involving a wide range of viral and host proteins, first by budding at the inner nuclear membrane and later fusing with the outer nuclear membrane. At this point, tegument proteins bind to the nucleocapsid in order to form the envelope so the fully functional virion can be assembled. It will later be released by infected cells during the lytic phase of the herpesvirus life cycle. In contrast, the latent phase is characterized by almost no gene expression and lack of virion formation [(5)]. Figure 1 depicts several of the previously mentioned characteristics.

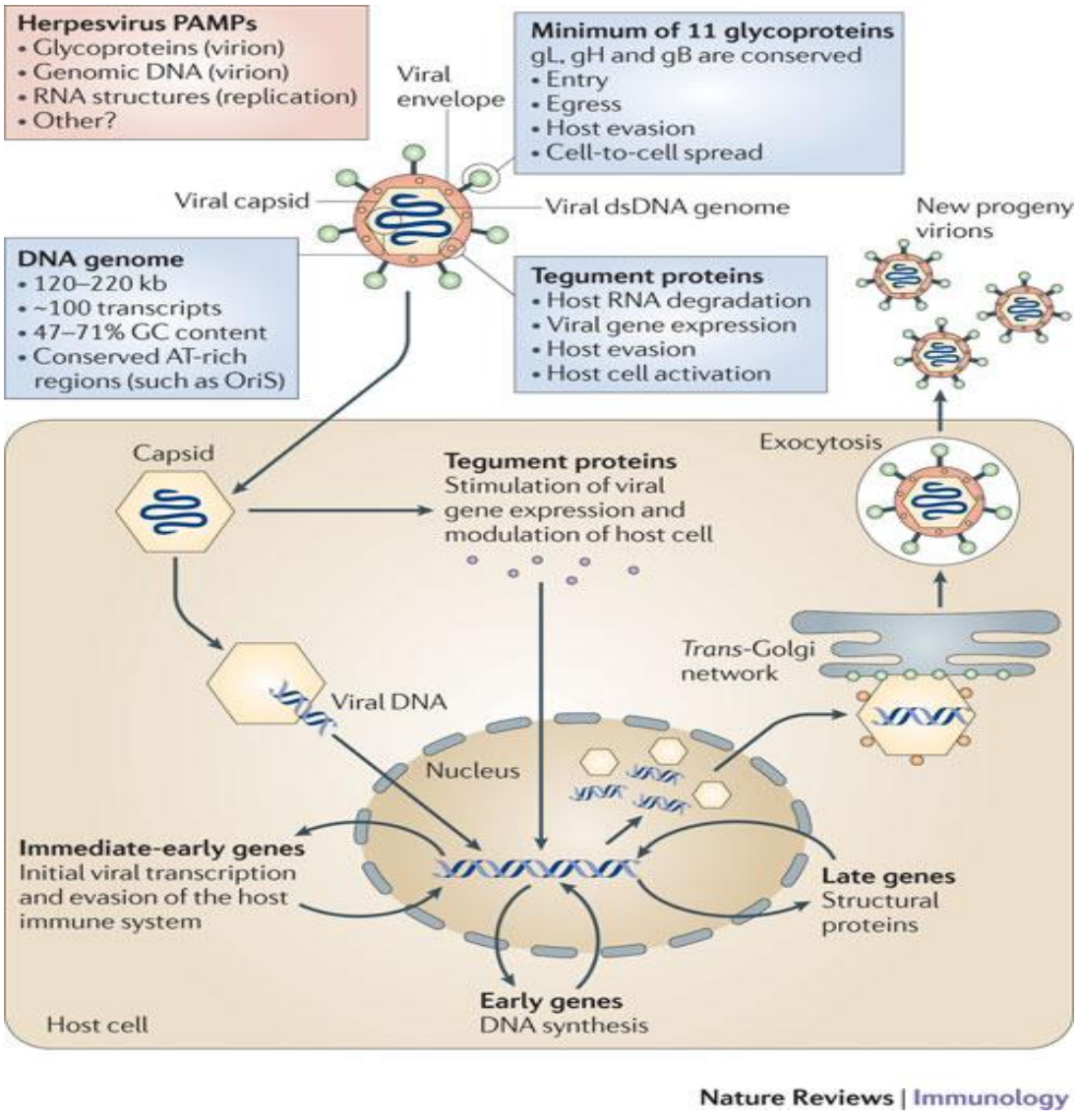


**Figure 1** – Herpesvirus life cycle and characteristics (adapted from Paludan, 2001).

According to taxonomical classification regarding herpesviruses, there are three subfamilies within the family *Herspesviridae* which comprise all known herpesvirus, defined according to their biological properties such as host range and growth kinetics. These are divided into the alpha, gamma and beta *herpesvirinae* subfamilies, which contain viruses responsible for infecting mammals, reptiles and birds. When it comes to human hosts, eight different herpesviruses have been identified, present in all three subfamilies [(6)]. All sub-families share a set of approximately forty conserved core genes which have been shown to be mostly associated with essential aspects of lytic replication [(7)]. Regardless of the common features of all members pertaining to the three subfamilies of herpesviruses, they all differ drastically when it comes to establishing latency and in which cell type they do it in, being that the reactivation of a persistent infection is responsible for the varied pathologies observed with these types of viruses.

## 2) HCMV

My work has been focused exclusively on the human herpesvirus 5, commonly known as the human cytomegalovirus (HCMV), the representative of the beta subfamily and the largest known human herpesvirus, with a linear double-stranded DNA genome of about 230kbp [(8)]. It represents an interesting case study for its high prevalence in human populations and for being the major pathogen responsible for congenital viral infection in humans as well as the leading cause in many birth defects.

Even though primary infection with HCMV is usually asymptomatic in healthy individuals, infection or reactivation in immunocompromised individuals can result in severe of fatal illness [(9)]. Its designation stands for the unique cytopathogenic effect it exerts in infected cells, which end up with a round and enlarged morphology designated cytomegaly [(10)]. HCMV is capable of infecting nearly every organ of the human host since viral replication involves a wide range of human cells including epithelial cells, endothelial cells, fibroblasts and smooth muscle cells [(11)]. According to various studies, when it comes to latency and reactivation, HCMV achieves this through infection of cells belonging to the myeloid progenitors cell line, designated CD34+ [(12)]. Reactivation of the virus is closely associated with differentiation of the myeloid progenitors since viral lytic gene expression only occurs when CD34+ cells commit to the dendritic cell or macrophage cell line pathway [(13)].

### 3) Manipulation of host cell biology and immunity by HCMV

As was previously mentioned, the ability of herpesviruses to remain hidden from the host defenses is indeed what makes the study of their host evasion strategies crucial to understanding their pathogenesis. Host evasion by herpesviruses includes manipulation of cell biology at transcriptional as well as translational levels and manipulation of intracellular structures and cell cycle. As the host mounts its defenses to eliminate the pathogen, its defensive strategies are thwarted by the virus through deviation and inhibition of the host cell biology and innate as well as adaptive immunity. It thus becomes essential to identify and characterize HCMV host evasion genes which might be responsible for counteracting the host immune defenses. Identifying and understanding the functions of such host evasion strategies may lead to the development of vaccines and therapeutic tools to combat both acute and persistent viral infection. Many host evasion genes have been identified through their homology to cellular genes. However, of the large number of the pathogen evasion genes that lack homology, some will have also evolved for host manipulation. Identification of these non-homologous genes can only be achieved through functional assays.

#### 3.1 Impact on the Cell Cycle

One function associated with HCMV, and one that is conserved among all herpesvirus, pertains to the ability of these viruses to modulate the cell cycle. For example, the nuclear localizing ORF20 protein from MHV-68 [(14)], when expressed in human and murine cells, induces cell cycle arrest at the G2/M phase, followed later by apoptosis. During the G2 phase, the cyclin B/Cdc2 complex is kept in an “off” state since the Cdc2 protein is in the inactive phosphorylated form [(14)]. Although the exact mechanism by which herpesviruses induce cell cycle arrest is yet to be fully understood, recent work has revealed that the UL76 protein from HCMV causes chromosomal aberrations and DNA damage, which may explain how UL24 genes might lead to cell cycle arrest, for example, through induction of the DNA Damage Response, leading to induction of a checkpoint at the G2/M transition [(15)].

Cell cycle arrests are crucial for the cell to monitor genome integrity so it can be repaired. If the damage is so extensive that is essentially beyond repair, this in turn can eventually lead to apoptosis [(16)]. Induction of cell cycle arrest at the G2/M interphase might favor virus replication and assembly by providing the virus with an organized intracellular system, a structure that is eventually lost during mitosis.

### 3.1.1 Induction and manipulation of the DNA Damage Response

The DNA Damage Response is a conserved signaling cascade responsible for detecting DNA damage and activating the appropriate responses such as cell cycle arrest and, if the damage is extensive, suicide of the cell through apoptosis [(17)]. Activation of the DNA Damage response is characterized by expression of the phosphorylated form of a specific histone called H2A.X, and phosphorylation of the signal transducer ATM kinase, as well as the downstream signaling proteins Chk2 and p53. ATM exists in resting cells as an inactive dimer that is activated upon DNA Damage by autophosphorylation at Ser1981 and dissociation of the dimer, thus exposing its active site [(18)]. A key component in the ATM-dependent pathway is the serine/threonine kinase Chk2 which, after activation by phosphorylation at Thr68 by ATM [(19)], will then phosphorylate p53, thus initiating the DNA Damage induced apoptosis [(20)].

In order to establish a successful infection, HCMV must find a way to escape the consequences of the DNA damage response, and it does this by mislocalizing the checkpoint proteins away from the damaged site, thereby inhibiting their function [(21)]. Furthermore, HCMV infection has been shown to stimulate the homology-directed repair pathway, which might indicate that HCMV exploits the cellular components involved in cellular DNA repair to enhance its own genome replication [(22)].

## 3.2 Manipulation of the interferon response

Another way by which HCMV controls the cellular environment for its own benefit involves the modulation of the interferon response as the first line of defense against viruses. Viruses have evolved several strategies to down regulate the impact of interferon mediated responses, such as the inhibition of IFN production and IFN-mediated signaling pathways as well as blocking the effects of IFN induced anti-viral proteins. During primary infection herpesviruses induce expression of type I interferon [(23)]. Thus, in the case of HCMV, a productive infection and consequent latency, will only be sustained by evasion of type I interferon mediated responses.

## 3.3 Manipulation of the chemokine response

Evasion of the immune system by viruses also depends on control of chemokine expression. These molecules control the traffic and activation of leukocytes through interaction with their transmembrane receptors. Since viruses evolved

conjointly with their respective hosts, they have developed several mechanisms to control these molecules and their receptors. Herpesviruses encode chemokine homologues which act by recruiting leukocytes, hijacking these molecules and exploiting their natural function in order to facilitate growth and viral dissemination. In addition of chemokine homologues, viruses are also able to induce or inhibit the expression of many chemokines. One such example is a pro-inflammatory chemokine called Interleukin-8, specifically induced by HCMV [(24)].

### 3.4 Induction of expression of IL-8

This chemokine plays a role in acute inflammation by attracting neutrophils, monocytes and cytotoxic T cells through interaction with the chemokine receptors CXCR1 and CXCR2 [(25)]. IL-8 also plays a role in angiogenesis and inhibition of the IFN-α response [(26)]. Although its expression remains low under normal conditions, it is rapidly induced by viruses such as HCMV and other external stimuli, such as TNF-α [(27)]. Low levels of IL-8 expression are partly due to transcriptional repression of its promoter, which contains an NF-κB element. Transcriptional activation of IL-8 expression is critically dependent on the NF-κB transcription factor as well as the additional factors AP-1 and NF-IL-6, which may contribute to optimal expression, depending on the stimulus or cell type [(28)]. IL-8 is also regulated at the mRNA level since its expression is controlled post-transcriptionally by the p38 MAPK pathway which works to stabilize the aforementioned mRNA [(28)]. The increase in IL-8 levels may contribute to aggravate infection since it enhances HCMV replication by attracting leukocytes such as neutrophils to infected endothelial cells. These infected cells then transmit the virus to the neutrophils which later disseminate HCMV throughout the body via the bloodstream [(29)]. It stands to reason that the mechanism of neutrophil chemotaxis by IL-8 may become a novel target for therapy against HCMV.

#### 3.4.1 Activation of the NF-κB transcription factor

The NF-κB signaling pathway, crucial for IL-8 expression, plays a major role in inflammation, immunity, proliferation, differentiation and survival. It consists of a family of transcription factors comprised of p65, RelB, c-Rel, p50 and p52 which can associate between themselves and form different homo- and hetero-dimeric protein complexes [(30)]. In the absence of a stimulus, these complexes are kept in an inactive state through their non-covalent association with the IκB proteins. There are two main pathways by which this complex signaling cascade is activated, the so called canonical and non-canonical pathways and both are illustrated in Figure 2.

#### 3.4.1.1 Canonical pathway

The canonical pathway, induced by inflammatory stimuli such as TNF-α and IL-1β, leads to activation of the p65/p50 dimer. When cell receptors specific for these stimuli are engaged, it triggers a signal which leads to the phosphorylation of the IκB kinase complex, composed of IKKα, IKKβ and the regulatory protein NEMO. After activation, the IKK complex phosphorylates IκB, leading to its ubiquitination and degradation. This event releases the NF-κB subunits, p50 and p65, from the inhibitory grip of IκB and allows for them to translocate to the nucleus and activate transcription of genes [(30)].

#### 3.4.1.2 Non-canonical pathway

The non-canonical pathway is induced by stimulation of the CD40 and lymphotoxin-β receptors. This leads to a signal that activates the NF-κB-inducing kinase which in turn leads to the activation of the IKKα dimer. Afterwards, phosphorylation and proteolytic processing lead to the active form of p52 which results in the activation of all dimeric complexes containing p52 as its non-processed form has an inhibitory effect. Since distinct dimers bind to different target sequences, stimulation of both pathways ensures a broader response by activating a different set of gene promoters [(31)].

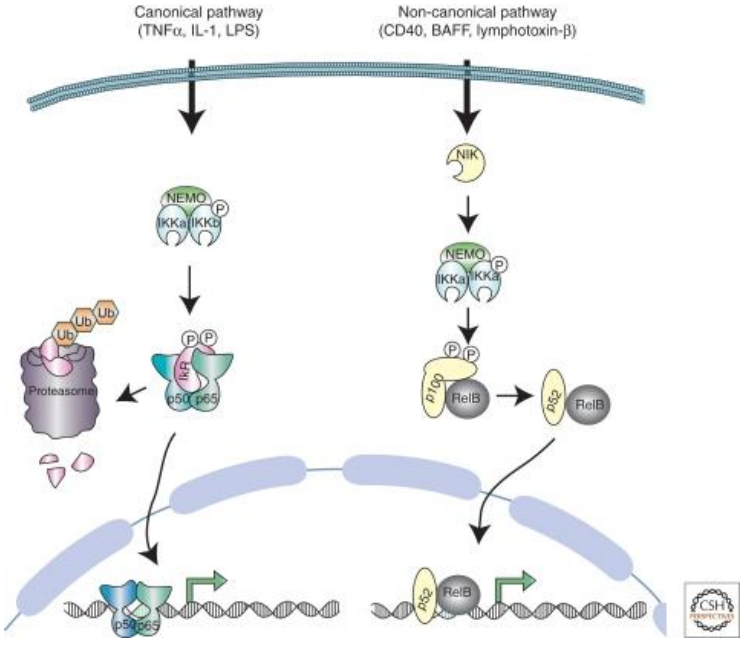


**Figure 2** – Canonical and non-canonical NF-κB pathways (adapted from Oeckinghaus and Ghosh, 2009).

### 3.4.1.3 Genotoxic pathway

Besides the previously mentioned pathways, in recent years another one has been described which does not rely on membrane receptor stimulation but rather on genotoxic stimuli which leads to a signal coming from the nucleus into the cytoplasm to converge on IKK complex activation as seen in the canonical pathway. Genotoxic stress is able simultaneously induce activation of ATM and translocation of NEMO to the nucleus where it is later sumoylated, phosphorylated and mono-ubiquilinated in an ATM-dependent manner. This processing eventually leads to the nuclear export of NEMO complexed with ATM which will be responsible for activating the IKK complex in the cytoplasm [(32)]. This atypical pathway presents a clear example of how induction of the NF-κB can lead to parallel activation of the DNA Damage Response[(33)].This particular pathway is shown in Figure 3.

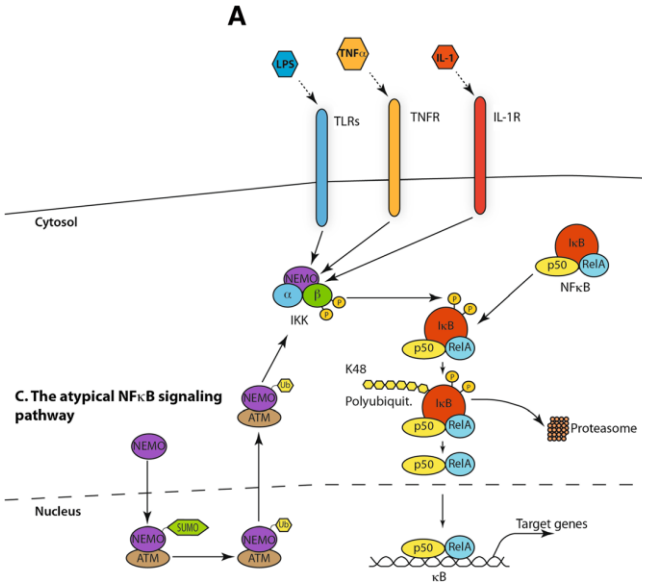


**Figure 3** – NF-κB genotoxic pathway (adapted from Hoesel & Schmid, 2013).

### 4) UL24

One example of a host evasion gene includes a particular non-homologous, unassigned host evasion gene family, conserved in all herpesviruses, the so called UL24 gene family. UL24 is present in the HSV-1 genome, and of the core herpesvirus genes, it remains the only one without an assigned function [(8)]. Its lack of homology with cellular genes combined with its degree of conservation seems to suggest a prominent role in the virus life cycle and host evasion mechanisms.

The HCMV homologue UL76 has been identified as a virion associated protein which indicates it is present within the cell at the very beginning of the infectious process [(34)]. Alignment of the predicted amino acid sequence of UL24 homologues has revealed five N-terminally located conserved regions with high similarity [(35)]. Aside from that, there have also been studies which have identified UL24 as a novel putative PD-(D/E)XK endonuclease [(36)]. However, until this day no endonuclease activity has been reported for UL24 or any of its homologues, including UL76. Previous work with UL24 has shown that deletion of this particular gene in HSV-1 resulted in a virus with reduced plaque size and diminished viral yield which suggests that its function, although not essential, is important for viral growth, at least in cell cultures [(37)]. The same holds true for the UL76 homologue of the AD169 strain of HCMV [(38)].

Absence of UL24 can also lead to a syncytial plaque phenotype observed for HSV-1 viral proteins, indicating that UL24 may also have a role in viral egress, a possible function which remains to be explored [(39)]. During viral infection, UL24 homologues are detected predominantly in the nucleus and transiently in the nucleolus [(15)]. It has been previously shown that UL24 can cause the redistribution of nucleolin and B23 in the nucleus [(40)]. Since deletion studies with UL24 resulted in a loss of nucleolin and B23 dispersal activity, this function might be conserved among all herpesviruses and therefore must be relevant for the pathogen's life cycle [(41)].

### 5) UL76, the UL24 homologue of HCMV

My particular objective was to characterize the function of the UL76, the HCMV UL24 homologue, a virion associated protein expressed with late kinetics during the lytic replication cycle [(42)]. The UL76 gene has five conserved domains in all of its homologues in the herpesvirus UL24 gene family as well as six putative nuclear localization sequences [(43)]. The UL76 protein has been shown to localize predominantly in the nucleus and nucleolus [(34)]. Previously known functions include the repression of replication genes as a way to inhibit viral production [(42)] as well as the regulation at a post-translational level of the UL77 gene [(44)].

It has also been reported that UL76 is involved in the regulation of ubiquitin proteasome pathway by eliciting novel aggresome formation through interaction with the ribosomal protein S5a, modulating the proteolytic function of this particular pathway and leading to the degradation of polyubiquitinated proteins [(43)].Other studies show that this particular homologue also induces and accumulates chromosome aberrations through DNA damage [(15)]. Furthermore, like all UL24 human homologues, UL76 also

induces cell cycle arrest at G2/M transition by inhibition of the mitotic Cdc2-cyclin B complex [45]. Moreover, UL76 is also specifically responsible for the induction of IL-8 through activation of the NF-κB pathway, which results in the translocation of p65 to the nucleus where it binds to the IL-8 promoter [24].

Induction of IL-8 mediated by UL76 also requires the activation of ATM and thus correlates with phosphorylation of NEMO on Ser85, indicating that UL76 activates the NF-κB pathway through the DNA Damage Response in a manner similar to the genotoxic stress pathway [24]. Activation of NF-κB by UL76 results from two separate events. Firstly, following DNA damage, ATM is activated through phosphorylation. Secondly, NEMO will be SUMOylated in an ATM-dependent manner at which point it will then be phosphorylated and ubiquitinated. After ubiquitination NEMO will form a complex with ATM and be exported to the cytoplasm where it will activate the IKK complex responsible for induction of the NF-κB pathway [32]. This mechanism explains why a nuclear protein such as UL76 can activate a cytoplasmic pathway like NF-κB.

● **Objectives**

The main objective is to identify the functional domains of UL76 responsible for IL-8 induction and for cell cycle arrest at G2M by deletion mutant analysis, and thus to further understand the dual activity of UL76. I will construct two deletion mutants, one coding for the conserved N-terminal region and the other coding for the variable C-terminal region. Each gene deletion mutant will then be cloned into the expression plasmid pcDNA3, incorporating haemagglutinin as an immunotag. After confirming the expression of both domains through western blot and immunfluorescence assay I will then determine which domain is responsible for IL-8 expression and which one induces cell cycle arrest at G2M. I will also try to determine the minimal functional domain required for each of the two functions by functional analysis of further deletion mutants, starting with deletion mutant analysis of the N-terminal region and its five homologous domains due to their high degree of conservation among all herpesvirus.

# 2) Materials and Methods

## 2.1 Cells

Human embryonic kidney 293T cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10 % fetal calf serum (Gibco) at 37 ºC in an atmosphere of 5 % $CO_2$ in air.

## 2.2 Plasmids

The UL76 gene and its derived constructs were all obtained by polymerase-chain reaction and cloned into the pcDNA3.1 plasmid fused in frame with an amino-terminal influenza haemaglutinin peptide (HA) "immunotag". The luciferase reporter construct containing human IL-8 promoter has been previously described [(46)]. The pCMVβ plasmid contains a β-galactosidase gene under the control of human cytomegalovirus immediate early promoter and serves as an internal control for variations in transfection efficiency. Lentiviral vector pHR-CMV-eGFP, the envelope HCMV-VSVG and packaging pCMVR8.9 have been described before [(47)]. The UL76 gene and its constructs fused with HA were excised from pcDNA3.1 by BamHI/XhoI digestion and cloned into the pHR-CMV-eGFP vector before lentiviral production.

## 2.3 Polimerase chain reaction (PCR) primers

PCR primers were designed (**Table 1**) for sequencing, amplification and molecular cloning of the two constructs belonging to the UL76 gene sequence, available online at the NCBI database, as well as three deletion mutants of the N-terminal region.

**Table 1** – PCR primers used for cloning of the UL76 constructs.

| PCR primer | Sequence |
|---|---|
| T7 | 5' AATACGACTCACTATAGGGAG 3' |
| SP6 | 5' CATTTAGGTGACACTATAGAATAG 3' |
| CMV Up | 5' CTGGATCCATGCCGTCCGGGCGTGGGG 3' |

| | |
|---|---|
| UL76 II Low | 5’ CCCTCGAGCA…AGACTC 3’ |
| C-terminal Up | 5’ CCGAATACGAGTCTGTTGCACACCTTTG 3’ |
| UL76 Low Total | 5’ CCCTCGAGCTATAAAGACCGTGTGGGAC 3’ |
| HD1+2 Low | 5’ CCCTCGAGACTCCAGAGTCCGGCGGT 3’ |
| HD2+3 Up | 5’ CCGAATTCTTGGGAGGCCTTTTCCCAC 3’ |
| HD1+2+3 Low | 5’ CCCTCGAGTCACCGCACGGACTGATCGTC 3’ |
| HD1 Low | 5’ CCTCGAGTCAGATGTCCAGGTGCTTGCG 3’ |

### 2.4 Molecular cloning of the constructs

The samples were first amplified through PCR under the conditions displayed below (**Table 2**). The PCR mixture, for a final volume of 25 µl, consisted of: 19.25 µl of miliQ water; 2.5 µl of Buffer Pfu; 1.5 µl of $MgCL_2$; 0.5 µl of dNTPs; 0.25 µl of Primer Up; 0.25 µl of Primer Low; 0.25 µl of Pfu DNA polymerase and 1 µl with 100 ng of DNA.

**Table 2** – Amplification PCR conditions.

| | |
|---|---|
| 95 ºC – 5 minutes | |
| 94 ºC – 1 minute | 30x |
| 45 ºC – 1 minute | |
| 72 ºC – 75 seconds | |
| 72 ºC – 10 minutes | |
| 4 ºC - Overnight | |

Afterwards, the PCR products were separated by 1.5 % agarose gel electrophoresis (Agarose Electrophoresis Grade, Invitrogen; diluted in Tris-acetate-EDTA (TAE) buffer) and the fragments with the correct size were then excised and the DNA extracted and purified with the Qiagen Gel Extraction Kit, following quantification by means of NanoDrop. The purified fragments were then sequenced through PCR for each of the six given sequences (**Table 3**), performed under the conditions displayed below (**Table 4**).

| **Table 3** – Gene sequence, predicted protein molecular weight and localization. | | | |
|---|---|---|---|
| **Sample** | **Nucleotide Sequence** | **Localization** | **Size (kDA)** |
| UL76 | 110327-111304 (975 bp) | Nucleus & Nucleolus | 36 |
| N-Terminal | 110327-110909 (582 bp) | Nucleus & Nucleolus | 22 |
| C-Terminal | 110910-111304 (393 bp) | Nucleus & Nucleolus | 15 |
| ΔHD1 | 110422-111304 (882 bp) | Golgi | 32 |
| HD1+2 | 110327-110577 (249 bp) | Nucleus & Nucleolus | 10 |
| HD2+3 | 110480-110673 (192 bp) | Cytoplasm | 8 |

The PCR mixture, for a total volume of 10 µl, consisted of: 2 µl of Buffer B; 2 µl of reaction mix; 1 µl of SP6 primer; 4 µl of miliQ water and 1 µl of DNA sample containing 250 ng. After the PCR was concluded, the DNA was precipitated by adding a mix to each sample, encompassing 10 µl of miliQ water, 2 µl of NaAC 3 M pH 4.6 and 50 µl of 95 % EtOH. The samples were then centrifuged at 16100 x g on a 5415R Microcentrifuge (Eppendorf) for 30 minutes at 4 ºC. After centrifugation, the supernatant was discarded and the samples were washed with 250 µl of 70 % EtOH, followed by another centrifugation at 16100 x g on a 5415R Microcentrifuge (Eppendorf) for 15 minutes at 4 ºC. The supernatant was discarded and the pellet was allowed to dry before delivering the samples for sequencing or storing at 4 ºC.

| **Table 4** – Sequencing PCR conditions. |
|---|
| 96 ºC – 1 minute<br>96 ºC – 10 seconds<br>50 ºC – 5 seconds (25x)<br>60 ºC – 4 minutes<br>4 ºC - Overnight |

The PCR products were next digested with the enzymes EcoRI and XhoI, 1 U of enzyme per µg of DNA, at 37 ºC for 2 hours, while at the same time the plasmid vector pcDNA3HA was digested under the same conditions. Both digestion reactions were inactivated at 65 ºC for 20 minutes and the DNA re-quantified and ligated. The ligation mix, for a total volume of 10 µl, consisted of: 1 µl of T4 DNA ligase; 1 µl of T4 DNA ligase buffer; 15 ng of sample DNA and 75 ng of pcDNA3HA. The ligation mix was incubated at room temperature for 1 hour, at which point it was directly used for

transformation of competent bacterial MH5α cells which had been previously thawed on ice after being kept at -80 ºC. The mixture was then incubated on ice for 30 minutes and heat-shocked at 42 ºC for 45 seconds before being incubated again on ice for another 2 minutes. After these steps, 100 µl of SOC were added to the mixture and the bacterial vials were shaken at 37 ºC for 1 hour before being placed on soft agar plates containing 100 ng/ml of ampicillin and incubated overnight at 37 ºC. The bacterial colonies obtained were then grown overnight at 37 ºC in selective Luria Broth medium supplemented with 100 ng/ml of ampicillin. The next day, an aliquot of the bacterial growth was saved for later production of glycerol stock before proceeding with the plasmid DNA extraction, according to the Qiagen Plasmid Mini Kit. The plasmid DNA obtained was then digested under the previously mentioned conditions in order to release the gene insert, which was then recovered by 1.5 % agarose gel electrophoresis. After confirming the presence of gene inserts, bacterial colonies positive for the recombinant plasmid were used to produce greater amounts of plasmid DNA through the Qiagen Plasmid Midi Kit.

**2.5 Lentivirus production and titration**

After cloning all of the constructs into pcDNA3HA, the resulting plasmids were digested with the enzymes BamHI/XhoI, 1 U of enzyme per µg of DNA, at 37 ºC for 2 hours and subcloned into the vector pHR-CMV-eGFP, upstream of an internal ribosome entry site-driven enhanced green fluorescent protein gene. After ligation under the same conditions as described before, competent bacterial cells were transformed and the subsequent procedure was carried out under the same conditions as mentioned for the cloning of the constructs into pcDNA3HA. Lentiviruses were produced by transient transfection of 293T cells, seeded at $3.5x10^5$ on 100 ml round plates the previous day, with a weight ratio of 3:1:1 of vector to packaging to envelope plasmids using Fugene 6 (Roche) according to manufacturer's instructions. Control lentiviruses were produced by co-transfection of the packaging plasmid pCMVR8.9 and the HCMV-VSVG envelope plasmid simultaneously with the empty pHR-CMV-eGFP plasmid. Supernatants of tranfected cells were collected at 48h post-transfection after ultracentrifugation at 25000 rpm for 4 h at 4 ºC on a Beckman XL-90 Ultracentrifuge with the SW28 rotor. Lentiviruses pellets were resuspended in fresh culture medium and frozen at -80 ºC. Lentivirus titers were determined by infection of 293T cells with a dilution factor of 4, followed by detection of eGFP positive cells through flow cytometry at 48 h post-infection.

### 2.6 Luciferase reporter assays

293T cells, seeded at $6x10^4$ cells/well in a 24 well plate one day prior to transfection, were co-transfected in triplicate with 100 ng of IL-8 luciferase reporter plasmid, 25 ng of the β-galactosidase internal control plasmid (pCMVβ) with the indicated amounts of either pcDNA3.1HA-UL76 or one of its constructs, according to the Lipofectamine 2000 (Invitrogen) protocol. Cells were lysed 24 h post-transfection with 100 µl of lysis buffer per well. Two different 96 well plates were used, one for the β-galactosidase control readings and another for the luciferase readings. B-galactosidase activity was measured using the Galacton-Plus kit from Tropix (Bedford, MA), with 10 µl of cell lysate as well as 40 µl of Galacton (Tropix), diluted (1:100) in Galacton Reaction Buffer Diluent (Tropix) being added to each well and incubated for one hour at room temperature, at which point 50 µl of Light Emission Accelerator (Tropix) were added to each well and the β-galactosidase activity was measured using a MicroLumatPlus LB96V Microplate Luminometer (EG&G Berthold) according to WinGlow program specifications. As for the luciferase readings, 15 µl of cell lysate was added to each well along with 75 µl of Luciferase Assay Substrate (Promega). Luciferase activity measurements were performed in the same conditions as in the β-galactosidase readings. The luciferase activity was then normalized relative to the β-galactosidase activity of each sample as a control of transfection efficiency.

### 2.7 Measuring of secreted IL-8 by Enzyme-linked Immunoabsorbent Assay

Supernatant of 293T cells, seeded at $1x10^5$ cells/well on a twelve well plate one day prior to transfection, which had been transfected with pcDNA3.1 (negative control) or either pcDNA3.1HA-UL76 plasmid or one of its constructs according to the lipofectamine 2000 (Invitrogen) were collected at 48 h post-transfection. The concentration of secreted IL-8 was determined following the manufacturer's instructions (IL-8 ELISA kit, BD Biosciences). Plates were analyzed at 450 nm using BioRad ELISA Reader (BioRad) and concentration of IL-8 was determined with reference to a standard curve obtained through a linear regression plot.

### 2.8 Western Blot

293T cells were seeded at $2x10^5$ cells/well on a six well plate. The following day the cells were transfected with the recombinant plasmid and Fugene 6 (Roche), according to manufacturer's instructions. One day later, the cells were recovered with

lysis buffer and washed in PBS 1 %. Samples of 15 µl were loaded on a 14 % sodium dodecyl sulfate polyacrilamide gel (SDS-PAGE). The separated proteins were transferred to a polyvinylidine difluoride (PVDF) membrane (GE Healthcare) and blocked with 5 % nonfat milk for 1 hour at room temperature. Afterwards the membranes were incubated with an anti-HA primary antibody (Roche) and left overnight at 4 ºC until the following day. Afterwards they were washed twice for 10 minutes with PBS 1% Tween 0,05 % and incubated with an anti-mouse secondary antibody for 1 hour. After another round of washing under the same conditions, the membrane was ready to be revealed at 700 nm and 800 nm using Odissey.

**2.9 Immunofluorescence assay**

293T cells were seeded at $2x10^5$ cells/well unto sterile glass coverslips and placed in six well culture plates. The next day they were transfected with pcDNA3.1HA-UL76 plasmid or one of its derived constructs as well as the control pcDNA3.1 plasmid according to the Fugene 6 (Roche) protocol. At 24 h post-transfection, cells were washed with PBS 1 % and fixed with 3.7 % paraformaldehyde for 10 minutes. Fixed cells were then permeabilised with PBS 0.1 % Triton X-100 for 10 minutes. After washing twice for 5 minutes with PBS 1 %, cells were blocked with PBS 0.05 % Tween 20 containing 5 % normal goat serum for 1 hour. The samples were later incubated with anti-HA FITC conjugated antibody (Sigma-Aldrich) for 1 hour to later visualize the HA-tagged protein. After washing and incubation with DAPI for 2 minutes, the coverslips were mounted on “Slow fade” (Invitrogen) and examined under a Leica $DMRA_2$ fluorescent microscope.

**2.10 Cell cycle arrest analysis**

293T cells were seeded at $1x10^5$ cells/well on a twelve well plate and infected with recombinant pHR-CMV-eGFP-UL76 or one its respective constructs as well as the control lentivirus the next day. Cells were collected by trypsinizing (Gibco BRL) 48h post-infection, washed once with PBS 1 % and fixed with 90 % EtOH overnight at 4 ºC. After fixation, cells were washed with PBS-1 %, resuspended in PBS-0.5 % Triton-X 100 and incubated with 50 U of DNAse-free RNAse A (Calbiochem) for 30 minutes at room temperature. After incubation, cells were stained with propidium iodide (Sigma). Flow cytometry analysis was performed using FACS Calibur instrument (Becton Dickinson) and cell cycle analysis was performed using CellQuest software.

# 3) Results

## 3.1 Preparation and expression of UL76 deletion mutant constructs

The two deletion mutants corresponding to the conserved N-terminal region and the non-conserved C-terminal region were cloned by PCR as described in Materials and Methods (Section 2.4). The wild type protein and both the N-terminal and C-terminal regions depicted in Figure 1 show the conserved domains in red as well as the putative nuclear localization signals in green. Figure 2 illustrates the results corresponding to the cloning of UL76 and its deletion mutants into pcDNA3HA.


**Figure 1** – Wild type UL76 protein and the corresponding deletion mutants. The UL76 protein is comprised of 325 amino acids while the N-terminal region has the first 194 amino acids and the C-terminal region is made up of the rest of the amino acids. In red it is possible to see the five N-terminally located conserved homologous domains while the putative nuclear localization signals are marked in green.

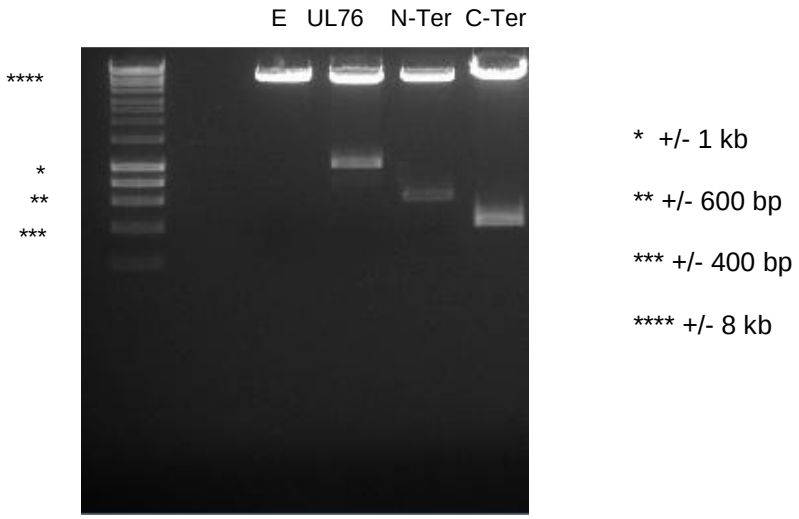


**Figure 2** – Band fragments from agarose gel electrophoresis after digestion from pCDNA3HA with EcoRI/XhoI enzymes. The top four bands are pcDNA3HA, the first well is an empty vector, the second, third and fourth are UL76, N-terminal and C-terminal sequences.

These constructs were then recovered by an agarose gel electrophoresis and subsequently sub-cloned into pSIN (Materials and Methods, Section 2.4) for posterior lentiviral production in order to over express the aforementioned mutants for the subsequent functional assays. The presence of an insert was confirmed by agarose gel electrophoresis. The following image presents those results (Figure 3).

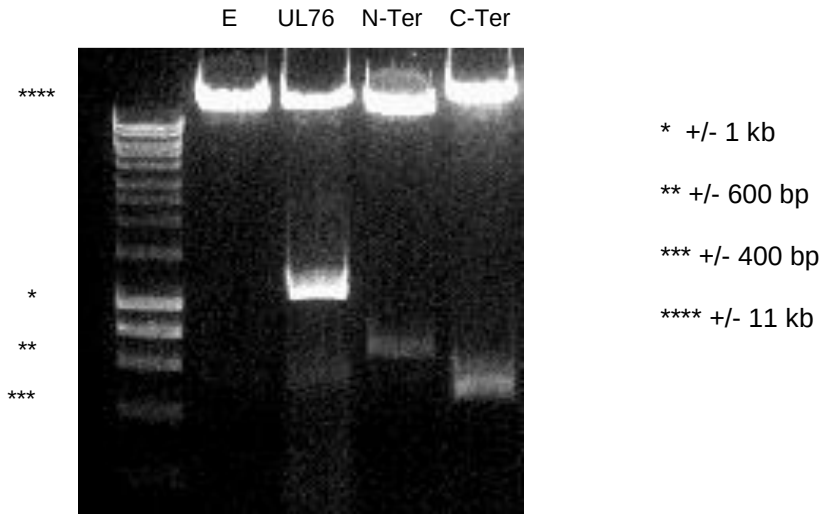


**Figure 3**– Band fragments from agarose gel electrophoresis after digestion from pSIN with BamHI/XhoI enzymes. The four top band fragments represent the expression vector. The first well marked with an E stands for the empty control while the next one is the UL76 gene. The remaining two wells are the deletion mutants for the N-terminal and C-terminal regions.

Finally, 293T cells transfected with recombinant lentiviruses were examined by Western Blot and Immunofluorescence assay (Materials and Methods, Section 2.8 and 2.9, respectively) in order to confirm expression of the predicted products. Positive and negative controls were provided by similar analysis with the entire UL76 HA-tagged protein and an empty expression vector for positive and negative control, as shown in Figure 4. The results of the Western Blot reveal the presence of 3 bands with molecular weights of 37 kDA, 24 kDA and 13 kDA, corresponding to the wild type protein, the conserved N-terminal and the non-conserved C-terminal regions, respectively.

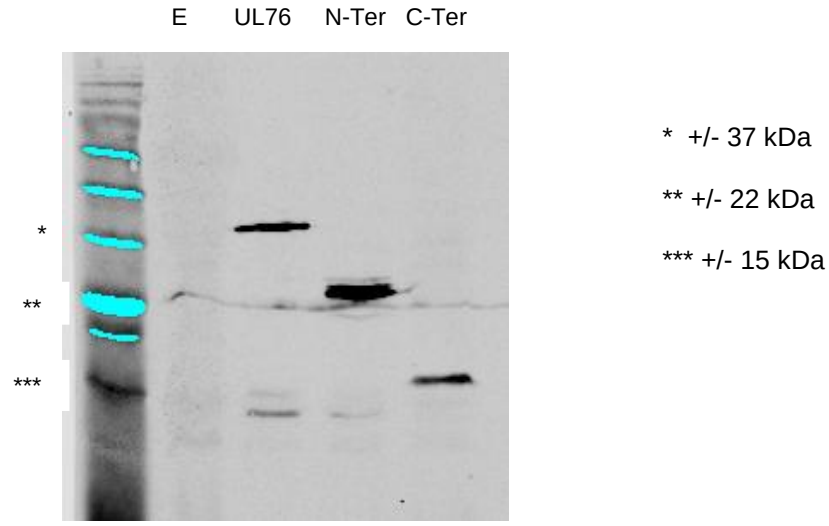


**Figure 4** – Western Blot depicting bands corresponding to the wild type protein and both deletion mutants. The first well represents the empty control while the second well shows the wild type protein UL76. The third and fourth wells stand for the N-terminal and C-terminal regions, respectively.

Afterwards we performed a western blot using a set of antibodies comprised of an anti-β-actin primary antibody (Cell Signaling) made in mouse and an anti-mouse secondary antibody which permitted analysis under the same conditions as mentioned before, in order to have a loading control, as shown below in Figure 5.

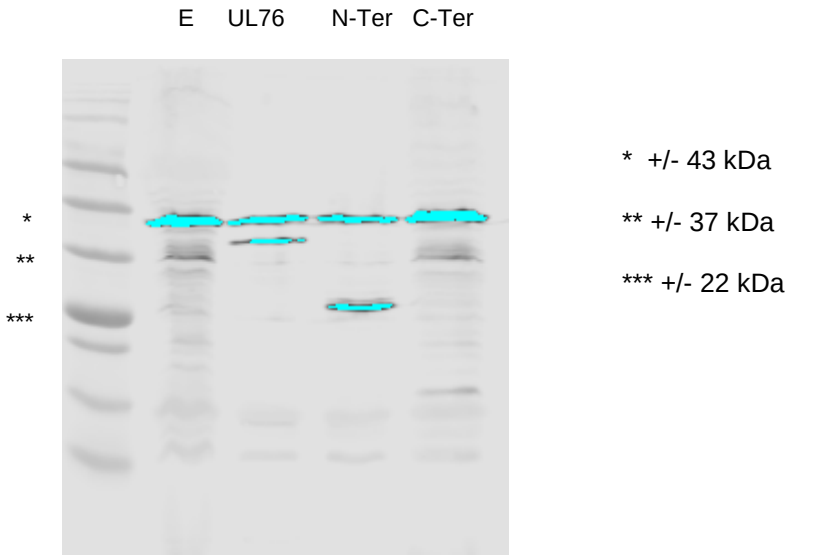


**Figure 5** – Western Blot depicting bands corresponding to β-actin, which represents a loading control, as well as the wild type protein and the N-terminal region.

To validate the results obtained from the western blot we performed immunofluorescence assays to see if the deletion mutants could also be observed by this particular method of detection. What the results showed us corroborated our previous findings since both the N-terminal and the C-terminal HA-tagged regions proved to be visible under a fluorescence microscope. Those two samples, as well as an empty expression vector and UL76 HA-tagged samples, for negative and positive control, had been previously incubated with an anti-HA-FITC conjugated antibody made in mouse (Sigma-Aldrich) that allowed for visualization through use of a LEICA $DMRA_2$ fluorescence microscope. The images obtained are depicted in Figure 6.

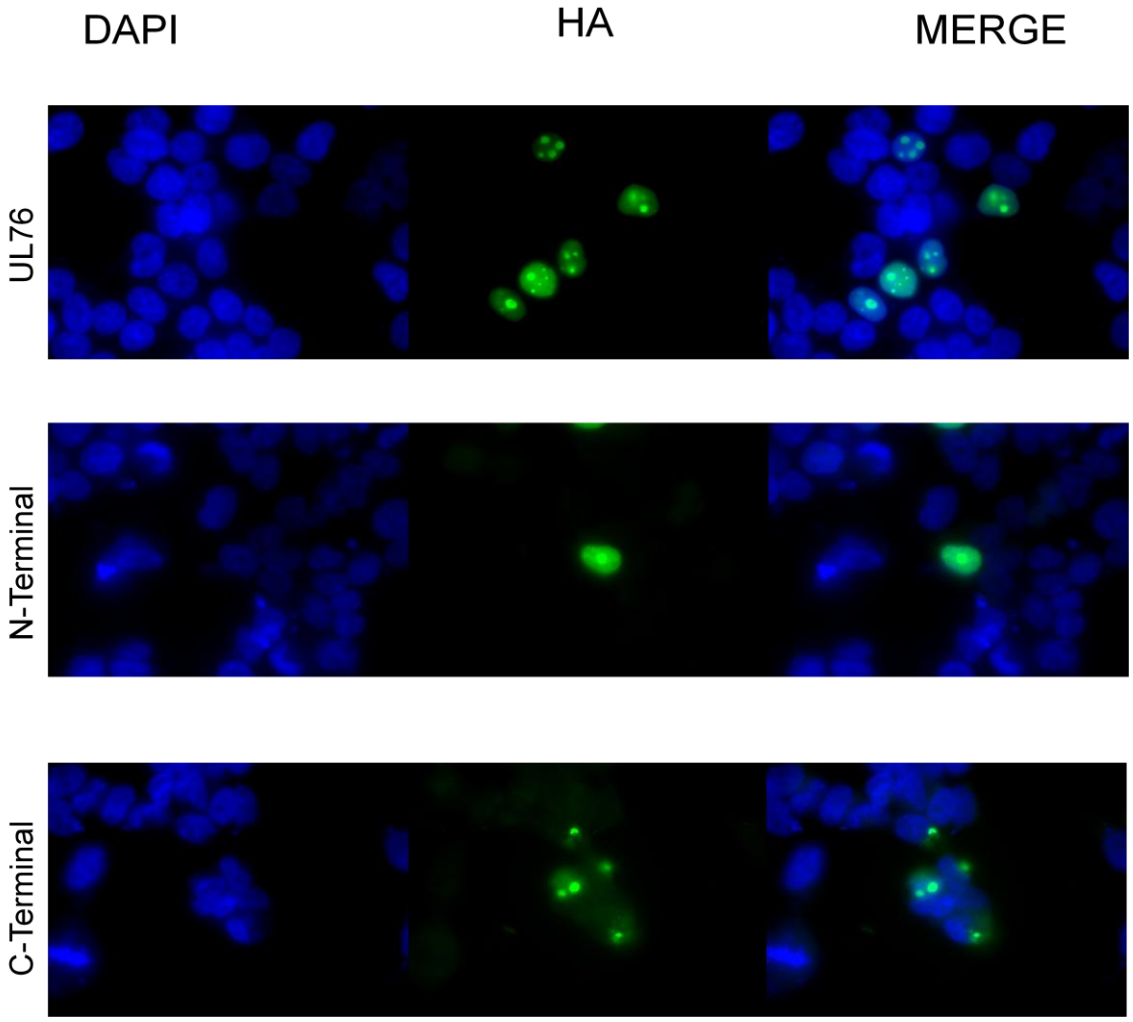


**Figure 6** –Immunofluorescence assays showing cellular localization of wild type UL76 and both mutants. With the DAPI staining one can see the fixed cells due to nuclear staining. A conjugated anti-HA-FITC antibody was used to bind to the HA tag on UL76 and its deletion mutants in order to see their localization, which from the results gathered appears to be strictly nuclear and nucleolar, for the wild type protein as well as the N-Terminal and the C-terminal regions. The MERGE allows us to see both stainings combined.

### 3.2 Functional analysis of UL76 deletion mutants constructs

Having demonstrated expression of the cloned N-terminal and C-terminal constructs, the next step was to use these in functional assays for IL-8 expression and cell cycle arrest.

#### 3.2.1 Induction of IL-8 expression

Induction of IL-8 is an essential step in HCMV infection due to its impact on viral replication and virion production [46]. UL76 induces this particular cytokine through a newly characterized NF-κB alternative pathway called the genotoxic pathway. This particular pathway is activated by DNA damage, thus providing us with a clear assumption that both IL-8 induction and cell cycle arrest are dependent on activation of the NF-κB pathway. A luciferase reporter construct containing the IL-8 promoter sequence was used to ascertain the transcriptional impact our mutants had on the IL-8 gene, as was previously detailed in Murayama et al, 1997. As positive and negative controls, luciferase activity was also determined for the entire UL76 sequence expression vector and the empty plasmid. The results in Figure 7 show that expression of UL76 and the non-conserved C-terminal region clearly stimulate the transcriptional activation of the IL-8 promoter in a significant manner while the conserved N-terminal region does not induce the activation of the IL-8 promoter.

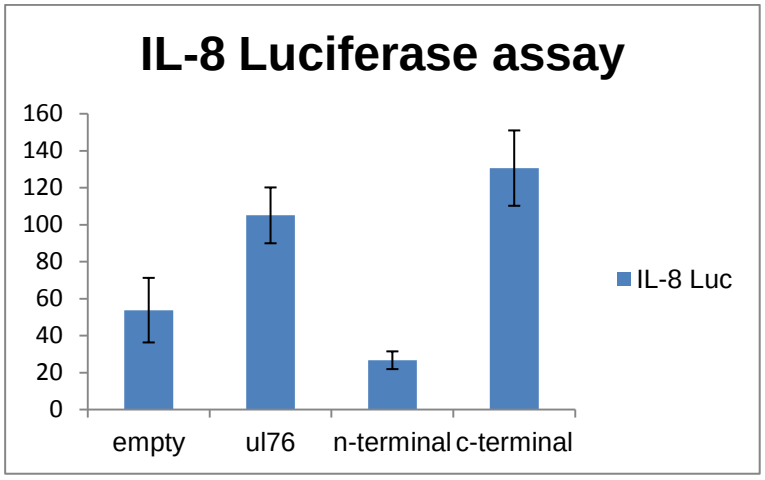


**Figure 7** – Luciferase reporter assay of IL-8 transcriptional activation. It is possible to see that the levels of promoter activation regarding IL-8 transcription are only relevant for the wild type UL76 and the C-Terminal region. The N-terminal region has a level which happens to be lower than the empty or negative control, thus leading us to hypothesize that induction of IL-8 transcription rests solely on the variable region.

To confirm those results at the level of protein expression we determined the amount of IL-8 secreted into the supernatants of similar transfected cells by Enzyme-Linked Immunosorbent Assay (ELISA). The results obtained (Figure 8) are consistent with the up-regulation of IL-8 at a transcriptional level observed in the previous assay since there was a clear rise in secretion of IL-8 in cells expressing both the UL76 protein and the C-terminal region, when compared to the control plasmid, while the conserved N-terminal region does not induce any remarkable expression. In summary, we may conclude that the non-conserved C-terminal region is necessary and sufficient for UL76 to induce expression of IL-8 at a transcriptional and protein level.

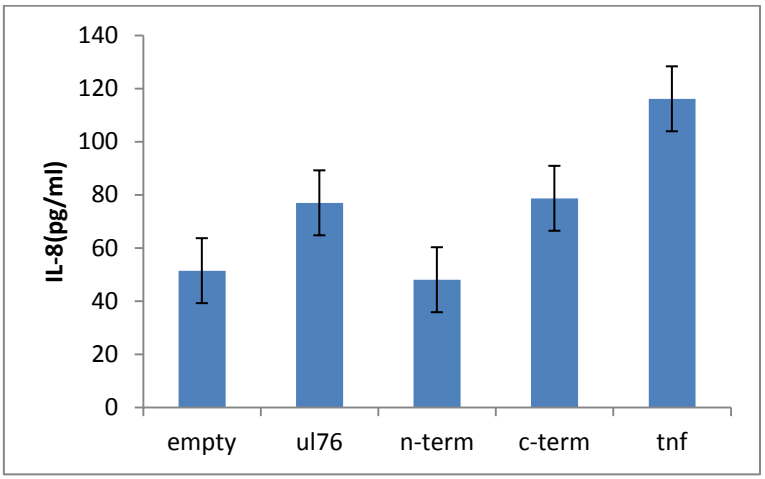


**Figure 8** – ELISA for IL-8 secretion. To further corroborate the previous essay these results demonstrate that IL-8 protein levels only reach significant levels in the two positive controls comprising the UL76 and TNF samples as well as in the C-terminal variable region, thus establishing that this region is responsible for an induction of IL-8 also at a protein level.

### 3.2.2 Induction of cell cycle arrest

Cell cycle arrest at G2/M is a conserved function among all UL24 family gene homologues [(45)] and is dependent on activation of the NF-κB pathway by the DNA Damage Response (Nascimento unpublished results). While there is evidence for the role of UL76 regarding the activation of the DNA Damage Response through the phosphorylation of H2A.X as a result of double-stranded breaks [(15)] and ATM as well as p53 at Ser15 (Nascimento unpublished results), there is still no consensus on how this specific mechanism is employed. One possible explanation might be related with findings about a putative novel PD-(D/E)XK endonuclease domain made up of three conserved signature amino acids, encoded by the UL24 gene family [(36)]. However,

recent work has shown that the cell cycle arrest caused by UL76 is not dependent on the putative endonuclease activity by assaying the impact on cell cycle arrest of a mutant UL76 gene which had those three critical amino acids changed to glycine. The outcome of expressing the UL76 endonuclease mutant still resulted in a G2/M arrest similar to the wild type UL76 [(24)]. In order to text the capacity of the N-terminal and C-terminal regions to induce G2M cell cycle arrest, 293Tcells were transfected as described in Materials and Methods Section 2.10 and processed for cell cycle analysis. The results (Figure 9) demonstrated that only the N-terminal conserved region induced a cell cycle arrest at G2/M similar to the wild type protein while the C-terminal non-conserved region was without impact.

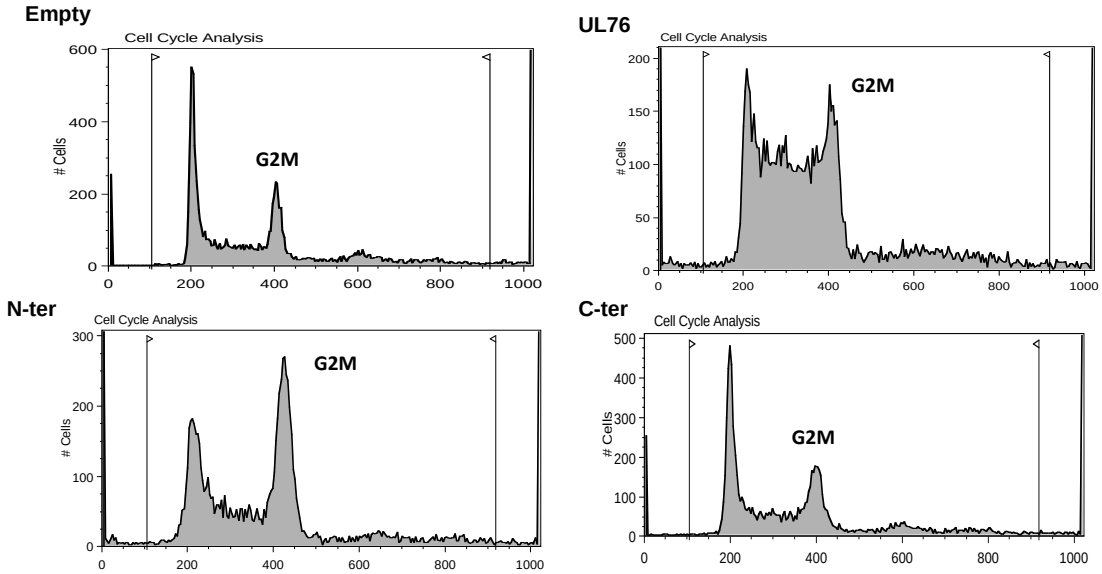


**Figure 9** – FACS analysis of cell cycle arrest essay. The upper left graph shows the FACS analysis for the empty vector with a majority of cells in G1 phase and a small peak at G2 and thus no cell cycle arrest. On the upper right side we have the graph corresponding to UL76 showing a cell cycle arrest since the G2 peak is basically the same as the G1. On the left bottom side there is the graph for the N-Terminal region clearly showing a cell cycle arrest as the bigger peak corresponds to the G2 phase. Finally, on the bottom right side we have the FACS analysis graph for the C-terminal region and here no arrest is observed since a great number of cells remain in G1.

# Discussion

Herpesviruses are a perfect model for the study of host evasion mechanisms. Understanding their ability to establish long term latency is key, since this is a crucial aspect of their survival strategy and highly relevant to their control, providing a clear cut example of how pathogens and their correspondent hosts adapt to each other over the course of evolution. This specific group of pathogens possesses a wide array of conserved genes solely responsible for manipulating the host cell biology and immunity in order to reproduce. Study of the proteins encoded by these genes is of the utmost importance for its potential benefits towards the development of an efficient vaccine against herpesviruses. For example, an attenuated vaccine containing a genetic mutant virus without one of these evasion genes would be expected to be less pathogenic and thus might be a protective vaccine against this important group of human pathogens. Until now, genes responsible for manipulating host cell biology and immunity have been largely identified through their homology with cellular proteins. Previous findings notwithstanding, there are still an undefined number of genes coding for viral proteins whose function remains to be characterized, mainly because these particular coding sequences lack any homology with cellular genes and do not code for either essential proteins or enzymes involved in replication. The only way to characterize such non-homologous genes is through functional assays.

This project focuses on one such virus host evasion gene, belonging to the non-homologous UL24 gene family, which is conserved amongst all herpesviruses subfamilies and indeed remains the only core herpesvirus gene without an assigned function. This gene, named UL76, is the UL24 homologue present in the human cytomegalovirus, the prototype of the β-herpesvirus subfamily. Its lack of homology, along with the fact that it is conserved in all subfamilies, points to a presumptive role in host cell manipulation and evasion possibly due to parallel evolution within the designated host. To support this suggestion, previous reports demonstrate that UL24 α-, β- and γ-herpesvirus homologues are able to induce cell cycle arrest at G2/M phase in mouse and human cells [(45)]. The cell cycle arrest at G2/M is induced by the DNA damage response which in the case of HCMV infection might be triggered by double stranded breaks [(15)]. This response is indicated by the phosphorylation of ATM and H2A.X as well as Chk2 and p53. Previously, it had already been reported that HCMV caused specific breaks at chromosome 1[(48)].

Another function recently demonstrated for UL76 is the induction of Interleukin-8, a property only described for the UL76 member of the UL24 family [24]. Significantly, this proinflammatory cytokine favors HCMV replication due to its ability to attract neutrophils, which will then become infected and serve as a means to disseminate the virus throughout the human body [46]. Past work comparing IL-8 secretion levels between wild type HCMV and a UL76 mutant show that the absence of UL76 results in a drastic reduction of IL-8 secretion by infected cells [24]. It is also noteworthy to mention that UL76 also has a positive effect on HCMV replication since infection by a UL76 deleted virus results in a small plaque phenotype and diminished viral replication, compared with the wild type [38]. A recent report highlighting the determinant regions of UL76 and their potential role eliciting aggresome formation through interaction with S5a of the ubiquitin proteasome system based on multiple protein sequence alignments of the UL24 family illustrate the presence of five conserved amino acid regions located in the conserved N-terminal of the protein, while the C-terminal was shown to contain a variable sequence [43].

In regards to IL-8 induction, past reports proved that UL76 induced IL-8 through the NF-κB pathway due to its requirement for IKKβ, degradation of IkB and subsequent translocation of p53 to the nucleus [24]. These results however, present an interesting conundrum since NF-kB activation normally starts in the cytoplasm in response to membrane bound receptor stimulation and not in response to nuclear stimuli, where UL76 is localized. Luckily, in recent years a new pathway for NF-kB, dependent on DNA damage, has been identified, thus offering an explanation as to how a nuclear signal can elicit such a cytoplasmatic response [32]. As UL76 has been shown to induce DNA damage through double-stranded breaks [15], this clearly became an obvious suspect for NF-kB mediated IL-8 induction. Specifically, in this more recent model of NF-kB activation, a key step is the activation of ATM, leading to a series of post-translational modifications of nuclear NEMO [24].

Thus, the main goal of this project was to determine which of the conserved N-terminal or the non-conserved C-terminal regions was responsible for the two major functions associated with UL76, the cell cycle arrest at G2/M or the induction of IL-8. We accomplished this by constructing 2 deletion mutants corresponding to the N-terminal conserved region and C-terminal variable region. These constructs were then characterized through functional assays, such as IL-8 activation luciferase reporter assays and ELISAs for IL-8 as well as cell cycle arrest assays. These tests were all performed after expression of the mutant constructs had been confirmed by both

western blot and immunofluorescence assay. Prior to the assay, our prediction was that the N-terminal conserved region would induce cell cycle arrest, since it was common to all α-, β- and γ-herpesvirus, while the C-terminal region would induce IL-8, a property restricted to UL76 of HCMV. Indeed, our results proved that the cell cycle arrest function shared by all UL24 family homologues is in fact located at the conserved N-terminal region and not at the variable region. Importantly, with the C-terminal region, no such arrest was observed. In contrast, both the luciferase reporter assay and the ELISA results indicated that expression of IL-8 is influenced at both transcriptional and protein level by the variable C-terminal region and not the conserved region, thus explaining why induction of IL-8 is not shared by all UL24 homologues.

These results present us with an interesting dilemma. Both the N-terminal and C-terminal truncated proteins appear to activate the DNA damage response via different signaling pathways and thus with different consequences, that is, induction of cell cycle arrest or induction of IL-8. Thus, the C-terminal region induces IL-8 through the DNA damage response, yet fails to cause cell cycle arrest, thereby suggesting that the ATM-dependent damage response pathway may not follow its traditional signaling pathway towards p53 activation, but instead goes through the NF-kB pathway, leading to IL-8 induction [(49)].

At the moment we are trying determine if there is any visible change in the levels of both γH2A.X and γATM when cells are transfected with the non-conserved C-terminal region, thereby formally proving that it does induce an ATM- dependent DNA damage response. Present preliminary work from our group (Nascimento, unpublished work), may be relevant to defining the signaling pathway manipulated by the N-terminal mutant. These results indicate an intermediary between UL76 and the L7a ribosomal protein, raising the possibility that L7a may act as an intermediary between UL76 and p53, thus bypassing the previous steps of the DNA damage pathway and leading directly to p53 and p21 activation, thereby explaining why both regions induce a response based on DNA damage but have different consequences.

In recent years it has been shown that activation of the DNA damage signaling pathway can occur in the absence of evident DNA lesions [(50)], perhaps providing a target for the impact of the N-terminal region. As mentioned above, a direct approach to confirm this possibility is to determine by western blot if cells maintain their basal levels of phosphorylated H2A.X and ATM when transfected with the N-terminal region.

If the levels remain unchanged then it is clear that this conserved region does not elicit the early steps in the DNA damage response. If, on the other hand, western blots of the same lysates also reveal a rise in p53 levels, and thus p21 activation, then the mechanism by which the UL76 conserved N-terminal region induces cell cycle arrest in the absence of DNA damage would be explained. As a footnote, in regards to the deletion mutant analysis of the constructs belonging to the N-terminal region I should mention that the results obtained from assays with those mutants were not shown here due to their inconclusive results. We hypothesize that these mutants lack the overall tridimensional structure needed for the protein to perform its select function and that is why they do not behave as expected.

Taken together, this work provides a logical platform to explain how a conserved, non-homologous herpesvirus host evasion gene has evolved to serve two distinct functions, both depending on a differential impact on the DNA damage signaling pathway.

# Concluding Remarks and Future Perspectives

Having established that the UL76 host evasion gene mediates cell cycle arrest through its conserved N-terminal region, on the one hand, and induction of IL-8 via the more variable C-terminal region, on the other, more detailed structure-function correlation should be investigated by examining smaller deletion and replacement mutants. For example, to define which of the five conserved amino acid sequences of the N-terminal region are necessary and/or sufficient for causing a G2/M arrest. If no conserved sequence is capable of eliciting cell cycle arrest by its own then the next step would be the construction of different combinations of deletion mutants.

Defining the specific region responsible for IL-8 induction is more complicated since the C-terminal region is essentially a variable sequence with no homology to other genes from the UL24 family. This makes a strategy for deletion/replacement mutant analysis a harder task, yet I believe it remains the best course of action.

Finally, unpublished preliminary evidence from our group reveals an interaction between UL76 and the ribosomal protein L7a offers yet another piece in the complicated puzzle provided by the mechanisms of the conserved non-homologous herpesvirus host evasion gene UL76.

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