Master Thesis:

Constitutively active Akt1 interferes with HCMV infection by inactivating EZH2

Η συνεχώς ενεργή μορφή Akt1 ρυθμίζει την μόλυνση από τον ανθρώπινο κυτταρομεγαλοϊό (HCMV) μέσω απενεργοποίησης EZH2

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This Master Thesis marks the end of my studies in the University of Crete in Heraklion and the commencement of a new chapter in my life overflowing with challenges and new experiences.

Summary

Human Cytomegalovirus (HCMV, HHV5) is a double stranded DNA beta-herpesvirus, which infects 50–90% of general population. HCMV infection has severe side-effects and correlated mortality in organ-transplant recipients, immunodeficient humans and newborns (congenital infection (cCMV)). Herein, it is investigated the epigenetic modulation of HCMV latency. The pioneer seeds of this project were the findings of a paper published by Sourvinos *et al.*,2014(226), indicated that immediate-early gene transcription and **HCMV** infection of human foreskin fibroblasts (HFFs) are dependent on histone H3K27 trimethylation, which is under the tight regulation of **EZH2**, JARID2, NDY1/KDM2B and JMJD3.

This project demonstrated for the very first time the interplay between Akt, EZH2 and GFI-1 in the context of HCMV infection. These scientific data represent that constitutively active Akt1 inhibits the infection of HCMV- infected HFFs, as opposed to active Akt3 which increases HCMV titer, favouring in this way the HCMV infection. Moreover, it is suggested the possible mechanism through which the constitutively active Akt1 blocks HCMV infection. This is owing to the inactivation of EZH2, via phosphorylation at Ser21 residue. It has already been indicated that Akt phosphorylates EZH2 at Ser21 and suppresses its methyltransferase activity by impeding EZH2 binding to histone H3, which results in a decrease of lysine 27 trimethylation and derepression of silenced genes.(225)

Moreover, Western Immunoblotting reveals that the mock cells own active Akt1 levels. In parallel, it is validated the phosphorylation of Akt3 in HCMV-infected HFFs expressing Akt3, Akt3 + myc EZH2 and Akt3 + myc EZH2 + shEZH2 3'UTR. Supporting the evidence of (226), we experimentally demonstrated that the mock cells possess elevated levels of the transcriptional repressor **GFI-1**. In contrast, GFI-1 is downregulated in HCMV-infected HFFs, irrespective of their EZH2 silencing status. Taken all the above into consideration, GFI-1 could be easily considered as a promising biomarker for HCMV infection. Targeting this molecule, especially upregulating GFI-1 expression might have therapeutic applications in HCMV infection in the forthcoming years.

Περίληψη

Ο ανθρώπινος κυτταρομεγαλοϊός (HCMV, HHV5), είναι ένας διπλής έλικας DNA β-ερπητοϊός, που μολύνει 50–90% του γενικού πληθυσμού. Η HCMV μόλυνση έχει σοβαρές επιπτώσεις και θνησιμότητα σε δέκτες μοσχεύματος οργάνων, ανοσοκατεσταλμένα άτομα και νεογνά (εκ γενετής CMV, cCMV). Εδώ, διερευνάται, η επιγενετική ρύθμιση της λανθάνουσας φάσης του HCMV. Έναυσμα της παρούσας εργασίας ήταν τα ευρήματα ενός paper που δημοσιεύθηκε από Sourvinos *et al.*,2014(226), που απέδειξε ότι η μεταγραφή των immediate early γονιδίων και η HCMV μόλυνση των ανθρώπινων ακροπόσθιων ινοβλαστών (HFFs) εξαρτώνται από την τριμεθυλίωση ιστόνης H3K27, η οποία ρυθμίζεται από τα ακόλουθα μόρια **EZH2**, JARID2, NDY1/KDM2B and JMJD3.

Αυτή η εργασία απέδειξε για πρώτη φορά την αλληλεπίδραση μεταξύ Akt, EZH2 και GFI-1 στα πλαίσια της HCMV μόλυνσης. Αυτά τα επιστημονικά δεδομένα αποδεικνύουν ότι η συνεχώς ενεργή μορφή Akt1 αναστέλλει την μόλυνση των HCMV-μολυσμένων HFFs, σε αντίθεση με την ενεργή μορφή Akt3που αυξάνει τον HCMV τίτλο, ευνοώντας με αυτό τον τρόπο την HCMV μόλυνση. Επιπλέον, προτείνεται ο πιθανός μηχανισμός μέσω του οποίου η συνεχώς ενεργή μορφή Akt1 αναστέλλει την HCMV μόλυνση. Αυτό οφείλεται στην απενεργοποίηση του EZH2, μέσω φωσφορυλίωσης στο κατάλοιπο Ser21. Είχε ήδη αποδειχθεί ότι η Akt φωσφορυλιώνει την EZH2 στη Ser21 και καταστέλλει την ενεργότητα της μέθυλομεταφοράσης της, μέσω παρεμπόδισης της πρόσδεσης του EZH2 στην ιστόνη H3, που οδηγεί σε μείωση της τριμεθυλίωσης της λυσίνης 27 και αποκαταστολή των σιγημένων γονιδίων. (225)

Επιπλέον, τα αποτελέσματα από το Western Immunoblotting αποκαλύπτουν ότι τα mock κύτταρα έχουν ενεργή μορφή Akt1. Παράλληλα, επβεβαιώνεται η φωσφορυλίωση του Akt3 στους HCMV-μολυσμένους HFFs που εκφράζουν Akt3, Akt3 + myc EZH2 και Akt3 + myc EZH2 + shEZH2 3'UTR. Τα αποτελέσματα της παρούσας εργασίας συνηγορούν με τα αποτελέσματα (226) και αποδεικνύουν ακόμα ότι τα mock κύτταρα έχουν αυξημένα επίπεδα του μεταγραφικού καταστολέα GFI-1. Ενώ, το GFI-1 ρυθμίζεται αρνητικά στους HCMV-μολυσμένους HFFs, άσχετα από τα επίπεδα σίγησης του EZH2. Λαμβάνοντας υποψιν τα ανωτέρω, το GFI-1 θα μπορούσε εύκολα να θεωρηθεί ένας πολλά υποσχόμενος βιοδείχτης για HCMV μόλυνση. Στοχεύοντας αυτό το μόριο, ειδικότερα η θετική ρύθμιση του GFI-1 θα μπορούσε να έχει θεραπευτικές εφαρμογές στην μόλυνση από HCMV τα επόμενα χρόνια.

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Introduction

Human cytomegalovirus (HCMV) infects most human beings globally, mainly without generating visible symptoms. HCMV discovered for the very first time in 1910, when the doctors observed the typical owl's eye inclusions (**Figure 1**) in newborns (1910) and over again in 1964 in individuals subjected to novel organ transplantation (1)

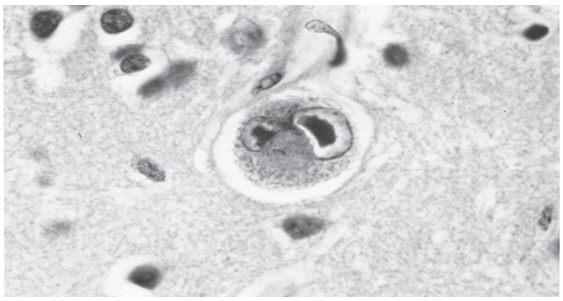

Figure 1. Owl's eye inclusion. A single epithelial cell bearing an intranuclear inclusion is shown in the choroid plexus. Reprinted from [92]. Reprinted from Griffiths,'Cytomegalovirus' in *Infections of the Central Nervous System*, Scheld WM, Whitley RJ, Marra CM (eds). Copyright 2014 Wolters Kluwer Health.

More up to date diagnostic approaches indicate that HCMV is considered an usual opportunistic infection in embryos, in allograft recipients, in bone marrow transplant individuals and in AIDS patients (**Table 1**). Apart from generating obvious end-organ ailment (straightforward symptoms), it is linked statistically with incidental post-transplantation responses, like graft rejection and cardiovascular disease (CVD) (2). Most lately, the lifelong effects of HCMV are owing to an excellent cell-mediated and humoral immune response and it has been correlated with increasing levels of morbidity in humans (3), so this virus is not regarded as harmless as it initially might be emerged

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Table 1. List of diseases associated with HCMV	
Congenital infection	Commonest virus
Transplant patients	Commonest opportunist
AIDS patients	Significant contributor to mortality
Intensive care	Associated with increased hospitalization, pneumonia
Elderly	Contributes to immunosenescence
General population	Increased mortality

1.1 Epidemiology of HCMV

Antibodies of IgG class, demonstrating the pre-infection stage, are detected in almost 60% of adults in developed world and 100% in developing regions. Infection could be acquired at various stages of development, as a fetus, a neonate, a toddler, a child or even an adult. Generally, individuals derived from poor socio-economic background infect with HCMV earlier than other groups. This implies that significant percentage of humans persist uninfected until they gain primary infection at a time period of vital medical interest. Reactivation of latent phase of virus and/or reinfection with a novel HCMV strain may also take place, as mentioned below.

About 2% of seronegative pregnant women have seroconverted to seropositive ones by the time of baby birth. The dominant origin of HCMV for these females is young children, mainly toddlers, whose saliva and urine possess elevated levels of HCMV (4,5). Among primary infected pregnant women, 32% propagate virus through placenta to bring out intrauterine infection (6). Unfortunately, this is not detected previous delivery, owing to a typical asymptomatic mother's phenotype, although retrospective examination can reveal symptoms of fever and discomfort (7). If symptoms are examined throughout pregnancy, primary infection can be recorded either by seroconversion or by the existence of IgM, accompanied by low-voracity IgG antibodies. Mothers undergo amniocentesis for diagnosic purposes of intrauterine infection and testing of amniotic fluid for HCMV DNA through PCR. This test is considered trustworthy in case that it is implemented after 21 weeks of pregnancy and at least 6 weeks post to the speculated time of uterine infection (8). This depicts that time frame is demanded for HCMV to cross from the uterine regions of infection to arrive to placenta and for prenatal kidney function to become successfully established for viruria to be measurable by testing amniotic fluid. The risk of serious disease provoked by intrauterine infection is approximately 20%, so a great deal of mothers chooses to go on with gestation unless structural impairs to neonatal are detected by ultrasonography (9,10).

The embryo can additionally be suffered from reactivation of latent maternal infection or by uterine reinfection with a novel HCMV strain (11,12).These mothers are asymptomatic and there are no diagnostic tests available to monitor these infections. Almost 1% of females who are seropositive before pregnancy deliver babies with congenital HCMV (cCMV) infection. Nevertheless, the prosperity of seropositive women in the group before baby birth indicates that, still in developed world, more congenitally suffered babies are delivered to 'immune' females than to those undergoing primary HCMV infection throughout gestation (13).

Throughout solid organ transplantation, seropositive donors often (about 78%) spread HCMV to seronegative recipients (14). Nearly 40% of seropositive recipients reactivate latent HCMV when administered immunosuppressive drugs and those with seropositive donors can also be reinfected with novel strains of HCMV (14,15). These infections can be identified and cured by monitoring recipients from the onset of transplantation by PCR for *CMV* DNA in whole blood or plasma and administering Ganciclovir (or its prodrug Valganciclovir) to those with a viral load above a determined level (3000 genomes/ml, equivalent to 2520 IU/ml whole blood).

Such treatment is very fruitful at preventing HCMV end-organ ailment, including gastrointestinal ulceration, hepatitis, pneumonitis or retinitis, which are provoked by viraemic propagation of virus. Equally successful technique is antiviral prophylaxis (16) where Valganciclovir is administered for a determined time frame (100 or 200 days) after transplantation. However some individuals emerge late-onset illness since prophylaxis is finished. It happens especially in donor-positive, recipient-negative individuals, where HCMV is propagated from a seropositive donor without immunity in the recipient. It is assumed that the refusal of antigen presentation to the immune system, promoted by the antiviral medicine, under immunosuppression is demanded to block graft rejection, damages the generation of high-affinity B and T lymphocytes potent to monitor HCMV replication in long term. Late-onset illness is seldom observed post preventive treatment, possibly due to excellent antigen presentation to the immune system (17). Moreover, some transplant individuals, for example those who are donor-positive recipient-negative, emerge second or third episodes of

viraemia, in cases where the first one has been dealt preventively. These are treated by extra cycles of pre-emptive therapy, resulting to full suppression of measurable viraemia.

After bone marrow transplantation, preventive treatment is implemented often due to the elevated haematological toxicity of Ganciclovir in these individuals. The epidemiology of HCMV is adverse to that discovered post solid organ transplant; almost all incidents of viraemia happen to seropositive recipients, with very few ones of HCMV propagation originated from the donor (18). Moreover, it is demonstrated that seropositive donors adoptively transfer some immunity to seropositive recipients (19).

In AIDS individuals, HCMV was a commonplace opportunistic infection in those whose CD4 counts dropped below 100. In 85% of incidents, the end-organ ailment was retinitis, while this constitutes less than1% of the end-organ illness observed post solid organ transplant. Approximately all HIV-positive humans are seropositive for HCMV before end-organ disease, so the retinitis ought to be caused by reactivation or reinfection. The gold standard prophylaxis for HCMV retinitis is antiretroviral approach to keep the CD4 count above 100, denoting that persistent immune surveillance is demanded to monitor obvious HCMV. HCMV retinitis still happens to AIDS patients who have not received antiretroviral medication, either from choice, from deprivation of consciousness about HIV infection or because they can't afford financially these drugs. Prior to the presence of antiretroviral treatment, the existence of HCMV viraemia was correlated with extreme morbidity as an indication of unintended impact of HCMV (20). Despite HCMV retinitis has vanished in patients delivered with gold-standard antiretroviral medication, this indirect influence on fatality still exists (21).

1.2 HCMV-General Characteristics

Human Cytomegalovirus (HCMV), also named as Human HerpesVirus 5(HHV5), is a double stranded DNA beta-herpesvirus, a member of Herpesviridae family, subfamily Betaherpesvirinae. Other members of this subfamily are the human herpes viruses 6 and 7 (HHV-6 and HHV-7). It is a ubiquitous prototypical herpesvirus which infects 50–90% of general population. The frequency of infection is determined by a great deal of agents like age of population, socioeconomic status and geographical regions (22,23). A noteworthy feature of HCMV is its ability to infect a broad range of cells within its host, namely fibroblasts, hematopoietic, endothelial, epithelial, smooth muscle and neuronal cells (24). As regards the healthy humans HCMV - infected, the majority of them has few if any implications. In case of symptoms, these individuals appear implications of mononucleosis, like fatigue, fever and muscle aches (23). However, HCMV infection in organ-transplant recipients, immunodeficient humans and newborns (congenital infection (cCMV)) has severe side-effects and correlated mortality (25,26).

HCMV is an enveloped DNA virus, and its viral genome is located within a capsid and surrounded by a protein layer named tegument. This structure includes proteins that are transferred to cells upon infection and can function prior to the beginning of viral gene transcription. The interplay of HCMV with its host initiate upon physical contact of virus with the cell membrane. In turn, this stimulates an increasing group of pattern recognition receptors that promote intracellular signaling cascades, expression

and release of various immunologically active cytokines and chemokines. Following this attachment, the virus penetrates into the cell and the capsid (containing viral DNA) is then translocated to nucleus. In nucleus, antiviral host responses block the initiation of viral gene expression, which starts fast utilising cellular factors like nuclear domain 10- associated proteins (reviewed in (27)) . HCMV can hijack this inhibition, and intranuclear transcription of viral genes begins (with aid from host transcription factors). Later, in the stage of infection, viral DNA is replicated and newly generated capsid proteins move to nucleus and packed with newly synthesized viral DNA. After envelopment at a juxtanuclear area, defined as viral assembly compartment, the enveloped virion can fuse with plasma membrane and new virion is emitted from the host cell.

HCMV possesses a large genome (~240 kb) (nearly half the size of the simplest free-living bacterium), rendering it the largest known human virus, which owns 165–252 open reading frames (ORFs) (28-30). Nevertheless, current research making use of ribosomal profiling and mass spectrometry analysis determined the existence of a great deal of undetermined ORFs and alternative splice sites (31). These ORFs are expressed in a special fashion resulting to two specialised infection patterns, namely, lytic and latent infection. Moreover, HCMV encodes for 14 microRNAs (miRNAs) and a great deal of long noncoding RNAs (lncRNAs). Its large genome size and its probable intricacy facilitate the virus to acquire, ameliorate, and utilise multifaceted strategies to implement various cellular machineries and signaling pathways that are essential to its efficiency and enable it to infect many cell types and to take place both productive and latent infections. The case of aberrant function of these cellular pathways seems to be a well-developed and strictly balanced viral technique to yield the expected implications in each infected cell type. Currently, it was demonstrated that also in cancer cells many pathways, which are regulated throughout HCMV infection, are deregulated, indicating the increasing level of HCMV sophistication (32). This finding is especially noteworthy in the context of the established link between HCMV and glioblastomas (33). However, there are many questions to be answered about the case of direct implication of HCMV in oncogenesis, at this stage.

The HCMV genome is comprised of unique short (US) and unique long (UL) segments both of which are surrounded by inverted repeats (23). Viral gene expression, throughout HCMV infection, takes place in a impermanently modulated way and is featured by three sequential and interdependent transcriptional waves. The first one regards the robust transcription of Immediate-Early (IE) genes IE1-72 KDa and IE2-86 KDa, which oppose and inactivate the host defenses and simultaneously upregulate the expression of early viral genes. The early genes (E), expressed in the second wave of transcription, promote viral DNA replication, a condition potent for the upregulation of late (L) genes. The latter encode viral structural proteins and are demanded for virion assembly and virion emit from infected cells. Viral progeny has been detected in biological fluids like urine, saliva and breast milk in the course of primary infection accompanied by reactivation recommends that various tissues serve as active sites of viral infection (34-36). HCMV utilizes cellular transcriptional stimulators and blocks cellular transcriptional repressors targeting the major immediate-early promoter (MIEP**),** in order to allow the initiation of transcription of immediate-early (IE) genes, (37).

Latency is considered a viral strategy of sustaining a persistent infection (38-40). Upon primary infection, HCMV establishes lifelong latency in bone-marrow-derived mononuclear cells (1 in 104–105 infected cells) featured by the persistence of viral

genome in absence of virion generation (41). Moreover, 2–13 copies of HCMV have been monitored per latently infected cells (41). It is worth mentioned that HCMV is in circular form in latently infected cells **(Figure 2)** (42). Until now, there are no reports supporting the integration of HCMV genome into host chromatin. Despite HCMV enormous cell tropism, the cells of myeloid origin, especially CD14+ peripheral blood mononuclear cells (PBMCs) and CD34+ hematopoietic progenitor cells (HPCs) have been identified as regions of HCMV latency (43-46). The reactivation of latent HCMV in immunosuppressed or immunodeficient individuals is the leading cause of mortality correlated with HCMV infection. The molecular mechanisms implicated in reactivation of HCMV latency have not already been delineated.

Nowadays, despite the genetic predisposition, intensive research is performed in terms of the epigenetic modulation of HCMV latency. Epigenetics concerns the heritable alterations regulating the gene transcription, remaining intact the DNA sequence (47). Epigenetic mechanisms are widely involved in cell development, differentiation and maintenance of genomic stability in eukaryotes (48). In cases of deregulation of the cellular epigenetic machinery, this commonly drives to pathological state, like cancer (49). The enzymes implicated in DNA methylation, histone alteration (acetylation/deacetylation, methylation/demethylation, sumoylation), chromatin remodeling and noncoding RNAs especially microRNA (miRNA) are characterized as vital leading molecules (38,50-53).

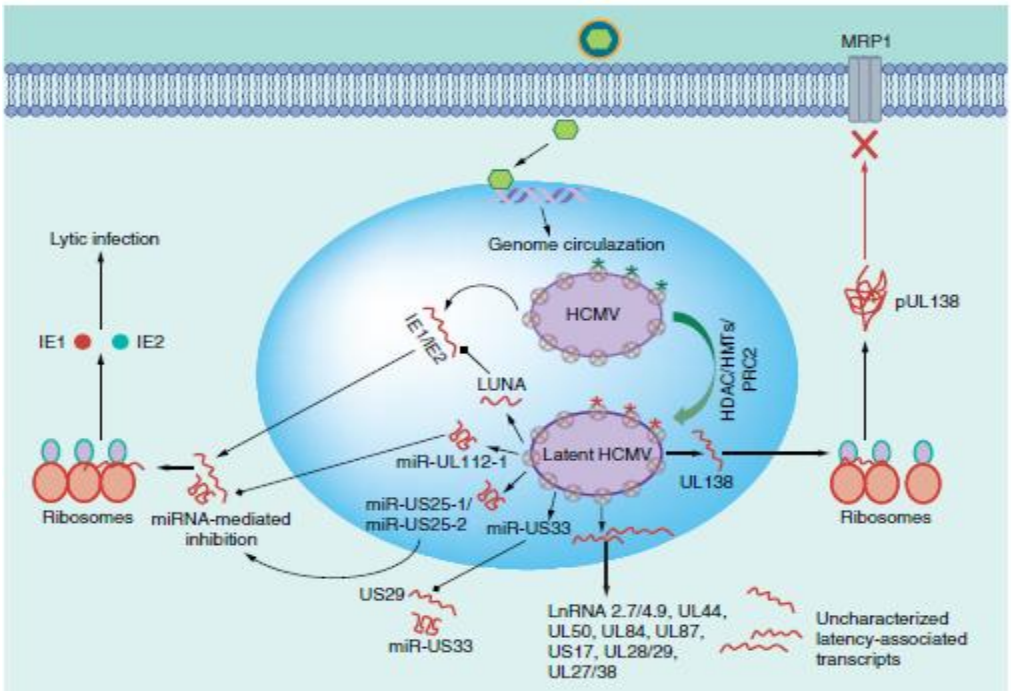

Figure 2. Summary of mechanisms implicated in modulating human cytomegalovirus (HCMV) latency. Once entering into the susceptible cell, HCMV is transferred to the nuclear pore where it delivers the viral genomic DNA into the nucleus. In nucleus, HCMV DNA circularized and along with cellular histone resulting to the generation of 'minichromosomes' . In latent phase, suppression of HCMV replication and transcription is performed by many epigenetic chromatin modifying molecules, namely HMTs and HDACs, PRC2 and microRNAs . Finally, many latency-correlated transcripts have been illustrated**.**
HCMV: Human cytomegalovirus; **HDAC**: Histone deacetylase; **HMT**: Histone methyltransferase; **PRC2**: Polycomb repressive complex 2.

1.3 Epigenetic mechanisms involved in HCMV latency

Establishment of HCMV latency

Upon entrance into the susceptible cell type, HCMV capsid with viral genome is transferred to the nuclear pore where it carries viral genomic DNA into the nucleus (54). **(Figure 2)** In nucleus, HCMV DNA is fast circularized and comes in touch with cell-encoded histone driving to the generation of chromatin scaffold (reviewed in (55,56). **(Figure 2)** In productive lytic infection *de novo* expression of IE proteins (IE1, IE2) has been performed from major IE enhancer/ promoter (MIEP) (57). Expression of IE proteins is executed by early and late proteins and is responsible whether the virus will subject to lytic or latent infection. Many reviews demonstrate that in latent phase the suppression of MIEP is characterized by many epigenetic chromatin modifying molecules, namely methyltransferases and histone deacetylases **(Figure 3)** (58-62). HCMV latent infection takes place in a few cells *in vivo*. However, it is hard to comprehend the latency in natural infection setting. Consequently, we ought to base on attaining knowledge for achieving HCMV latency that has been originated from the experimental models. These experimental models represent the most approximate species of natural latent infection; for example, *ex vivo* infected culture of CD14+ monocytes and CD34+ HPCs and less authentic differentiated or undifferentiated THP-1 cells.

Post-translational histone modifications take place in other members of Herpesviridae. It is apparent that viruses exploit the histone modifying machinery likewise as used by their host for homeostasis and tissue specific expression. These latency-correlated transcript areas have H3K4me2 and H3K9, K14 acetylation (63). Likewise, a great variety of chromatin modification marks has been demonstrated in histone-linked latent genome of Kaposi's sarcoma-associated herpesvirus virus (KSHV) and Epstein–Barr virus (EBV) (64). EBV and KSHV own methylated form of their promoter to promote latency (64), which is not well known in HCMV.

Methylation of histone tails in the promoter region, or into a gene, plays a pivotal role in modulation of gene expression. Histones subject to lysine mono-, di-, or tri methylation at various sites and the functional implications of histone methylation are site-based. Thus, tri-methylation of promoter-correlated histone H3 at K4 is a feature of active (open) chromatin, whereas dimethylation and tri-methylation of histone H3 at K9, or trimethylation at K27 are traits of inactive (closed) chromatin. Furthermore, mono-, di- and tri-methylation at other sites, such as K36 into a gene, maybe influence the transcriptional elongation and/or RNA splicing (reviewed in (65)). Methylation of core histones at multiple sites is performed by a host of site-based methyltransferases. For instance, tri-methylation of histone H3 at K27 is taken place by EZH2, a constituent of the polycomb repressor complex 2 (PRC2) (66,67), whose function is controlled by many co-factors, namely the jumonji domain-containing proteins JARID2 (68) and NDY1/KDM2B (69).

Histone methylation is reversible, with demethylation being performed by a host of site-specialised histone demethylases. The pioneer histone demethylase to be determined (LSD1) throws away H3K4me1 and H3K4me2 methyl groups, via an oxidative chemical reaction that utilizes FAD as a co-factor and yields an inconstant imine intermediate (70). The large family of jumonji domain-including histone demethylases deducts lysine methyl groups from a great deal of mono-, di- or tri-

methylated sites, during an oxidative reaction that uses Iron (FeII) and a ketoglutarate as co-factors and gives rise to a temporary hydroxymethyl intermediate. Demethylation of histone H3K27me3 is executed enzymatically by the jumonji domain demethylases, UTX/KDM6A, its homolog UTY, and JMJD3/ KDM6B (Reviewed in (71)).

Polycomb repressive complex 2 (PRC2) is a methyltransferase which stimulates tissue-specific differentiation (72,73) and enhances pluripotency in embryonic stem cells . (74) PRC2 is an indispensable cellular constituent, playing pivotal role in cell development and homeostasis. PRC2 functions in catalytic reaction of histone H3 trimethylation on lysine 27 residues of heterochromatin (74). PRC2 is composed of SET domain including EZH2 which has catalytic methyltransferase activity (HMT) accompanied by zinc finger protein SUZ12 and WD40 repeat (75-77). For example, current research from Kulesza laboratory indicates the effect of PRC2 in promoting latency by repressing the HCMV lytic genes' expression in HCMV model cell lines for latency (THP 1 and NT2D1). **(Figure 3)** They scientifically revealed that the blockage of PRC2 ended to an upregulation in the expression of HCMV viral transcripts and downregulation in H3K27me3 levels **(Figure 3)**. Similar results have been formerly demonstrated from Kulesza laboratory in murine cytomegalovirus (MCMV) (75).

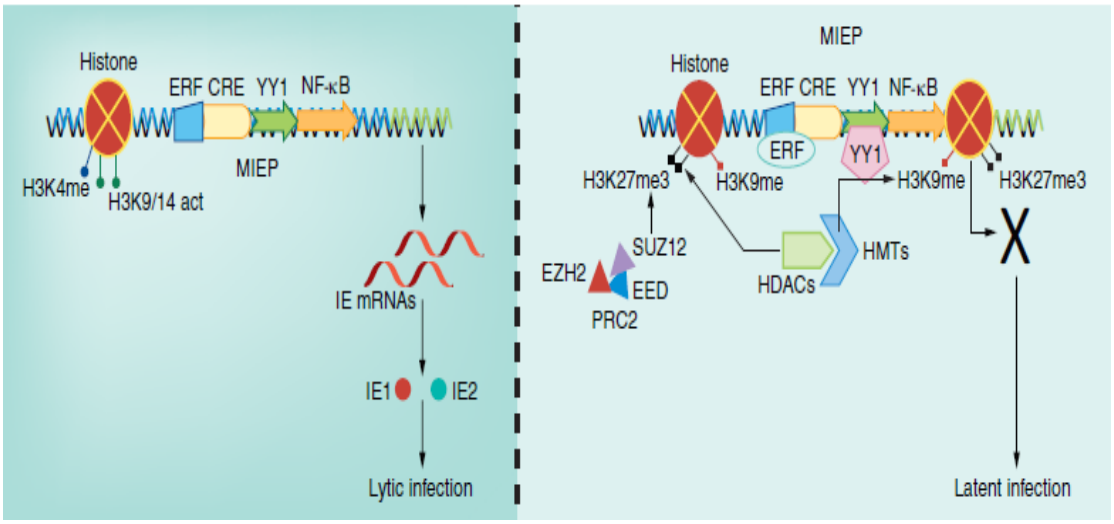

Figure 3. Shift of lytic to latent infection by chromatin remodeling of major immediate early enhancer/promoter.

Major immediate early enhancer/promoter (MIEP) owns binding sites for many transcriptional modulators containing ERF, CRE, YY1 and NF- κB. In lytic phase, histones connected to MIEP are in euchromatin form of configuration featured by an elevation in H3K4 methylation and H3K9/14 acetylation. On the other hand, in latent phase histones are methylated at K9 (H3K9me) and K27 (H3K27me3) resulting to the generation of transcriptional repressive structure. These signaling events are characterised by many molecules like HMTs, HDACs and PRC2. The function of YY1 and ERF have been demonstrated in giving rise to latency. Additionally, a great deal of novel mechanisms has been currently demonstrated that controls the transition of latent into lytic infection.

CRE: Cyclic AMP response elements**; ERF**: Ets-2 repressor factor; **PRC2**: Polycomb repressive complex 2; **YY1**: Yin Yang 1.

Disturbance of this epigenetic balance is significantly linked to carcinogenesis (78). For example, overexpression of EZH2, a main member of Polycomb repressive complexes (PRCs) that has inner histone methyltransferase (HMTase) activity (79,80) has been involved in cancer progression and metastases in various cancer types (81-83).

1.4 HCMV latency signature

Despite not all potent latent sites *in vivo* are well identified, monocytes seem to be dominant cells where HCMV establishes latency *in vivo* (45). HCMV has been recovered from monocytes extracted from healthy donors upon reactivation (42,84,85). Apart from monocytes, latent HCMV has been observed in granulocyte-macrophage progenitor cells (86) and myeloid progenitor cells (44,87). The differentiation of CD14+ or CD34+ cells into macrophages or dendritic cells can undergo transition from latent infection into lytic phase (44,88-91). CD14+ or CD34+ cells have been widespread utilised as a model for HCMV latency establishment and reactivation (38,92-94). HCMV has been demonstrated to effectively replicate (with hallmark of latency infection) in CD34+/CD38- (primitive hematopoietic cell) (95). On the other hand, in mature CD34+/c-kit+ cells HCMV infection results to expression of restricted groups of proteins (95). The cellular environment is of utmost significance in modulating latency (95).

Most of the knowledge concerning HCMV biology has been relied upon the laboratory strains. These laboratory strains have been largely passaged in fibroblasts. It was demonstrated that extensive passaging of HCMV in fibroblasts drives to the removal of 13–15 kb of genome which is otherwise existing in the low passage or clinical strains. This removed region named 'ULb' region is of significant importance in immune invasion and promotes the establishment of latency (89). UL138 is one of the ORF into the 'ULb' region (89). Goodrum *et al.* suggest the demand for UL138 ORF for latent infection in CD34+ cells (89). Moreover, currently the function of pUL138 protein in promoting latency in HPCs has been revealed (96). Petrucelli and colleagues detected only partial loss of latency-linked features in HPCs infected with recombinant HCMV (HCMV UL138Stop and UL138-null). Their scientific findings illustrate the need of other UL138 transcripts in upregulating latency in HPCs. Moreover, pUL138 is located in Golgi bodies of the infected cells and upregulates the expression of gene from MIEP locus (96). pUL138 is of highly importance in establishing latency in a cell-type specific manner. For example, in endothelial cells UL133-UL138 locus is highly demanded for successful replication of the virus, while in fibroblasts this locus is disposable (97).

HCMV encodes a great deal of immune evasion molecules during viral life cycle. These immune evasion mechanistic pathways target both innate and adaptive immunity, containing a coordinated attempt to block antigen presentation to T cells (reviewed in (23,98-100). The US2-11 region of HCMV functions at multiple stages to inhibit loading of viral peptides onto MHC molecules, along with the downregulation of cell-surface levels of MHC molecules (101-104). These actions are enhanced by virally expressed cytokines and chemokines, which either function as mimics of cellular immunosuppressants (ie viral IL-10) or as chemokine sinks to impede normal immune actions (eg US28 and RANTES binding) (reviewed in (105)). Moreover, the impact on antigen presentation is enhanced by also influencing on the expression of co-stimulatory molecules vital for T cell activation, accompanied by major cellular receptors demanded for the migration of antigen-presenting cells (106-108). Finally, the adaptive response is targeted by a great deal of viral actions (**Figure 4**). However, the downregulation of class I MHC molecules is a key problematic state by placing infected cells to the role of NK cells (due to the 'missing self' hypothesis). Finally, HCMV encodes many genes that also downregulate NK-stimulatory ligands from the surface of infected cell, whereas simultaneously promoting prohibitory ligands, like the expression of a viral form of HLA-E (UL40) (109).

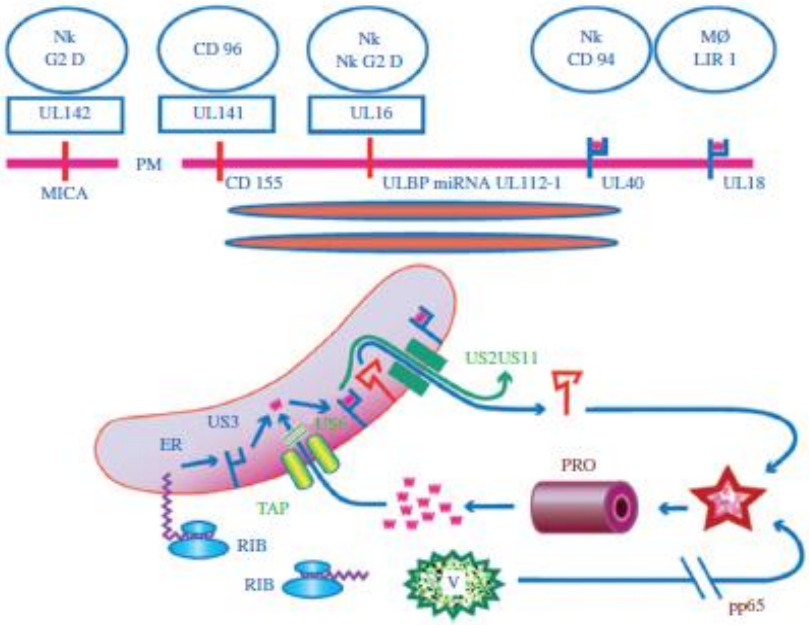

Figure 4. Mechanistic basis utilized by HCMV to interplay with cell-mediated immunity. Synthesis of a protein on a ribosome (RIB) is depicted, accompanied by digestion in the proteasome (PRO), transfer of peptides into the endoplasmic reticulum (ER) by the transporter linked with antigenic peptides (TAP) and expose, along with class I HLA molecules, at the plasma membrane. HCMV proteins pp65, UL97, US3, US6, US2 and US11 decline the exposal of mature class I complexes. HCMV proteins UL18 and UL40 exhibit decoy signals to block immune attack. HCMV proteins UL16, UL141, UL142 plus micro-RNA UL112-1 inhibit the presence of stress ligands, which would trigger immune attack. Reprinted from Griffiths, 'Cytomegalovirus', in *Principles and Practice of Clinical Virology*, Sixth Edition, Copyright © 2009 John Wiley & Sons, Ltd. Published by John Wiley & Sons Ltd with permission.

1.5 Pathogenesis of HCMV

The major principles of HCMV pathogenesis that connect the various disease correlations are viraemia, the threshold link between viral load and disease and immune pressure enabling HCMV to insist in sanctuary regions.

The threshold association (**Figure 5**) was initially determined in 1975 and consists the pioneer citation connecting the viral load of any virus to clinical setting in individuals (110). Endpoint titration of sequential urine specimens exhibited that asymptomatic patients with congenital HCMV characterized , on average, one log more increasing amount of viruria compared to those with perinatal infection (clinically benign). Moreover, the average level of viruria was an extra 1 log more elevating in newborns with symptomatic cCMV infection. Following 3–6 months, the levels of viruria in two cohorts of congenitally infected incidents became identical and, with the lapse of a year, included the cases of perinatal HCMV in bearing low-level insistent infection **(Figure 5).**

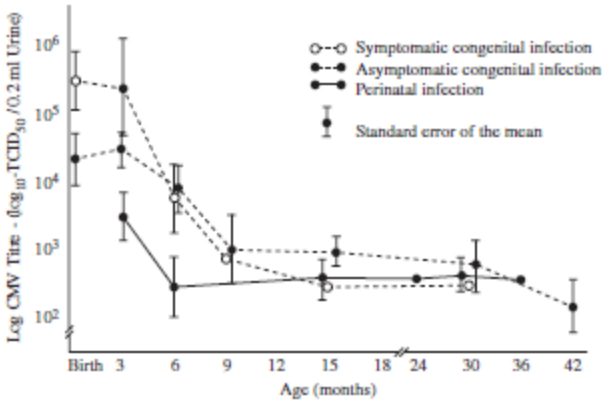

Figure 5. The pioneer report connecting HCMV viral load with clinical setting. Reprinted from (110)**.**Sergio Stagno, David W. Reynolds, Alex Tsiantos, David A. Fuccillo, Walter Long, Charles A. Alford, 'Comparative Serial Virologic and Serologic Studies of Symptomatic and Subclinical Congenitally and Natally Acquired Cytomegalovirus Infections', *Journal of Infectious Diseases*, 1975, Vol. 132, Iss.5, pages 568–577, by permission of Oxford University Press.

Then, the consequences for HCMV pathogenesis are illustrated on **Table 2**. It is worth mentioned, that all of these implications have proved to be veritable and offer the scientific rationale for treatment (111).

Table 2. Implications for viral pathogenesis

• HCMV is a systemic infection
• Sampling urine provides information about clinically inaccessible sites, eg inner ear
• HCMV is a chronic infection in neonates, so there may be a HCMV-specific immune defect
• HCMV may cause disease once a threshold value of viral load is exceeded
• Treatment may be beneficial if viral load is kept below this threshold value
• Postnatal treatment may be beneficial, even if short-term

This threshold link has been observed in groups of renal transplant individuals and in incidents of cCMV with sensorineural hearing impair of diverse sensitivities, as illustrated in **Figure 6**, which moreover, depicts the long-standing 'bath' model of viral pathogenesis, where an elevating viral load stimulates consecutive actions, which rise on the risk of end-organ ailment. It is of utmost significance that in multivariable models, the majority of immunosuppressive medication brings out HCMV end-organ illness by elevating the viral load, whereas steroids are responsible for pathological setting by decreasing the demanded viral load threshold. (112-115). The middle panel of **Figure 6** exemplifies the reason for the success of pre-emptive medication. This is owing to the fact that the patients are encouraged to trigger their immune system with a small dose of antigen, after that antiviral therapy is extended to cease the viral load, up to levels needed to bring about end-organ illness. The robust link between end-organ disease and increasing viral load, accompanied by the prevention of end-organ disease by administering antiviral medication, which retards the viral replication, is constant with direct lysis of target cells in terms of a pathogenetic mechanism.

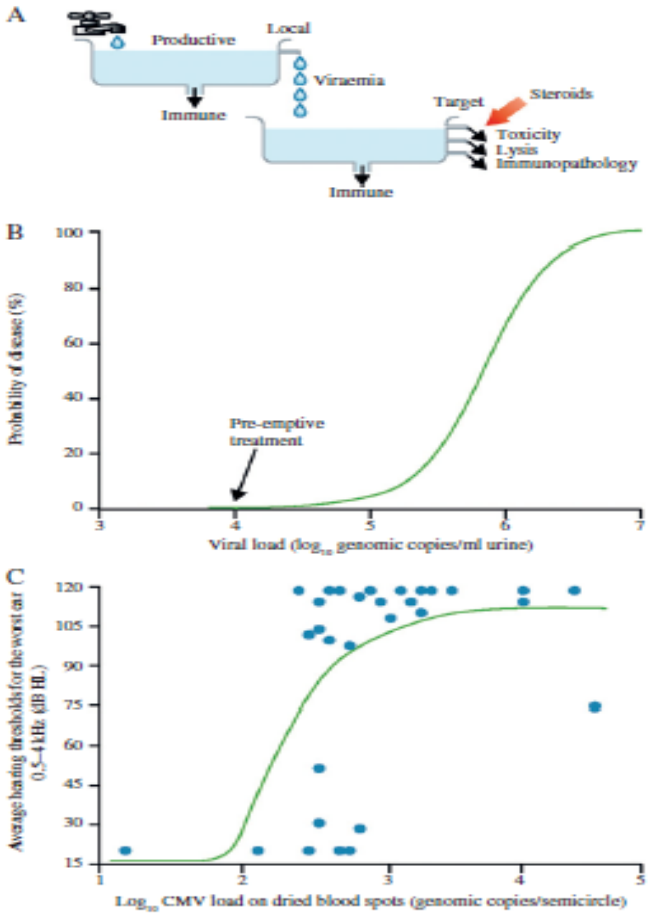

Figure 6. A multicomponent model of HCMV pathogenesis: (A) a bath model of viral dynamics; (B) the non-linear link between high viruria and HCMV end-organ disease after renal transplantation; (C) a likewise shaped curve associating viral load in dried blood spots taken at birth to the severity of sensorineural hearing impair detected consequently in kids with congenital HCMV infection**.** Reprinted from *The Lancet Infectious Diseases*, Vol. 10, Paul D Griffiths, 'Burden of disease associated with human cytomegalovirus and prospects for elimination by universal immunisation', Pages 790–798, Copyright 2012, with permission from Elsevier.

Reports of the replication rate of HCMV displayed that its dynamics is rapid, with a duplication time of viraemia of about 1 day (116). In other words, the doubling factors of primary HCMV infection in individuals are very like to those of primary HIV infection (116). The notoriety of HCMV as a moderately developing virus is invalid and is probably originated from the gradual evolution of cytopathic consequences in fibroblast cell cultures. It is well understood that strains of HCMV that duplicate in these cells have extended genetic alterations from the wild-type and are less pathogenic to humans than wild-type viruses (117). Epithelial and endothelial cells are more characteristic of HCMV infection in individuals. Thus, there is no place for sayings like the following 'studying the wrong virus in the wrong cell line utilising the wrong end point'.

The immune system employs more molecules to regulating HCMV in contrast to other viruses, owing to the fact that healthy seropositive humans usually have >1% of their peripheral blood T cells specific for one antigen of HCMV. Thus, in warfare terms, HCMV is considered to be in a 'stand-off' connection with the immune system, ready to duplicate fast if the established immune response is under threat. This may occur in patients administered immunosuppressive medication to impede

graft rejection, in HIV infected patients, or in humans with immature immune system (the fetus and bone marrow transplant individuals with recent engrafting marrow). Supposedly, it can also take place if the immune system is 'attacked' by a rapid shock efficient to accelerate the patient's access to the intensive care system. The assumed pathogenesis is HCMV duplication triggering the release of cytokines, which are afterwards noxious to the lungs. A recent controlled clinical trial is randomizing seropositive patients subject to intensive care administered with ganciclovir or placebo, to identify whether this supposed action can be under control.

In elderly patients, the agglomeration of years of lasting immune surveillance for HCMV in sanctuary regions may be extremely fatal in two major manners. Firstly, the declined amount of naive T cells might render seropositive humans less potent to reply to vaccines for influenza or pneumococcal infection, and this is disputable, based on findings from small studies (118). Second, the growing plethora of stimulated T cells might interfere with inflammatory attacks on bystander cells, for example those generating the endothelium, to increment the risk of cardiovascular disease (CVD).

Taken into consideration the main actions of HCMV on cell-mediated immunity, natural killer cells and cytokines, one might suppose that HCMV could influence overall lethality. The pioneer test of this assumption was published in 2011, and indicates a striking influence of HCMV even after well-known risk agents, like smoking and diabetes, were controlled for among the standardized group in USA (3). The findings were validated in a second population from UK (119). The outcomes that the unintented actions of HCMV are performing in cohorts to generate an additional risk of death should encourage the development of vaccines and their implement to disturb HCMV spread at the general society level (120).

2.1 AKT

Akt or PKB (Protein Kinase B) is a 56-kDa member of the AGC serine/threonine protein kinase family, identified in 1991 (121,122). In mammals, there are three genes, localised on different chromosomes, encoding for Akt1/α, Akt2/β, and Akt3/γ. Despite Akt1 and Akt2 are ubiquitously expressed (123), Akt3 is detected mainly in brain, kidney, and heart (124). Akt isoforms own increasing percentage of sequence homology in their catalytic domains, but differential in other domains of the protein, i.e. the C-terminus regulatory domain and the N-terminus pleckstrin homology (PH) domain (123) (**Figure 7**). The kinase catalytic domain, localised in the central domain of the protein, exhibits elevated level of similarity to those detected in protein kinase A (PKA) and protein kinase C (PKC), thus another term attributed to Akt, is protein kinase B (PKB) (125).

All three Akt isoforms recruit to the membrane via the N-terminus PH domain which binds to phosphatidylinositol 3,4,5-trisphosphate (PIP3) synthesized by phosphatidylinositol 3-kinase (PI3K), resulting to phosphorylation and upregulation of Akt kinase activity (126). The three isoforms possess same phosphorylation residues: Thr 308 (Akt1)/Thr 309 (Akt2)/Thr 305 (Akt3) and Ser 473 (Akt1)/Ser 474(Akt2)/Ser 472 (Akt3) (123) (**Figure 7**). The Thr residues are phosphorylated by phosphoinositide dependent protein kinase 1 (PDK1), which is also recruited to the plasma membrane by PIP3 (127,128). As opposed to, the Ser residues are under the

control of mammalian target of rapamycin complex 2 (mTORC2) (129). Despite this, the Ser residues can be phosphorylated by other kinases, like integrin-linked kinase (ILK) (130). Thus, the coordinated action of two phosphorylation facts enable the full upregulation of Akt, however, without the Ser 473 phosphorylation, Thr 308 p-Akt can still phosphorylate some, but not all, of its substrates (131).

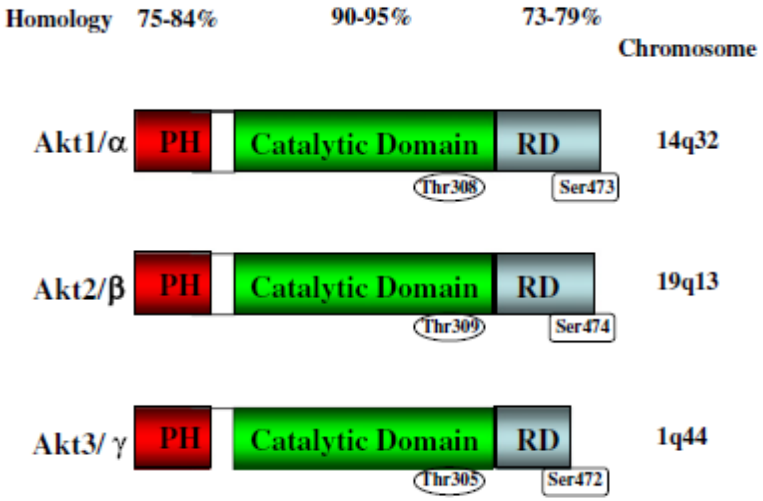

Figure. 7. Domain structure of Akt isoforms. All the Akt isoforms possess a catalytic (kinase) domain in the central region of the molecule. The PH domain acts as a phosphatidylinositol 3,4,5-trisphosphate-binding module. The RD is located at the Cterminus, adjacent to the kinase domain. Phosphorylation sites in the activation loop of the catalytic domain and in the RD are indicated. The percentage of sequence homology of the three isoforms as well as the chromosome location of the genes coding for the Akt isozymes are also highlighted. Abbreviations: PH, pleckstrin homology; RD, regulatory domain.

Despite robust findings that 3 Akt isoforms are functionally distinct, very little attention has been drawn to their nonredundant roles (132-136). Thus, only a handful of isoform-specific phosphorylation substrates have been determined to date (137,138).

Protein phosphatases inactivate Akt. The Thr residues are dephosphorylated by protein phosphatase 2A (PP2A). The core enzyme of PP2A is a dimer composed of a catalytic subunit (PP2A/Cα or β) and a regulatory/structural A subunit (PP2A/Aα or β). A third regulatory B subunit (PP2A/B), that identifies substrate specificity, can be connected with this core structure (139).

The Ser residues are targeted by the pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) family of isozymes. Three PHLPP isoforms have already been determined, the alternatively spliced PHLPP1α and PHLPP1β, and PHLPP2 (140). These PHLPP isoforms are implicated in the dephosphorylation of special Akt isozymes. PHLPP1 targets Akt2 and Akt3, whereas PHLPP2 dephosphorylates Akt1 and Akt3 (141).

Akt was initially identified for its function in modulating cell growth and survival. This could be owing to the direct or indirect actions of Akt on various cell proteins. The constant activation of Akt drives to cell cycle deregulation and impeding of pro-apoptotic pathways that are characteristic hallmarks of human cancers (142). However, overactivation of Akt signaling is oncogenetic (143). Thus, Akt activity is

enhanced in various types of neoplasia, where it is implicated in drug - resistance (144). Akt is considered as a promising therapeutic molecule for innovative cancer management, and some Akt inhibitors are currently being experimented in clinical trials in cancer patients (145,146).

Upon its activation, Akt isoforms translocate to diverse subcellular compartments, like endoplasmic reticulum, mitochondria, Golgi, and nucleus, where they phosphorylate substrates or come in touch with other cell constituents.

2.2 Nuclear Akt

The existence of active, phosphorylated Akt (p-Akt) into the nucleus has been described since the late 1990s. It is evident that, some of Akt substrates are localised in the nucleus, including the FOXO family of transcription factors (147) or the transcriptional coactivator p300 (148). Akt1, Akt2, and Akt3 have been referred to be located in the nucleus or to translocate into the nucleus after exposure to a wide variety of stimuli including insulin-like growth factor-1 (IGF-1), F(ab′)2 fragment of anti-mouse IgG acting on B-cell receptor, hypoglycemia, insulin, and nerve growth factor (NGF) (149,150).

The nuclear localization sequence (NLS) motif of Akt is unknown, however the proto-oncogene T-cell leukemia-1 (TCL1) protein family may be implicated in Akt nuclear localization, as initially reported in human T-cell leukemia (151). Three TCL1 isoforms have been determined in both the human and the mouse genome: TCL1, TCL1B, and MTCP1 (mature T cell proliferation 1) (152). TCL1 binds to the PH domain of Akt and participates in the generation, at the plasma membrane level, of TCL1-Akt high-molecular-weight protein complexes. Akt is afterwards preferentially phosphorylated and upregulated into these complexes (153). In two-cell mouse embryos that were genetically defective in TCL1, phosphorylated Akt had a striking cortical localization and was deprived of blastomere nuclei (154). Furthermore, when the levels of TCL1B were declined by siRNA in T47-D breast cancer cells, a drop in the levels of nuclear (but not cytoplasmic) p-Akt was noticed. In contrast, when TCL1B was overexpressed in either MCF-7 or T47-D cells, a rise on the levels of nuclear p-Akt was observed (155). This evidence pinpointed the significant role of TCL1 in nuclear targeting of Akt. However, additional research is essential to establish if the TCL1–Akt interplay is an ubiquitous mechanism needed for nuclear entry of Akt. Indeed, whereas TCL1 proteins are commonly expressed at increasing levels in embryonic/fetal cells and in diverse stages of B- and T-cell development, their levels in healthy adult tissues are declined, at least in mouse (152). However, the expression of TCL1 proteins is enhanced in many solid and hematologic cancer types (155,156). This evidence could be used to answer the growing nuclear placement of Akt in some cancer types.

Whether or not Akt ought to be phosphorylated for entrance into the nucleus is disputable. Indeed, it has been reported that unphosphorylated Akt could translocate into the nucleus in HEK293 cells overexpressing kinase-dead Akt mutants (T308A, S473A) that could not be phosphorylated, recommending that phosphorylation of Akt was not demanded for its nuclear placement (157). As opposed to, overexpression of similar mutants damaged Akt nuclear translocation after NGF trigger of PC12 cell (158). Thus, it was deduced that activity and phosphorylation of Akt kinase were implicated, to some extent, in Akt nuclear translocation (158). It is obvious that these variations are based on the diverse cell types/stimuli utilised. Another argumentative topic concerns whether or not Akt can be phosphorylated into the nucleus. The

nucleus owns all the machinery vital for phosphorylating Akt at Thr 308, like PI3K (159), PIP3 (160), and PDK1 (161,162). Furthermore, mTORC2 has been denoted to locate into the nucleus in aggressive variants of papillary thyroid carcinomas, subsequently with elevated levels of Ser 473 p-Akt. However, it has been assumed that mTORC2 could phosphorylate Akt at Ser 473 into the nucleus of thyroid neoplastic cells (163). Given this link, it is striking that mTORC2 has been localized to both the cytoplasm and the nucleus, while mTORC1 is mainly cytoplasmic (164), even if anmTORC1 constituent, Raptor, has been nowadays determined as a phosphoprotein implicated in rDNA transcription in nucleoli (165,166).

Some findings demonstrate that Akt translocates to the nucleus after having been phosphorylated at the plasma membrane (167,168) and that nuclear PDK1 is not implicated in phosphorylating Akt at Thr 308. But it is possible that PDK1 nuclear transport may be a mechanism to sequestrate it from activation of cytosolic signaling pathways (169). However, this event is regarded as highly disputable (170).

All three Akt isoforms are characterized by a classic, leucine rich, leptomycin sensitive nuclear export sequence (NES). According to this, the overexpression of Akt1 exhibiting a non-functional NES, ended to incessant nuclear localization of Akt1 and increasing cell translocation *in vitro* of Akt1−/− fibroblasts (157).

2.3 Functions of Akt

Akt is considered to be an essential node in diverse signaling cascades downstream of growth factor receptor tyrosine kinases and G protein-coupled receptors. Akt isoforms have utmost functions in cell survival (143), proliferation (171), growth (172), migration (173), polarity (174), insulin-stimulated glucose transfer (175), glucose and lipid metabolism (176,177), contractility in both skeletal muscle (178) and cardiomyocytes (179), angiogenesis (180), and self-renewal of stem cells (181-183). Damaged Akt activity has been correlated not only with oncogenesis (184-186), but also with other disorders, including type 2 diabetes, cardiovascular and neurodegenerative diseases, and muscle hypotrophy (187,188).

2.4. Functions of nuclear Akt

2.4.1. Cell cycle progression

Taken into consideration the utmost function of Akt in cell cycle modulation, it is expected that Akt is translocated to the nucleus during early G1 phase of the cell cycle (189). Upregulation of cyclin D1 by nuclear Akt may be tightly correlated with the fact that Akt favours the histone acetyltransferase (HAT) activity and transcriptional activity of p300 (148) which plays role in the histone acetylation at the cyclin D1 promoter and cyclin D1 gene transcription (190).

When a fast removal of nuclear p Akt was stimulated by extracellular ATP or statins in insulin-triggered A549 cells, PHLPP1 and PHLPP2 located to the nucleus. PHLPP1 and PHLPP2 were contributory to Akt dephosphorylation, which, however, needed nuclear translocation of the PIP3 phosphatase, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (191). PTEN is a tumor suppressor, because it negative regulates the oncogenic PI3K/Akt signaling cascade by dephosphorylating PIP3 (192). Upon nuclear Akt dephosphorylation and removal,

proliferating cell nuclear antigen (PCNA) and p21Waf1/Cip1 moved to the nucleus where they generated a complex. It is established that nuclear PCNA/p21Waf1/Cip1 complexes promote cell cycle arrest (193,194). p110β PI3K was of utmost importance in nuclear export of p-Akt in insulin-triggered cells posed to either statins or increasing levels of extracellular ATP (195). This evidence is constant with a research which has demonstrated a link between p110β PI3K and Akt at the nuclear level (196). Nuclear p110β PI3K is implicated in DNA duplication, as its catalytic function was highly demanded for controlling the nuclear upregulation of Akt during the S phase of the cell cycle and consequently the phosphorylation of the cell cycle progression inhibitor, p21Waf1/Cip1 (196).

2.4.2. Cell survival

Akt signaling cascade impedes cell death both by mediating on the cytoplasmic apoptotic machinery and by involving the expression of genes implicated in cell death and survival. Therefore, there is robust data demonstrating a more direct contribution for nuclear Akt in apoptosis repression. This role of Akt has been widely investigated in PC12 neural cells treated with NGF and primary cardiomyocytes.

2.4.3. DNA repair

Akt is upregulated after exposure to DNA damaging factors like ionizing radiation (IR) (197). Active Akt stimulates the DNA damage-prompted transcription and favours cell survival (198). IR causes DNA double strand breaks (DSB) (199). It is striking that, DNA-dependent protein kinase (DNA-PK),which is implicated in DSB repair (200), has been determined as a supposed Ser 473 Akt kinase (201). Another main molecule in DSB repair is the ataxia-telangiectasia-mutated (ATM) kinase (202). It has been demonstrated that a subset of Ser 473 p-Akt aggregated in the nucleus at IR-stimulated foci where it co-localized with the DBS markers, γ-H2AX and Ser 1981 p-ATM (203). Ser p-473 Akt activation was irrespective of both PI3K and DNA-PK, but was downstream of meiotic recombination 11 (MRE11)-dependent ATM activation and ring finger protein 168 (RNF168)-dependent histone ubiquitinylation. MRE11, accompanied by RAD50 and Nijmegen breakage syndrome 1 (or NBS1, also known as nibrin), generate the MRE11 complex, a sensor of DSB that regulates the DNA damage reply by controlling the upregulation of ATM (204). RNF168 is an E3 ubiquitin ligase which is implicated in histone H2A and γ-H2AX non-proteolytic poly-ubiquitinylation of the DSB flanking chromatin, a key process for restitution of genome stability (205). However, these data correlated nuclear p-Akt with DSB repair and cell survival. But, it is vague until now, which protein kinase would phosphorylate Akt at the DSB foci, taken into consideration that ATM is unable to directly phosphorylate Akt (206).

2.4.4. RNA export

Many types of RNA are moved from the nucleus, like tRNA, rRNA, snRNA, and mRNA. A major molecule in mRNA nuclear movement is the protein termed as REF/Aly, which is recruited to mRNA in splicing process(207). Microinjection of antibodies to Aly inhibited mRNA translocation remaining intact other movement mechanisms (208). Aly was found on nuclear speckles where it inteplayed with PIP3 (209). Nuclear Akt phosphorylated Aly on Thr 219 and this phosphorylation was essential for Aly binding to PIP3. Removal of Aly through siRNA ended to declined

cell proliferation and mRNA movement, and these two procedures needed both Aly phosphorylation by Akt and Aly interplay with PIP3 (209). So, Aly phosphorylation could be regarded as an additional mechanism by which nuclear Akt regulates cell cycle, mainly by favouring the movement of mRNAs encoding for proteins that are implicated in cell cycle progression.

2.4.5. Cell differentiation

Akt signaling cascade is of pivotal importance in insulin-dependent adipogenic differentiation (210). A novel approach has been recently generated for the estimation of the signaling functions of Akt detected at diverse subcellular regions during adipogenic differentiation. Selective Akt inhibitor peptides were created exploiting the variations of the Akt substrate recognition sequence (R-X-R-X-X-S/T) which included alanines instead of the target serines or threonines, cloned in-frame with the cDNA coding GST. A myc epitope tag was also contained, for enabling the peptide immunoobservation (211). Furthermore, the prohibitory peptides were engineered in-frame with peptides aiming to distinct subcellular regions. All in all, for creating a selective inhibitor of nuclear Akt, the SV40 large T antigen amino acids 126–132 were utilised. Selective blockage of nuclear Akt, influenced the location and the transcriptional expression of FOXO3a in 3T3L1 preadipocytes prompted to differentiate into adipocytes via induction with insulin and dexamethasone. This ended to inhibition of adipogenic differentiation, as reported by analysis of Oil Red O aggregation (211).

2.4.6. Tumorigenesis

The existence of nuclear p-Akt has been demonstrated in lung, breast, prostate, and thyroid cancer, along with in AML (212-216). The functions of nuclear Akt in cancer have not been delineated until now, however, it seems to be that once Akt is located in the nucleus, it would be implicated in the modulation of signaling cascades of cancer cell proliferation and survival. This principle is significantly augmented by the data that the promyelocytic leukemia protein (PML) tumor suppressor plays a role in dephosphorylating p-Akt in the nucleus (217). Thus, it is regarded worthinvestigated whether that continuous treatment with increasing concentrations of statins of A549 lung neoplastic cells, brought about a drop in nuclear Thr 308 p-Akt levels (218). Taken all the above into consideration, it has been speculated that the cancer prevention actions of statins (219,220), could be somehow associated with negative modulation of nuclear p-Akt levels.

3.1. HCMV microinfections are often detected in inflamed tissues

Although organ-specific implications, inflammatory diseases commonly generate same histopathological alterations and share various risk agents, like genetic and epigenetic ones, smoking, infections, and food. Despite the significant advancement in curing the implications of inflammatory diseases, the causal agents of these states remain a mystery.

It had already been demonstrated that almost 90% of individuals with inflammatory bowel diseases are characterized by an active HCMV infection in their bowel (221). The infected cells were seldom and exist in the deep mucosa of the bowel—but exclusively in inflamed regions. Cells in healthy bowel tissues from the same human being were sporadically positive for HCMV DNA, but indications of an active infection and expression of viral proteins had lost.

In purposes of analysis tissue samples derived from humans with other inflammatory diseases, they upgraded the novel sensitive technology so as to monitor low-grade HCMV infection in cancer (222). In inflamed regions of the bowel, they observed disperse HCMV infection, which failed to be detected by the standard immunohistochemical assays. In initial research, they have monitored the virus in humans with rheumatoid arthritis, Sjogren's syndrome, dermato- and polymyositis, psoriasis, Wegener's granulomatosis, ulcerative colitis, and Crohn's disease (unpublished data). In all these incidents, HCMV reactivation was obvious only in inflamed tissues but not in noninflamed tissue samples from the same human or healthy controls. So, active low-grade viral infection in these individuals was constantly linked with disease setting and inflammation.

The term utilized for this active, low-grade HCMV infection is "microinfection," as it can be detected only with highly sensitive staining techniques. Strikingly, the profile of expression of distinct IE proteins diversed from that noticed by traditional immunohistochemistry, both in high grade infections *in vivo* (commonly in immunodeficient humans) and in infected cells *in vitro*, because IE expression is commonly noticed in the cytoplasm of infected cells. It is not established whether an HCMV microinfection is an epiphenomenon of inflammation or a causal agent in inflammatory diseases (223,224). Therefore, the existence of an active HCMV microinfection in inflamed tissues may be a state- of- the- art that will launch the road for exploring common causal factors that might accelerate or deteriorate inflammatory diseases.

4.1. A novel study

A pioneer study, which prompted us to be engaged with this project, is a paper published on Science in 2005, which indicates that the HMTase activity of EZH2 is essential for H3 K27 trimethylation and is modulated by the Akt signaling pathway via phosphorylation of EZH2(225) They demonstrated that Akt phosphorylates EZH2 at serine 21 and represses its methyltransferase (HMT) activity by inhibiting EZH2 binding to histone H3, which drives to a drop in lysine 27 trimethylation and derepression of silenced genes, disturbing, in this way, the gene silencing and may drive to malignant transformation. Moreover, S21D-EZH2 stimulated cell growth in culture and tumor growth in animals, and phosphorylated EZH2 also connected with Ki67, a proliferative biomarker in primary tumor tissues (225).

Despite Akt induced the phosphorylation of EZH2, it decreases its affinity toward histone H3, remaining intact the PRC constitution, which is established for its methyltransferase activity. In other words, the subcellular localization of EZH2 or its interaction with Polycomb group (PcG) proteins, Suz12 and Eed did not alter after Akt-mediated EZH2 phosphorylation. Thus, it is implied that the phosphorylated-EZH2 complex, may also aim nonhistone substrates that are responsible for oncogenesis. In this context, the plastic alterations in EZH2 substrate affinity mediated by Akt may be a potent reply to the question concerning the decreased HMTase activity of EZH2 toward histone H3 in case of tumors, where EZH2 is overexpressed. Taken all the above into consideration, their findings indicate that Akt controls the methylation activity, through phosphorylation of EZH2, which may lead to tumorigenesis (225).

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5.1. The pioneer seeds for this research project …

A widely cited and novel paper published by Sourvinos *et al.*,2014(226), demonstrated that immediate-early gene transcription and **HCMV** infection of human foreskin fibroblasts (HFFs) are dependent on histone H3K27 trimethylation, which is under the tight regulation of **EZH2**, JARID2, NDY1/KDM2B and the histone demethylase JMJD3. They revealed the EZH2/NDY1/KDM2B/JARID2/JMJD3 axis which downregulates **GFI1,** a repressor of the MIEP of HCMV. Blockage of this pathway enhances GFI1 expression and impedes the MIEP activation and HCMV infection. Once the virus enters into the virus-infected cells, UV-sensitive virus-related agents favour MIEP activation by promoting the negative modulation of GFI1 in both wild-type and NDY1/ KDM2B, EZH2 or JARID2 knockdown cells. Therefore, since the levels of GFI1 in the latter cells post the infection are more elevated than in wild-type cells, the HCMV-triggered GFI1 degradation does not succeed in modulating negatively GFI1 to levels favourable for MIEP activation and viral infection (226).

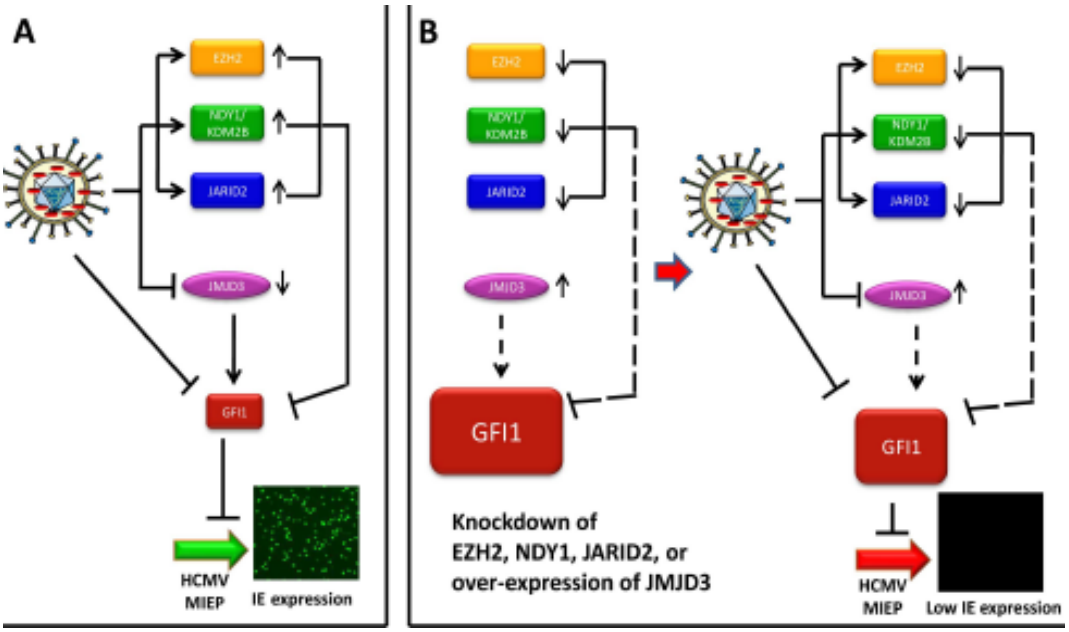


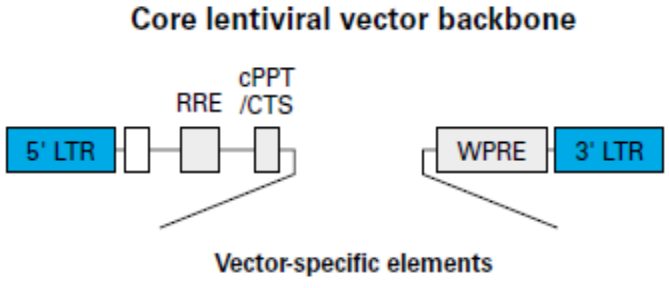
Figure 8. Infection by HCMV relies on the negative regulation of GFI1, a repressor of immediate-early gene transcription. The evidence is recapitulated on the model depicting the interplay between HCMV and the host. (Panel A) This panel describes the infection of wild type HFFs. HCMV immediately downregulates GFI1 to favour the activation of the MIEP of HCMV, and viral infection. Moreover, virus infection modifies the expression of NDY1/ KDM2B, EZH2, JARID2 and JMJD3. The solid lines from these molecules to GFI1 exhibit that they intensively negative modulate GFI1 both prior and post infection, although owing to HCMV-triggered alterations in their expression, the suppression is stimulated post infection. (Panel B, Left) The suppression of GFI1 in uninfected cells was impeded by the knockdown of NDY1/KDM2B, EZH2 or JARID2 and by the overexpression of JMJD3, driving to great positive regulation of GFI1 (dotted lines). (Panel B, Right) indicates the infection of HFFs in the left side of panel B. HCMV remains in degrading GFI1. Therefore, the negative regulation of GFI1 by HCMV is inadequate to downregulate it to levels that permit the activation of the MIEP and viral infection. doi:10.1371/journal.ppat.1004136.g007

In order to be retained the downregulation of GFI1, HCMV also commences an NDY1/EZH2/JARID2/ JMJD3-based pathway, which suppresses GFI1 during the infection cycle. The silencing of EZH2 may lead to the suppression of MIEP, also by regulating the levels of histone H3K27me3 and H3K4me3 in the immediate early region of HCMV in the initial three hours from the onset of HCMV infection. Finally, they deduced that HCMV infection is based on both EZH2NDY1/ /JARID2/JMJD3-dependent and independent mechanisms which are triggered by the virus and modulate the expression of GFI1, a SNAG domain - containing transcriptional repressor of the immediate early region of HCMV. All these data is illustrated on the above **Figure 8.** EZH2-based epigenetic mechanisms also regulate histone modifications in the immediate-early region of HCMV that may lead to promotion of the MIEP in the very initial stages of infection. Blockage of the signaling axis may have preventive or therapeutic applications in viral infection, whereas selective trigger of the pathway may have therapeutic applications in Oncology (226).

Materials and Methods

Gene Transfer and Expression Using Recombinant Lentiviruses

Recombinant lentiviral vectors are powerful and efficient tools for transferring heritable genetic material into the genome of virtually any cell type (Ausubel *et al.*, 1995; Coffin *et al.*, 1996). Lentiviruses are perhaps the most versatile of retroviruses since they are able to infect, transduce, and sustain expression in almost any mammalian cell, including dividing and nondividing cells, stem cells, and primary cell cultures. In the Lenti-X systems, high titers of recombinant, replication incompetent virions are easily generated using a **Lenti-X HTX Packaging System** (Cat. Nos. 631247, 631249 or 631251), in which a Lenti-X expression vector containing your gene of interest (GOI), is cotransfected along with a Lenti-X HTX Packaging Mix into the **Lenti-X 293T Cell Line** (Cat. No.632180). The lentiviral supernatants produced by the transfected packaging cells can then be used to infect and transduce target cells to express your GOI, fusion protein, or shRNA.



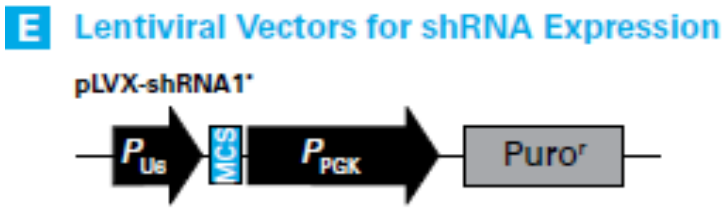
All pLVX vectors possess the requisite HIV-1 LTRs and the lentiviral packaging signal (Ψ) as well as other elements to improve transgene expression, viral titer, and overall vector function.

• **WPRE**: A woodchuck hepatitis virus posttranscriptional regulatory element prevents poly A site readthrough, promotes RNA processing and maturation, and increases nuclear export of RNA (Zufferey *et al.* 1999; Higashimoto *et al.*, 2007). It works in the context of viral genomic transcripts in packaging cells to enhance vector

packaging and increase the viral titers. In addition, the WPRE boosts expression of your GOI in transduced target cells by facilitating the maturation of mRNA transcripts produced by the vector's internal promoter (e.g. *P*CMV or *P*Tight).

• **cPPT/CTS**: A central polypurine tract/central termination sequence creates a "DNA flap" that increases nuclear importation of the viral genome during target cell infection. The cPPT/CTS element improves vector integration and transduction efficiency (Zennou *et al.*, 2000).

• **RRE**: A Rev response element helps to increase titers by promoting the nuclear export of unspliced viral genomic RNA (Cochrane, *et al.*, 1990).



P_{U6}: human U6 shRNA promoter (RNA Pol III), **MCS**: multiple cloning site, P_{PGK}: phosphoglucokinase promoter, **Puro** ^{r}: Puromycine resistant

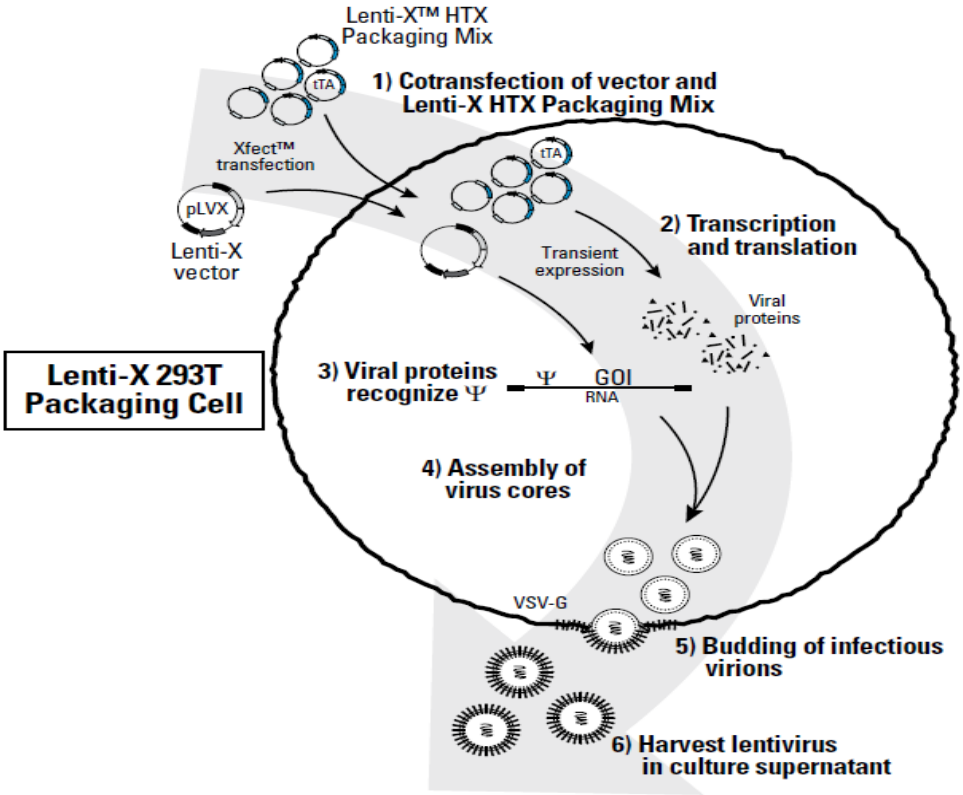


Figure 2. Lentivirus production with the Lenti-X HTX Packaging System and Lenti-X 293T cells. Initially, cotransfection of a Lenti-X vector and the Lenti-X HTX Packaging Mix (Step 1) results in the production of the corresponding recombinant lentiviral genomic RNA transcript and viral packaging proteins (Step 2). A vector in the packaging mix encodes the Tet-Off transactivator (tTA), which produces extra-high expression of specific packaging proteins via Tet-Off transactivation. Recognition of the packaging sequence (Ψ) on the recombinant viral RNA genome by the packaging proteins (Step 3) results in the assembly of viral cores, which are transported to the cell membrane (Step 4). Cores are then enveloped by cellular membrane containing aggregated VSV-G or ecotropic/gp70 envelope proteins. Mature, infectious virions then bud from the cell (Step 5) and are collected in the medium (Step 6). While the virions are infectious, they lack several critical genes required for their subsequent replication and production in target cells. The use of multiple plasmids with which to express the viral proteins adds a strong measure of safety to virus production since several low-frequency recombination events would need to occur in order to regenerate a replication-competent viral genome.

Protocol for lentiviruses production

1. Plating HEK 293T

- Remove supernatant of 100 mm plate
- 1 PBS wash
- Add 7 ml Dulbecco's modified Eagle's minimal essential medium (DMEM) high glucose supplemented with 10% fetal bovine serum(FBS; Biosera),, penicillin (100 units/ml) /streptomycin(100 μg/ml), L-glutamine and non-essential amino acids (Biosera)
- Excellent suspension for single cells production
- Share 6 ml DMEM and 1,5 ml cells for next day transfection in fresh 100 mm plates and 7 ml DMEM and 1ml cells for cell culture
- Incubate cells under 5% CO_2 in a humidified incubator at 37°C

2. Transfection HEK 293T –Turbofect protocol

- Name new tubes (for each virus have 2 tubes, the first for OPTIMEM and the second for the rest reagents)
- Add 900 μl OPTIMEM (Reduced Serum Media is a modification of Eagle's Minimum Essential Media, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factors)
- Add 3 μg retroviral vector of interest
- Add 2 μg p.Δ891 packaging plasmid
- Add 1 μg pMD2.G packaging plasmid
- Add 18 μl Turbofect Transfection reagent (Fermentas, USA)
- Vortex the tube of reagents
- Transfer the amount of reagents in the tube of OPTIMEM, redilute and vortex
- Incubate for 15-20 mins, to be conducted the reaction
- Share dropwise the material of tube on the HEK 293T cells and shake crossed the 100 mm plate so as to be as homogenized as better
- Incubator 37^{o} C

On the next day after transfection of HEK 293T cells, remove the material of 100 mm plate very carefully and then add slowly 10 ml DMEM.

On the next day (48 hours after transfection) the virus harvested, centrifuged for 10 mins at 1800 rpm and then aliquots of the supernatant lentivirus stored at -80 ^{o} C.

Retroviruses

A **retrovirus** is a single-stranded positive-sense RNA virus with a DNA intermediate and targets a host cell. Retroviruses function differently – their RNA is reverse-transcribed into DNA, which is integrated into the host cell's genome (when it becomes a provirus), and then undergoes the usual transcription and translational processes to express the genes carried by the virus. So, the information contained in a

retroviral gene is used to generate the corresponding protein via the sequence: RNA → DNA → RNA → polypeptide. This extends the fundamental process identified by Francis Crick (one gene-one peptide) in which the sequence is: DNA → RNA → peptide. Retroviruses are valuable research tools in molecular biology and have been used successfully in gene delivery systems.

Protocol for retroviruses production

1. Plating HEK 293T

- Remove supernatant of 100 mm plate
- 1 PBS wash
- Add 7 ml Dulbecco's modified Eagle's minimal essential medium (DMEM) high glucose supplemented with 10% fetal bovine serum(FBS; Biosera), penicillin(100 units/ml) /streptomycin(100 μg/ml), L-glutamine and non-essential amino acids (Biosera)
- Excellent suspension for single cells production
- Share 6 ml DMEM and 1,5 ml cells for next day transfection in fresh 100 mm plates and 7 ml DMEM and 1ml cells for cell culture
- Incubate cells under 5% CO_2 in a humidified incubator at 37°C

2. Transfection HEK 293T –Turbofect protocol

- Name new tubes (for each virus have 2 tubes, the first for OPTIMEM and the second for the rest reagents)
- Add 900 μl OPTIMEM (Reduced Serum Media is a modification of Eagle's Minimum Essential Media, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factors)
- Add 3 μg retroviral vector of interest
- Add 1 μg AmphoPack packaging plasmid
- Add 18 μl Turbofect Transfection reagent (Fermentas, USA)
- Vortex the tube of reagents
- Transfer the amount of reagents in the tube of OPTIMEM, redilute and vortex
- Incubate for 15-20 mins, to be conducted the reaction
- Share dropwise the material of tube on the HEK 293T cells and shake crossed the 100 mm plate so as to be as homogenized as better
- Incubate cells under 5% CO_2 in a humidified incubator at 37°C

On the next day after transfection of HEK 293T cells, remove the material of 100 mm plate very carefully and then add slowly 10 ml DMEM. On the next day (48 hours after transfection) the virus harvested, centrifuged for 10 mins at 1800 rpm and then aliquots of the supernatant retrovirus stored at -80 ^{o} C.

To be more specific, I familiarized with the production of **lentiviruses** such as **pLKO.1 puRo** (U6-driven shRNA empty vector with puro resistance) **pLKO.1 shEZH2** (U6-driven shRNA empty vector, includes a stuffer for easy cloning, silencing EZH2 expression of 13th clone) and **pLKO.1 shEZH2 3' UTR** (U6-driven shRNA empty vector, includes a stuffer for easy cloning, silencing endogenous EZH2

expression of 13th clone, exogenous EZH2 expression remains intact). The lentiviruses utilize the technique of silencing the gene expression through shRNA and own 2 packaging plasmids the pMDG2 plasmid, VSV-G-expressing envelope vector and pΔ891 plasmid.

Moreover, I engaged with the production of **retroviruses** such as: **pBABE puro** (Retroviral vector for cloning and expressing your gene of interest, Puromycin selection), **pBabe-puro-Myr-Flag-AKT1(**retroviral vector expressed in mammals incorporated with insert AKT1, which is a Myr-Flagged protein on the N-terminal of insert), **pBabe-puro-Myr-HA-AKT2**(retroviral vector expressed in mammals incorporated with insert AKT2, which is a Myr-Flagged protein on the N-terminal of backbone and HA-Flagged protein on the N-terminal of backbone), **pBabe-puro-Myr-HA-AKT3**(retroviral vector expressed in mammals incorporated with insert AKT3, which is a Myr-Flagged protein on the N-terminal of backbone and HA-Flagged protein on the N-terminal of backbone)**, pSMP-EZH2** (retroviral RNAi wild type vector expressed in mammals with selectable markers for puromycin, incorporated wt EZH2 gene), **pcDNA3-3myc-6His-EZH2 21A** (vector expressed in mammals owning selectable markers for neomycin, incorporating EZH2 (Enhancer of Zeste Homolog 2 and an additional mutation of S21A, Ser 21 was replaced by Ala. It has 2 tags: 3 Myc and 6 His on the N terminal on insert. The retroviruses overexpress their gene of interest and are proved to be valuable research tools in molecular biology and have been used successfully in gene delivery systems. Moreover they possess only one amphotropic packaging construct the so called AmphoPack.

In general, Human Foreskin Fibroblasts (HFFs) were used for the propagation of HCMV virus and HEK 293T cells were transfected to package lentivirus and retrovirus constructs.

HFFs plating for the transduction of viruses

- Remove supernatant from 75cm^2 Corning® cell culture flask
- 1 PBS wash
- Add 0.25% Trypsin-EDTA (1x) for dissociation of cell monolayers, remove trypsin and then incubate cells in CO_2 incubator for 15-20 mins
- Add 7 ml Dulbecco's modified Eagle's minimal essential medium (DMEM) high glucose supplemented with 10% fetal bovine serum, penicillin/streptomycin, L-glutamine and non-essential amino acids (Biosera)
- Excellent suspension for single cells production
- Share 6 ml DMEM high glucose and 3 ml HFFs for next day process of addition of produced viruses (supernatants) in fresh 100 mm plates of HFFs and in parallel keep the same flask adding 7 ml DMEM high glucose and 1ml HFFs for cell culture
- Incubate cells under 5% CO_2 in a humidified incubator at 37°C

On the next day, the produced viral supernatant is added into HFFs in presence of polybrene (1/1000) (Sigma-Aldrich, USA)., a highly efficient infection reagent used to introduce retroviral vectors into mammalian cells. After 2 days, we refresh the DMEM, putting 8ml fresh DMEM high glucose. The next day, we add puromycin (10 mg/ml) (Sigma-Aldrich, USA). in a proportion of 2 μl puromycin in 11 ml DMEM

high glucose in purposes of puromycin selection. Cells infected with multiple retrovirus or lentivirus constructs, were selected for these constructs sequentially. The alive monolayer adherent HFFs are resistant to puromycin, expressing the corresponding supernatant of lentivirus or retrovirus.

The final outcome is the **expansion** of myr-AKT1, myr-AKT2, myr-AKT3, EZH2Ser21A, pLKO.1, shEZH2 and shEZH2 3' UTR HFFs in **75cm^2 flasks**. They are maintained in **presence of puromycin**.

Basic Nucleofector® Kit for Primary Mammalian Fibroblasts (AMAXA)

Pre-Nucleofection

Remove media from the cultured HFFs and wash cells once with PBS. Then follows the process of trypsinisation of HFFs and excellent suspension making use of DMEM high glucose. Then centrifuge the well suspended HFFs for 10 mins at 1100 rpm. The pellet depicts the HFFs.

Nucleofection

Prepare 6-well plates by filling appropriate number of wells with 1.5 ml of supplemented culture media and pre-incubate/equilibrate plates in a humidified 37°C/5% CO_2 incubator.

Resuspend the HFFs pellet carefully in 100 µl room temperature Nucleofector® Solution (90 µl basic solution, 10 µl supplement solution) per sample. Please make sure that the entire supplement is added to the Nucleofector® Solution! Combine 100 µl of cell suspension with 8µg plasmid DNA, into certified cuvette. The sample must cover the bottom of the cuvette without air bubbles. Select the appropriate Nucleofector® program U-012. Insert the cuvette with cell/DNA suspension into the Nucleofector ® cuvette holder and apply the selected program. Take the cuvette out of the holder once the program is finished. Then, add 500 µl of the pre-equilibrated culture media to the cuvette and gently transfer the sample immediately into the 6-well plate (final volume 1.5 ml DMEM per well/sample). Use the supplied pipettes and avoid repeated aspiration of the sample.

Post-Nucleofection

Incubate the cells in a humidified 37°C/5% CO_2 incubator.

The Basic Nucleofector® Kit for Primary Mammalian Fibroblasts (AMAXA) consists an *in vitro* electroporation method. Nucleofection® is a technology based on the momentary creation of small pores in cell membranes by applying electrical pulse. The comprehensive way in which Nucleofector® Programs and cell type-specific solutions are developed ensures that nucleic acid substrates are delivered not only to the cytoplasm, but also through the nuclear membrane and into the nucleus. Transfected cells retain excellent viability and the function of intracellular systems is highly conserved.

Viral stocks were grown in Human Foreskin Fibroblasts (HFF) cells and titrated by **plaque assays** as described (227)

Serum starvation

Remove the supernatant, 1 PBS wash and add DMEM serum free with P/S

HCMV infection

Control (mock cells)

12-well plate

pLKO.1	**shEZH2**	**myr-Akt1**	**myr-Akt1 + myc EZH2**
myr-Akt1+myc EZH2 +shEZH2 3'UTR	**myr-Akt3**	**myr-Akt3+ myc EZH2**	**myr-Akt3 + myc EZH2 +shEZH2 3'UTR**

HCMV Infected cells

12-well plate

pLKO.1	**shEZH2**	**myr-Akt1**	**myr-Akt1 + myc EZH2**
myr-Akt1+myc EZH2 +shEZH2 3'UTR	**myr-Akt3**	**myr-Akt3+myc EZH2**	**myr-Akt3 + myc EZH2 +shEZH2 3'UTR**

Firstly, plate the corresponding type of cells pLKO.1, shEZH2, myr-Akt1 myr-Akt2 and myr-Akt3 HFFs in wells of 12-well plates. After 2 days, add 1,5 ml supernatant virus in presence of polybrene in specific wells (myc EZH2, shEZH2 3′UTR). In some wells, HFFs subject to double transduction (e.g myr-Akt1+myc EZH2 +shEZH2 3'UTR, myr-Akt2+myc EZH2 +shEZH2 3'UTR, myr-Akt3 + myc EZH2 +shEZH2 3' UTR). After 2 days, add puromycin in cells and check them in microscope.

For HCMV-infected 12-well plate prepare a mix which includes 8 ml DMEM high glucose and 40 μl wild type AD169 HCMV strain (use of filter tips). To infect HFFs with HCMV, cell monolayers were incubated with the virus at a multiplicity of infection (MOI) of 0.5 PFU/cell. Then share 1 ml mix per well and incubate for 4 hours at 37 ^{o} C. The inoculum was removed and replaced by fresh medium. Then follows the addition of puromycin in wells in purposes of puromycin selection.

Cell lysis and western blotting

The cells were washed in ice-cold PBS and were lysed with PBS-EDTA. The cell lysates from HCMV-infected and mock infected cells were centrifuged for 10 mins at 2.500g. The pellet of suspended cells resuspended with 60 μl M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA), enriched with the Halt-Protease Inhibitor Cocktail, EDTA-free (PIERCE, Rockford, IL, USA) and Phosphatase Inhibitor Cocktail. An additional centrifuge takes place at 14.000g for 15 mins to be removed the cell debris. Then the supernatant is transferred to a fresh tube for further analysis.

Proteins were separated on 10% polyacrylamide gels containing Sodium- Dodecyl Sulphate, and then transferred to nitrocellulose membranes. Western blots of the supernatants (soluble whole-cell lysates) were probed with the following antibodies.

- **Ezh2** rabbit monoclonal antibody (no. 4905, Cell Signaling, in a dilution of 1/1000
- **Phospho Akt1/2/3** Antibody: sc-7985-R p-Akt1/2/3 (Ser 473)- rabbit polyclonal IgG, from Santa Cruz Biotechnology, Inc in a dilution of 1/300
- **Total Akt1/2/3** Antibody (H-136): sc-8312 (H-136) is a rabbit polyclonal IgG from Santa Cruz Biotechnology, Inc in a dilution of 1/600
- **Gfi-1** (N-20) X, Goat Polyclonal IgG (sc-8558-X) from Santa Cruz Biotechnology, Inc in a dilution of 1/500
- **β-Actin** Antibody (C4): sc-47778 mouse monoclonal IgG_1 from Santa Cruz Biotechnology, Inc in a dilution of 1/5000

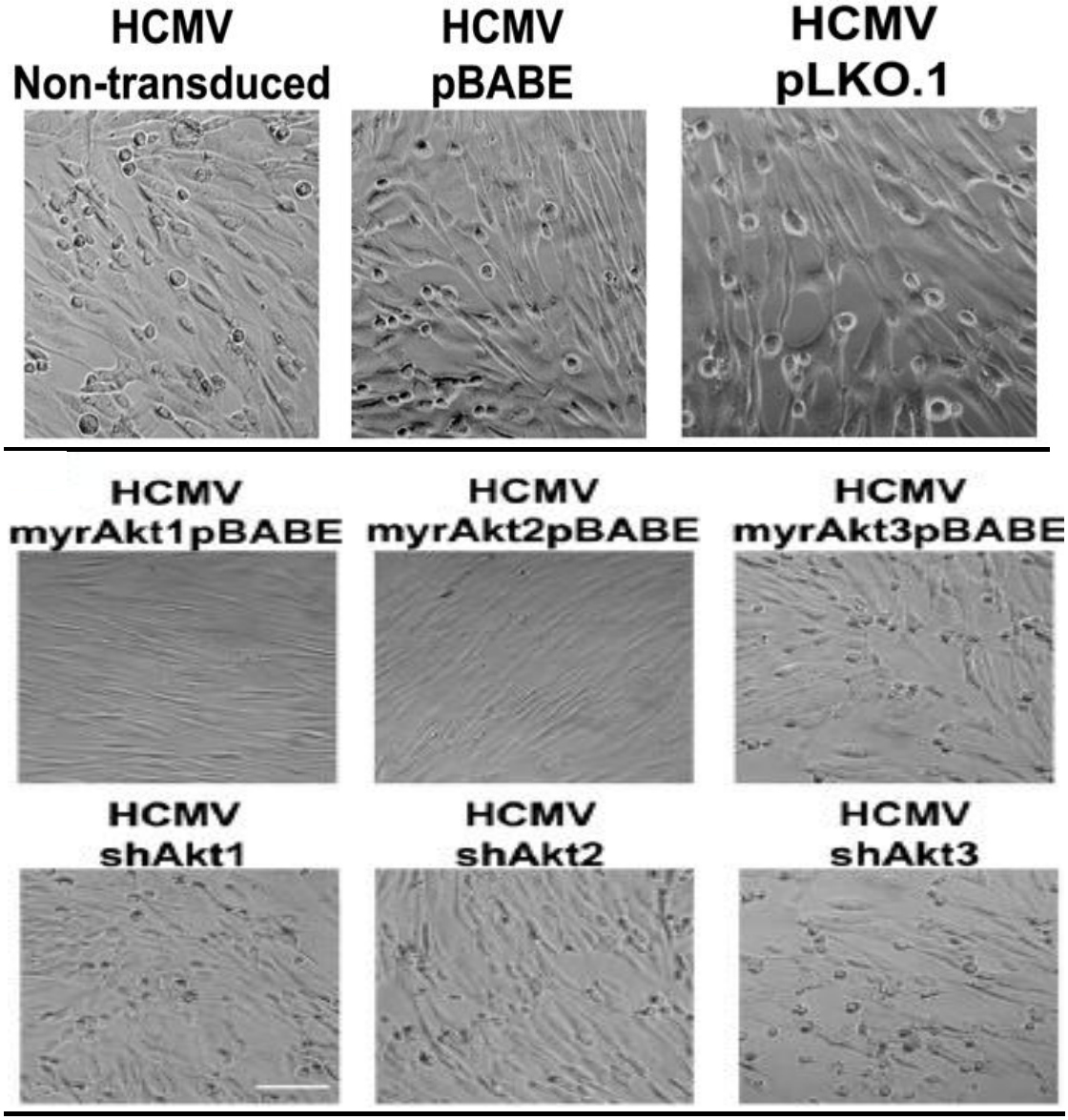
The Horseradish Peroxidase conjugated secondary antibodies were diluted either in 1% milk in TBS-T or in 1% BSA in TBS-T and incubated with the blots for 50 mins at room temperature. The Horseradish Peroxidase conjugated secondary antibodies are the following:

- **Donkey anti-Goat** IgG-HRP : sc-2020 Horseradish Peroxidase conjugated secondary antibody from Santa Cruz Biotechnology, Inc in a dilution of 1/5000
- **Goat anti-Mouse** IgG Antibody, Peroxidase Conjugated, H+L, AP124P ,Millipore-Sigma, in a dilution of 1/5000
- **Goat anti-Rabbit** IgG Antibody, Peroxidase Conjugated AP132P, Millipore-Sigma, in a dilution of 1/5000

The bound secondary antibodies were detected with WBLUF0100 | Luminata Forte Western HRP substrate. Digital images of the proteins were acquired using the LAS-4000 luminescent image analyzer (Fujifilm Life Science).

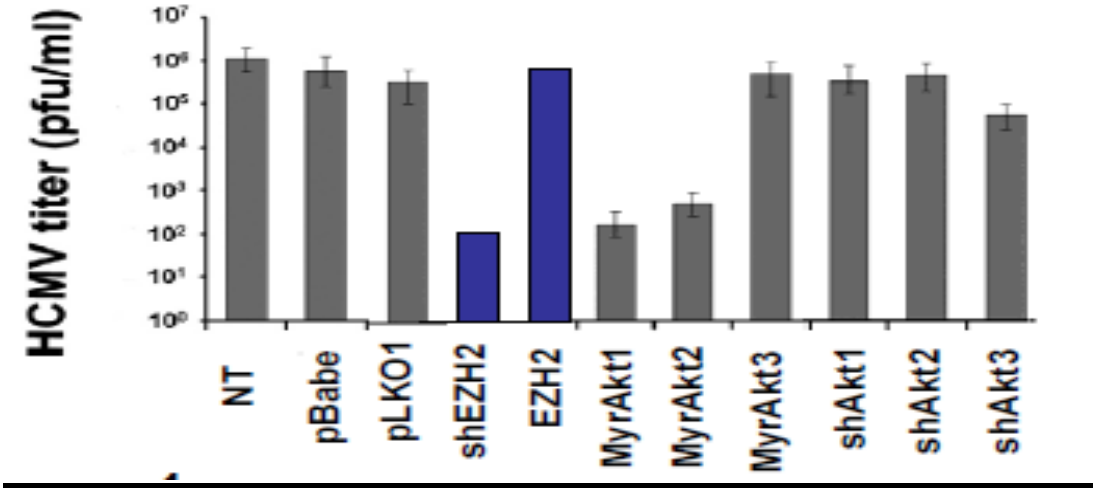
Results

1. Constitutively active Akt1 and Akt2, but not Akt3, inhibit the HCMV infection of HCMV-infected HFFs



Images acquired from optical microscopy, illustrated the detection of HCMV-infected HFFs. HFFs were either non transduced or transduced with pBABE, pLKO.1, pBABE-based retroviral constructs of myrAkt1, myrAkt2, myrAkt3 and pLKO.1-based lentiviral constructs of shAkt1, shAkt2, shAkt3. HFFs were infected with AD169 strain of HCMV (MOI 0.5 PFU/cell). The infected cells were monitored by light microscopy 5 days later.

2. EZH2 is required for HCMV infection and the constitutively active Akt1 and Akt2, but not Akt3, inhibit the HCMV infection of HCMV-infected HFFs



Titration of viral load in **plaque assay** experiments: HFFs were either non transduced or transduced with pLKO.1-based lentiviral constructs of shEZH2, shAkt1, shAkt2, shAkt3, or with the empty vector. Alternatively, HFFs were transduced with pBABE-based retroviral constructs of EZH2, myrAkt1, myrAkt2, myrAkt3 or with empty vector. HFFs were infected with AD169 strain of HCMV (MOI 0.5 PFU/cell). HCMV was harvested 5 days post infection from the transduced and non-transduced cells and titrated by plaque assay. The bars show the viral titers (mean 6 SD)

3. The constitutively active Akt1 blocks HCMV infection inactivating EZH2, via phosphorylation at Ser21 residue

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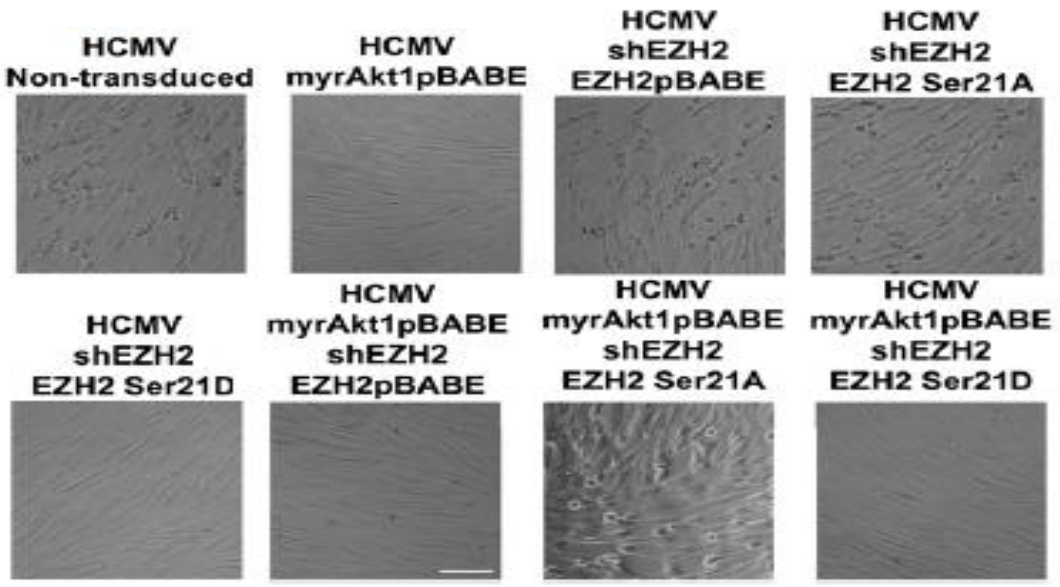
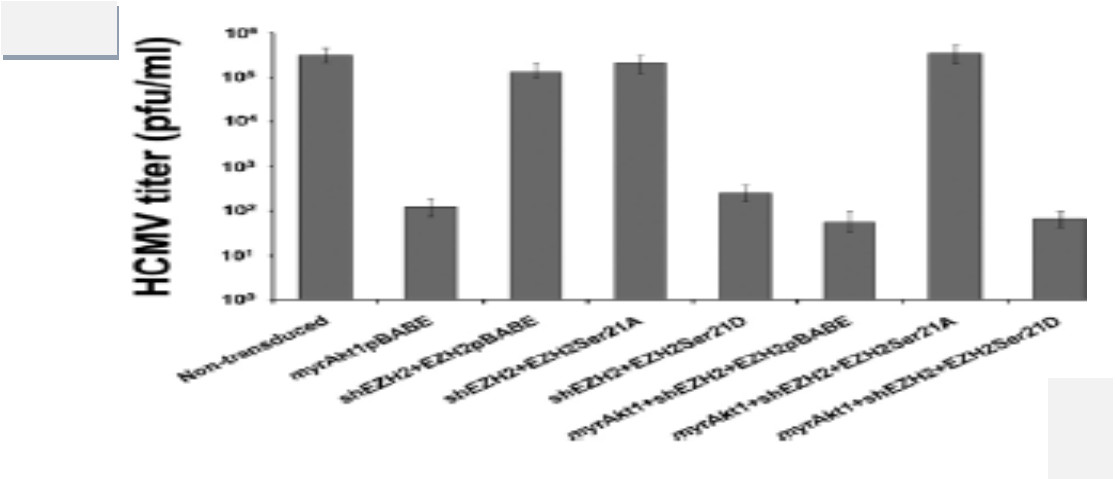


Images acquired from optical microscopy, illustrated the detection of HCMV-infected HFFs. HFFs were infected with HCMV (MOI 0.5 PFU/cell). The infected cells were monitored by light microscopy 5 days later. HFFs were either non tranduced or transduced with myr-Akt1 pBABE retroviral vector. Then some of these cultures also transduced with the pLKO.1-based construct of shEZH2. After this, some of these cultures were also transduced with pBABE-puro-based retroviral constructs of EZH2 like EZH2pBABE, EZH2S21A, EZH2S21D. Likewise, HFFS infected with HCMV (MOI 0.5 PFU/cell). Then they transduced with lentiviral based-PLKO.1. shEZH2 construct. Then, some of these cultures also transduced with pBABE-puro-retroviral constructs of EZH2pBABE, EZH2S21A, EZH2S21D. HCMV was harvested 5 days post infection from the transduced and non-transduced cells and titrated by a plaque assay.

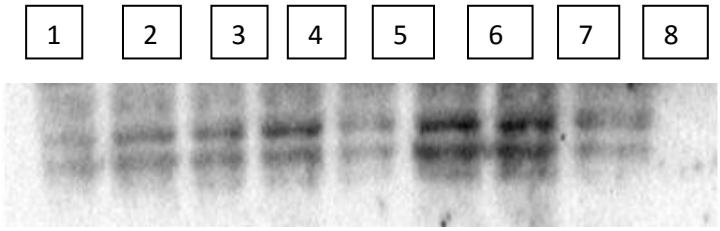


Titration of viral load in **plaque assay** experiments

Western Immunoblotting Results

1.Western Immunoblotting for the protein levels of EZH2 in mock cells

EZH2

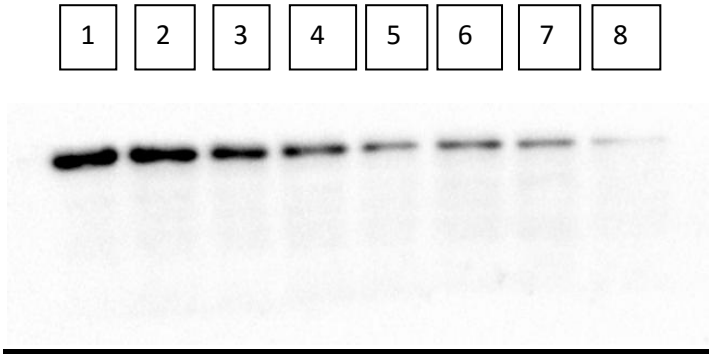


Mock cells

1. **PLKO.1**
2. **shEZH2**
3. **Akt1**
4. **Akt1 (myc EZH2)**
5. **Akt1 (myc EZH2, shEZH2 3'UTR)**
6. **Akt3**
7. **Akt3 (myc EZH2)**
8. **Akt3 (myc EZH2, shEZH2 3'UTR)**

2.Western Immunoblotting for the protein levels of total AKT in mock cells

Total AKT

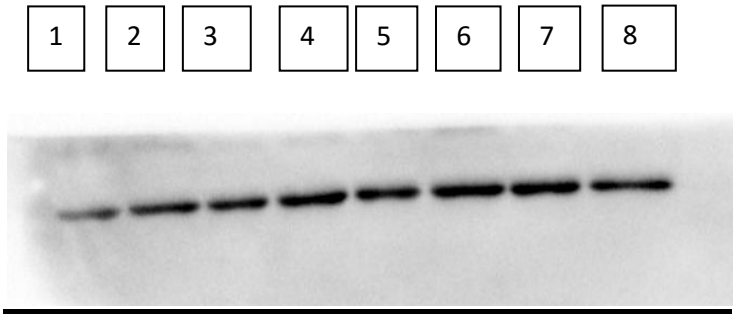


Mock cells

1. **PLKO.1**
2. **shEZH2**
3. **Akt1**
4. **Akt1 (myc EZH2)**
5. **Akt1 (myc EZH2, shEZH2 3'UTR)**
6. **Akt3**
7. **Akt3 (myc EZH2)**
8. **Akt3 (myc EZH2, shEZH2 3'UTR)**

3.Western Immunoblotting for the protein levels of β - actin in mock cells

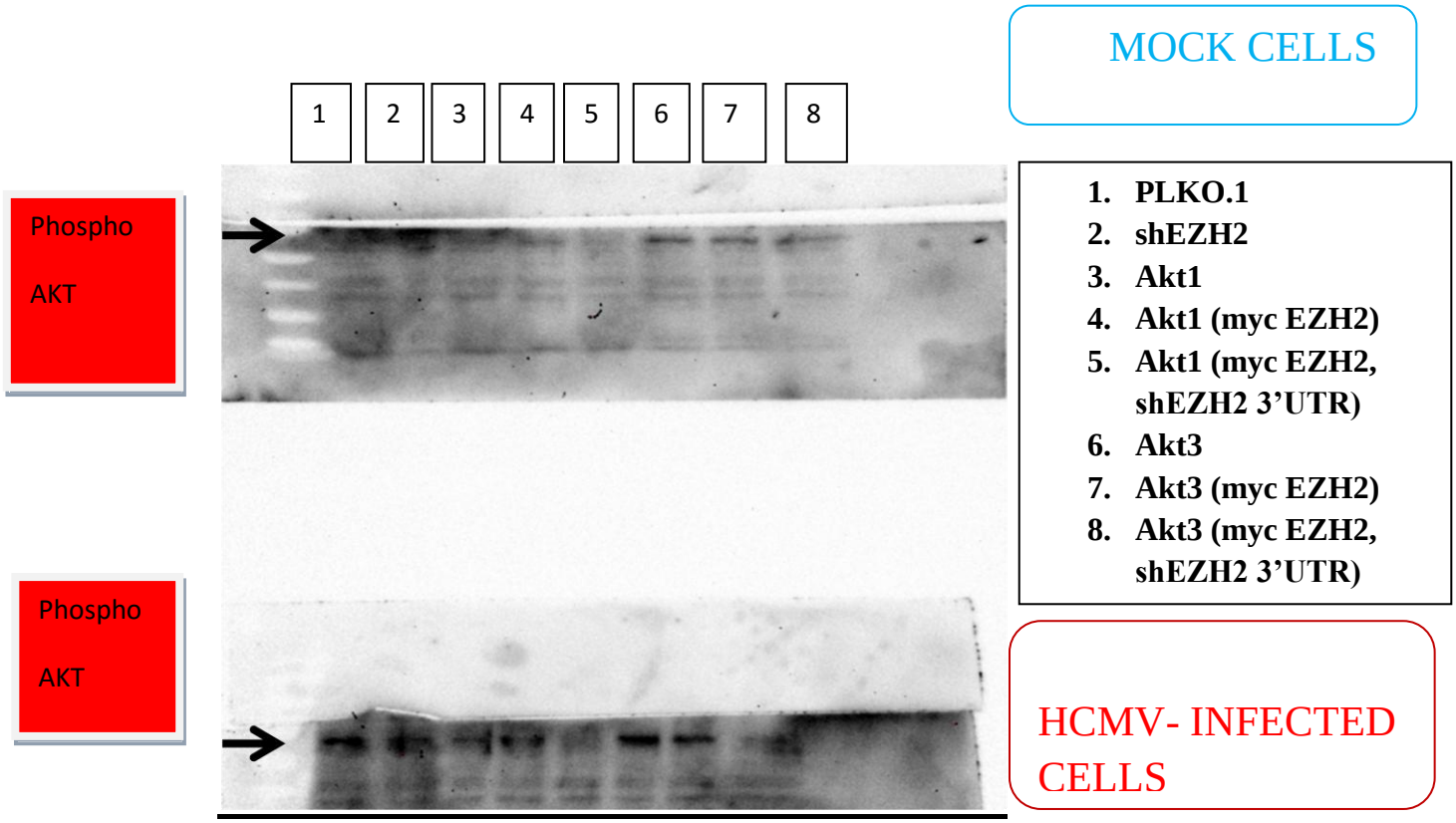
β-actin



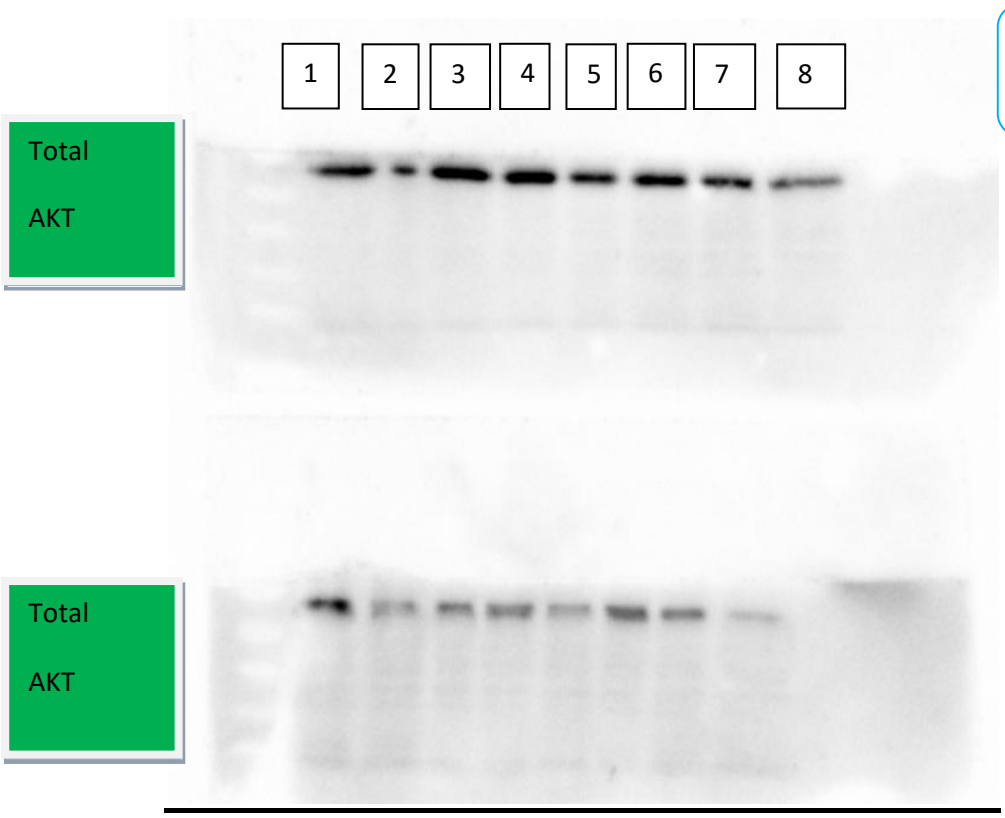
Mock cells

1. **PLKO.1**
2. **shEZH2**
3. **Akt1**
4. **Akt1 (myc EZH2)**
5. **Akt1 (myc EZH2, shEZH2 3'UTR)**
6. **Akt3**
7. **Akt3 (myc EZH2)**
8. **Akt3 (myc EZH2, shEZH2 3'UTR)**

4.Western Immunoblotting for the protein levels of phospho-AKT in mock and HCMV-infected cells



5.Western Immunoblotting for the protein levels of total AKT in mock and HCMV-infected cells

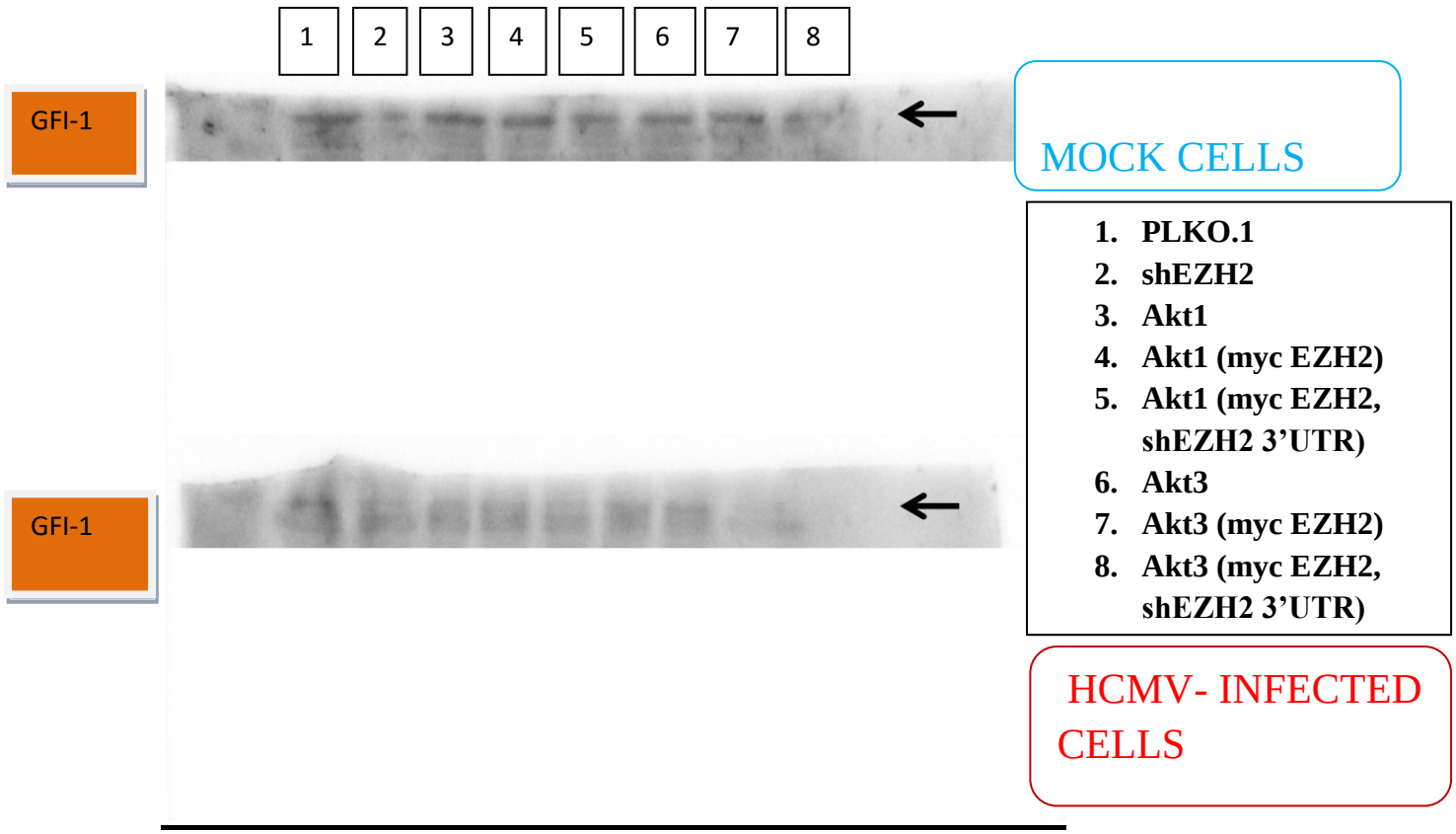


MOCK CELLS

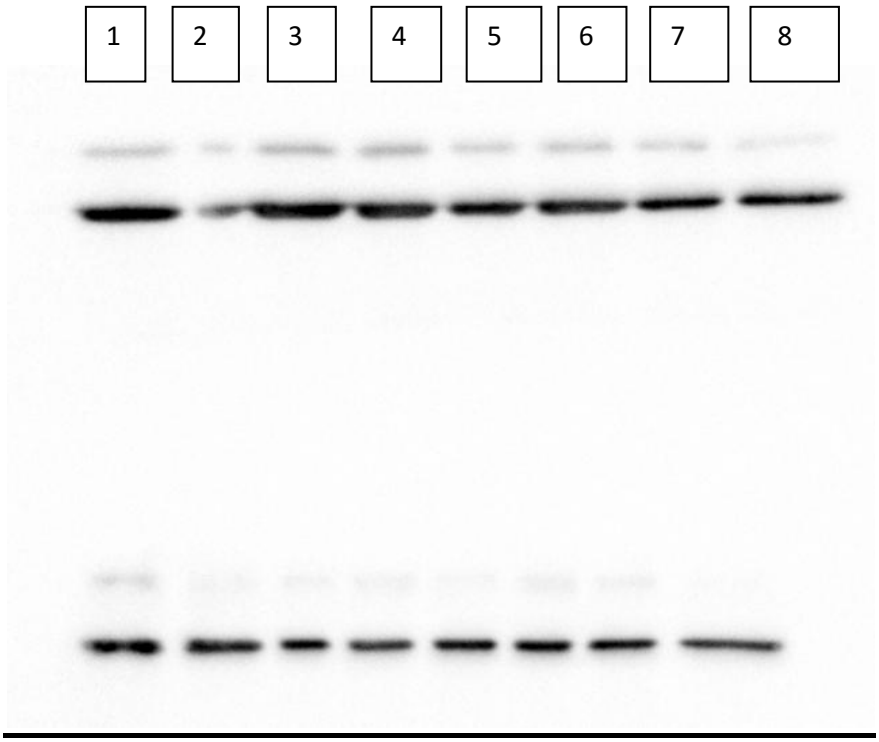
1. **PLKO.1**
2. **shEZH2**
3. **Akt1**
4. **Akt1 (myc EZH2)**
5. **Akt1 (myc EZH2, shEZH2 3'UTR)**
6. **Akt3**
7. **Akt3 (myc EZH2)**
8. **Akt3 (myc EZH2, shEZH2 3'UTR)**

HCMV- INFECTED CELLS

6.Western Immunoblotting for the protein levels of GFI-1 in mock and HCMV-infected cells



7.Western Immunoblotting for the protein levels of β-actin in mock and HCMV-infected cells



MOCK CELLS

1. **PLKO.1**
2. **shEZH2**
3. **Akt1**
4. **Akt1 (myc EZH2)**
5. **Akt1 (myc EZH2, shEZH2 3'UTR)**
6. **Akt3**
7. **Akt3 (myc EZH2)**
8. **Akt3 (myc EZH2, shEZH2 3'UTR)**

HCMV- INFECTED CELLS

Discussion

This project demonstrated for the very first time the interplay between Akt, EZH2, and GFI-1 in the context of HCMV infection. Firstly, it was indicated the differential potential of Akt isoforms in HCMV infection. To be more specific, data represent that constitutively active Akt1 inhibits the infection of HCMV - infected HFFs, as opposed to active Akt3 which increases HCMV titer, favouring in this way the HCMV infection. Then some additional findings validate the pivotal role of EZH2 in HCMV, supporting the published in detail evidence of (226), where they shed light to the fact that EZH2, NDY1/KDM2B, and histone H3K27 tri-methylation are required for the transcriptional activation of the major immediate-early promoter (MIEP) of HCMV and the initiation of HCMV infection. Moreover, it was suggested the possible mechanism through which the constitutively active Akt1 blocks HCMV infection. This is owing to the inactivation of EZH2, via phosphorylation at Ser21 residue. It had already been indicated that the Akt phosphorylates EZH2 at serine 21 and suppresses its methyltransferase activity by impeding EZH2 binding to histone H3, which results in a decrease of lysine 27 trimethylation and derepression of silenced genes.(225)

Then the Western Immunoblotting unravels significant data about the protein expression of EZH2, GFI-1 and the activation of Akt in mock and HCMV-infected HFFs. It is illustrated that both Akt3-transduced HFFs and Akt3 + mycEZH2-transduced HFFs possess elevated levels of EZH2. Whereas, the knockdown of EZH2 in 3' UTR leads to an expected and significant decrease in EZH2 expression in Akt3 + mycEZH2+ shEZH2 3'UTR - transduced HFFs. Likewise, the silencing of EZH2 in 3' UTR results in an expected and obvious drop in EZH2 expression in Akt1 + mycEZH2+ shEZH2 3'UTR - transduced HFFs compared to the EZH2 levels in both Akt1-transduced HFFs and Akt1 + mycEZH2-transduced HFFs. It is worth mentioned that it is demonstrated for first time that the mock cells expressing active Akt1 own decreased levels of EZH2 (inactivation of EZH2) as opposed to mock cells expressing active Akt3, where there is robust EZH2 expression in both Akt3-transduced HFFs and Akt3 + mycEZH2-transduced HFFs.

Further, it is validated the phosphorylation of Akt3 in HCMV-infected HFFs expressing Akt3, Akt3 + myc EZH2 and Akt3 + myc EZH2 + shEZH2 3'UTR. It is worth further investigated the evidence that Akt3 + myc EZH2 + shEZH2 3'UTR transduced HCMV-infected HFFs express lower levels of phospho - Akt3 as opposed to Akt3 and Akt3 +myc EZH2 transduced HCMV-infected HFFs. Similarly, for the phosphorylation of Akt1 in HCMV-infected HFFs. A possible explanation might be that knocking down EZH2, decreases HCMV infectivity and in turn leads to a decline of activation of both Akt1 and Akt3 in HCMV-infected HFFs. The mock cells own active Akt1.

Supporting the evidence of (226), we experimentally demonstrated that the mock cells possess apparent elevated levels of the transcriptional repressor GFI-1. In contrast, GFI-1 is downregulated in HCMV-infected HFFs irrespective of their EZH2 silencing status. This can be justified by the precise explanation of (226) where it was reported that during infection, the incoming HCMV rapidly negatively regulates the GFI1 protein in both wild-type cells and in cells in which EZH2 is knocked down. However, since the pre-infection levels of GFI1 in the latter cells are significantly higher, the virus fails to downregulate it to levels permissive for MIEP activation and

viral infection. Following the EZH2-NDY1/KDM2B-JARID2- independent downregulation of GFI1 in the early stages of infection, the virus also initiates an EZH2-NDY1/KDM2B-JARID2- dependent program that represses GFI1 throughout the infection cycle.(226)

Taken all the above into consideration, the evidence of this project identify a novel interplay of Akt , GFI-1 and EZH2 in HCMV infection. The differential function of Akt isoforms play significant role in the viral infection. The phosphorylation levels of Akt isoforms are epigenetically modulated according to EZH2 status, in HCMV-infected HFFs. In parallel, GFI-1could be easily considered as a promising biomarker for HCMV infection. Targeting this molecule, especially upregulating GFI-1 expression might have therapeutic applications in HCMV infection in the forthcoming years.

References

1. Hill, R. B., Jr., Rowlands, D. T., Jr., and Rifkind, D. (1964) INFECTIOUS PULMONARY DISEASE IN PATIENTS RECEIVING IMMUNOSUPPRESSIVE THERAPY FOR ORGAN TRANSPLANTATION. *The New England journal of medicine* **271**, 1021-1027
2. Rubin, R. H. (1989) The indirect effects of cytomegalovirus infection on the outcome of organ transplantation. *Jama* **261**, 3607-3609
3. Simanek, A. M., Dowd, J. B., Pawelec, G., Melzer, D., Dutta, A., and Aiello, A. E. (2011) Seropositivity to cytomegalovirus, inflammation, all-cause and cardiovascular disease-related mortality in the United States. *PloS one* **6**, e16103
4. Staras, S. A., Flanders, W. D., Dollard, S. C., Pass, R. F., McGowan, J. E., Jr., and Cannon, M. J. (2008) Cytomegalovirus seroprevalence and childhood sources of infection: A population-based study among pre-adolescents in the United States. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* **43**, 266-271
5. Cannon, M. J., Hyde, T. B., and Schmid, D. S. (2011) Review of cytomegalovirus shedding in bodily fluids and relevance to congenital cytomegalovirus infection. *Reviews in medical virology* **21**, 240-255
6. Kenneson, A., and Cannon, M. J. (2007) Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. *Reviews in medical virology* **17**, 253-276
7. Revello, M. G., Fabbri, E., Furione, M., Zavattoni, M., Lilleri, D., Tassis, B., Quarenghi, A., Cena, C., Arossa, A., Montanari, L., Rognoni, V., Spinillo, A., and Gerna, G. (2011) Role of prenatal diagnosis and counseling in the management of 735 pregnancies complicated by primary human cytomegalovirus infection: a 20-year experience. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* **50**, 303-307
8. Revello, M. G., Sarasini, A., Zavattoni, M., Baldanti, F., and Gerna, G. (1998) Improved prenatal diagnosis of congenital human cytomegalovirus infection by a modified nested polymerase chain reaction. *Journal of medical virology* **56**, 99-103
9. Dollard, S. C., Grosse, S. D., and Ross, D. S. (2007) New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection. *Reviews in medical virology* **17**, 355-363
10. Guerra, B., Simonazzi, G., Banfi, A., Lazzarotto, T., Farina, A., Lanari, M., and Rizzo, N. (2007) Impact of diagnostic and confirmatory tests and prenatal counseling on the rate of pregnancy termination among women with positive cytomegalovirus immunoglobulin M antibody titers. *American journal of obstetrics and gynecology* **196**, 221.e221-226
11. Huang, E. S., Alford, C. A., Reynolds, D. W., Stagno, S., and Pass, R. F. (1980) Molecular epidemiology of cytomegalovirus infections in women and their infants. *The New England journal of medicine* **303**, 958-962
12. Boppana, S. B., Rivera, L. B., Fowler, K. B., Mach, M., and Britt, W. J. (2001) Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity. *The New England journal of medicine* **344**, 1366-1371
13. de Vries, J. J., van Zwet, E. W., Dekker, F. W., Kroes, A. C., Verkerk, P. H., and Vossen, A. C. (2013) The apparent paradox of maternal seropositivity as a risk factor for congenital cytomegalovirus infection: a population-based prediction model. *Reviews in medical virology* **23**, 241-249
14. Atabani, S. F., Smith, C., Atkinson, C., Aldridge, R. W., Rodriguez-Peralvarez, M., Rolando, N., Harber, M., Jones, G., O'Riordan, A., Burroughs, A. K., Thorburn, D., O'Beirne, J., Milne, R. S., Emery, V. C., and Griffiths, P. D. (2012) Cytomegalovirus replication kinetics in solid organ transplant recipients managed by preemptive therapy. *American journal of transplantation : official journal of the American*

Society of Transplantation and the American Society of Transplant Surgeons **12**, 2457-2464

15. Grundy, J. E., Lui, S. F., Super, M., Berry, N. J., Sweny, P., Fernando, O. N., Moorhead, J., and Griffiths, P. D. (1988) Symptomatic cytomegalovirus infection in seropositive kidney recipients: reinfection with donor virus rather than reactivation of recipient virus. *Lancet (London, England)* **2**, 132-135
16. Owers, D. S., Webster, A. C., Strippoli, G. F., Kable, K., and Hodson, E. M. (2013) Pre-emptive treatment for cytomegalovirus viraemia to prevent cytomegalovirus disease in solid organ transplant recipients. *The Cochrane database of systematic reviews* **2**, Cd005133
17. Singh, N. (2006) Antiviral drugs for cytomegalovirus in transplant recipients: advantages of preemptive therapy. *Reviews in medical virology* **16**, 281-287
18. Boeckh, M., and Nichols, W. G. (2004) The impact of cytomegalovirus serostatus of donor and recipient before hematopoietic stem cell transplantation in the era of antiviral prophylaxis and preemptive therapy. *Blood* **103**, 2003-2008
19. Wimperis, J. Z., Brenner, M. K., Prentice, H. G., Reittie, J. E., Karayiannis, P., Griffiths, P. D., and Hoffbrand, A. V. (1986) Transfer of a functioning humoral immune system in transplantation of T-lymphocyte-depleted bone marrow. *Lancet (London, England)* **1**, 339-343
20. Webster, A., Lee, C. A., Cook, D. G., Grundy, J. E., Emery, V. C., Kernoff, P. B., and Griffiths, P. D. (1989) Cytomegalovirus infection and progression towards AIDS in haemophiliacs with human immunodeficiency virus infection. *Lancet (London, England)* **2**, 63-66
21. Deayton, J. R., Prof Sabin, C. A., Johnson, M. A., Emery, V. C., Wilson, P., and Griffiths, P. D. (2004) Importance of cytomegalovirus viraemia in risk of disease progression and death in HIV-infected patients receiving highly active antiretroviral therapy. *Lancet (London, England)* **363**, 2116-2121
22. Bate, S. L., Dollard, S. C., and Cannon, M. J. (2010) Cytomegalovirus seroprevalence in the United States: the national health and nutrition examination surveys, 1988-2004. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **50**, 1439-1447
23. Boeckh, M., and Geballe, A. P. (2011) Cytomegalovirus: pathogen, paradigm, and puzzle. *The Journal of clinical investigation* **121**, 1673-1680
24. Sinzger, C., Grefte, A., Plachter, B., Gouw, A. S., The, T. H., and Jahn, G. (1995) Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues. *The Journal of general virology* **76 (Pt 4)**, 741-750
25. Crough, T., and Khanna, R. (2009) Immunobiology of human cytomegalovirus: from bench to bedside. *Clinical microbiology reviews* **22**, 76-98, Table of Contents
26. Manicklal, S., Emery, V. C., Lazzarotto, T., Boppana, S. B., and Gupta, R. K. (2013) The "silent" global burden of congenital cytomegalovirus. *Clinical microbiology reviews* **26**, 86-102
27. Tavalai, N., and Stamminger, T. (2008) New insights into the role of the subnuclear structure ND10 for viral infection. *Biochimica et biophysica acta* **1783**, 2207-2221
28. Davison, A. J., Dolan, A., Akter, P., Addison, C., Dargan, D. J., Alcendor, D. J., McGeoch, D. J., and Hayward, G. S. (2003) The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome. *The Journal of general virology* **84**, 17-28
29. Murphy, E., Rigoutsos, I., Shibuya, T., and Shenk, T. E. (2003) Reevaluation of human cytomegalovirus coding potential. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 13585-13590
30. Murphy, E., Yu, D., Grimwood, J., Schmutz, J., Dickson, M., Jarvis, M. A., Hahn, G., Nelson, J. A., Myers, R. M., and Shenk, T. E. (2003) Coding potential of laboratory

and clinical strains of human cytomegalovirus. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 14976-14981
31. Stern-Ginossar, N., Weisburd, B., Michalski, A., Le, V. T., Hein, M. Y., Huang, S. X., Ma, M., Shen, B., Qian, S. B., Hengel, H., Mann, M., Ingolia, N. T., and Weissman, J. S. (2012) Decoding human cytomegalovirus. *Science (New York, N.Y.)* **338**, 1088-1093
32. Roy, S., and Arav-Boger, R. (2014) New cell-signaling pathways for controlling cytomegalovirus replication. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **14**, 1249-1258
33. Dziurzynski, K., Chang, S. M., Heimberger, A. B., Kalejta, R. F., McGregor Dallas, S. R., Smit, M., Soroceanu, L., and Cobbs, C. S. (2012) Consensus on the role of human cytomegalovirus in glioblastoma. *Neuro-oncology* **14**, 246-255
34. Meier, J., Lienicke, U., Tschirch, E., Kruger, D. H., Wauer, R. R., and Prosch, S. (2005) Human cytomegalovirus reactivation during lactation and mother-to-child transmission in preterm infants. *Journal of clinical microbiology* **43**, 1318-1324
35. Mehler, K., Oberthuer, A., Lang-Roth, R., and Kribs, A. (2014) High rate of symptomatic cytomegalovirus infection in extremely low gestational age preterm infants of 22-24 weeks' gestation after transmission via breast milk. *Neonatology* **105**, 27-32
36. Schleiss, M. R. (2013) Developing a Vaccine against Congenital Cytomegalovirus (CMV) Infection: What Have We Learned from Animal Models? Where Should We Go Next? *Future virology* **8**, 1161-1182
37. Castillo, J. P., and Kowalik, T. F. (2002) Human cytomegalovirus immediate early proteins and cell growth control. *Gene* **290**, 19-34
38. Liu, X. F., Wang, X., Yan, S., Zhang, Z., Abecassis, M., and Hummel, M. (2013) Epigenetic control of cytomegalovirus latency and reactivation. *Viruses* **5**, 1325-1345
39. Sinclair, J., and Sissons, P. (2006) Latency and reactivation of human cytomegalovirus. *The Journal of general virology* **87**, 1763-1779
40. Kumar, A., Abbas, W., and Herbein, G. (2014) HIV-1 latency in monocytes/macrophages. *Viruses* **6**, 1837-1860
41. Slobedman, B., and Mocarski, E. S. (1999) Quantitative analysis of latent human cytomegalovirus. *Journal of virology* **73**, 4806-4812
42. Bolovan-Fritts, C. A., Mocarski, E. S., and Wiedeman, J. A. (1999) Peripheral blood CD14(+) cells from healthy subjects carry a circular conformation of latent cytomegalovirus genome. *Blood* **93**, 394-398
43. Mendelson, M., Monard, S., Sissons, P., and Sinclair, J. (1996) Detection of endogenous human cytomegalovirus in CD34+ bone marrow progenitors. *The Journal of general virology* **77 (Pt 12)**, 3099-3102
44. Reeves, M. B., MacAry, P. A., Lehner, P. J., Sissons, J. G., and Sinclair, J. H. (2005) Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 4140-4145
45. Taylor-Wiedeman, J., Sissons, J. G., Borysiewicz, L. K., and Sinclair, J. H. (1991) Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *The Journal of general virology* **72 (Pt 9)**, 2059-2064
46. Tey, S. K., Goodrum, F., and Khanna, R. (2010) CD8+ T-cell recognition of human cytomegalovirus latency-associated determinant pUL138. *The Journal of general virology* **91**, 2040-2048
47. Egger, G., Liang, G., Aparicio, A., and Jones, P. A. (2004) Epigenetics in human disease and prospects for epigenetic therapy. *Nature* **429**, 457-463
48. Cavalli, G. (2006) Chromatin and epigenetics in development: blending cellular memory with cell fate plasticity. *Development (Cambridge, England)* **133**, 2089-2094

49. Feinberg, A. P., Ohlsson, R., and Henikoff, S. (2006) The epigenetic progenitor origin of human cancer. *Nature reviews. Genetics* **7**, 21-33
50. Fu, M., Gao, Y., Zhou, Q., Zhang, Q., Peng, Y., Tian, K., Wang, J., and Zheng, X. (2014) Human cytomegalovirus latent infection alters the expression of cellular and viral microRNA. *Gene* **536**, 272-278
51. Liu, X., Chen, X., Yu, X., Tao, Y., Bode, A. M., Dong, Z., and Cao, Y. (2013) Regulation of microRNAs by epigenetics and their interplay involved in cancer. *Journal of experimental & clinical cancer research : CR* **32**, 96
52. O'Connor, C. M., DiMaggio, P. A., Jr., Shenk, T., and Garcia, B. A. (2014) Quantitative proteomic discovery of dynamic epigenome changes that control human cytomegalovirus (HCMV) infection. *Molecular & cellular proteomics : MCP* **13**, 2399-2410
53. Pavelin, J., Reynolds, N., Chiweshe, S., Wu, G., Tiribassi, R., and Grey, F. (2013) Systematic microRNA analysis identifies ATP6V0C as an essential host factor for human cytomegalovirus replication. *PLoS pathogens* **9**, e1003820
54. Rode, K., Dohner, K., Binz, A., Glass, M., Strive, T., Bauerfeind, R., and Sodeik, B. (2011) Uncoupling uncoating of herpes simplex virus genomes from their nuclear import and gene expression. *Journal of virology* **85**, 4271-4283
55. Nevels, M., Nitzsche, A., and Paulus, C. (2011) How to control an infectious bead string: nucleosome-based regulation and targeting of herpesvirus chromatin. *Reviews in medical virology* **21**, 154-180
56. Nitzsche, A., Paulus, C., and Nevels, M. (2008) Temporal dynamics of cytomegalovirus chromatin assembly in productively infected human cells. *Journal of virology* **82**, 11167-11180
57. Stinski, M. F., and Isomura, H. (2008) Role of the cytomegalovirus major immediate early enhancer in acute infection and reactivation from latency. *Medical microbiology and immunology* **197**, 223-231
58. Groves, I. J., Reeves, M. B., and Sinclair, J. H. (2009) Lytic infection of permissive cells with human cytomegalovirus is regulated by an intrinsic 'pre-immediate-early' repression of viral gene expression mediated by histone post-translational modification. *The Journal of general virology* **90**, 2364-2374
59. Ioudinkova, E., Arcangeletti, M. C., Rynditch, A., De Conto, F., Motta, F., Covan, S., Pinardi, F., Razin, S. V., and Chezzi, C. (2006) Control of human cytomegalovirus gene expression by differential histone modifications during lytic and latent infection of a monocytic cell line. *Gene* **384**, 120-128
60. Nevels, M., Paulus, C., and Shenk, T. (2004) Human cytomegalovirus immediate-early 1 protein facilitates viral replication by antagonizing histone deacetylation. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 17234-17239
61. Reeves, M., and Sinclair, J. (2008) Aspects of human cytomegalovirus latency and reactivation. *Current topics in microbiology and immunology* **325**, 297-313
62. Reeves, M. B. (2011) Chromatin-mediated regulation of cytomegalovirus gene expression. *Virus research* **157**, 134-143
63. Bloom, D. C., Giordani, N. V., and Kwiatkowski, D. L. (2010) Epigenetic regulation of latent HSV-1 gene expression. *Biochimica et biophysica acta* **1799**, 246-256
64. Chen, H. S., Lu, F., and Lieberman, P. M. (2013) Epigenetic regulation of EBV and KSHV latency. *Current opinion in virology* **3**, 251-259
65. Suganuma, T., and Workman, J. L. (2011) Signals and combinatorial functions of histone modifications. *Annual review of biochemistry* **80**, 473-499
66. Muller, J., Hart, C. M., Francis, N. J., Vargas, M. L., Sengupta, A., Wild, B., Miller, E. L., O'Connor, M. B., Kingston, R. E., and Simon, J. A. (2002) Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. *Cell* **111**, 197-208

67. Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002) Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes & development* **16**, 2893-2905
68. Herz, H. M., and Shilatifard, A. (2010) The JARID2-PRC2 duality. *Genes & development* **24**, 857-861
69. Kottakis, F., Polytarchou, C., Foltopoulou, P., Sanidas, I., Kampranis, S. C., and Tsichlis, P. N. (2011) FGF-2 regulates cell proliferation, migration, and angiogenesis through an NDY1/KDM2B-miR-101-EZH2 pathway. *Molecular cell* **43**, 285-298
70. Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A., and Shi, Y. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**, 941-953
71. Kampranis, S. C., and Tsichlis, P. N. (2009) Histone demethylases and cancer. *Advances in cancer research* **102**, 103-169
72. Greijer, A. E., Dekkers, C. A., and Middeldorp, J. M. (2000) Human cytomegalovirus virions differentially incorporate viral and host cell RNA during the assembly process. *Journal of virology* **74**, 9078-9082
73. Sarcinella, E., Brown, M., Tellier, R., Petric, M., and Mazzulli, T. (2004) Detection of RNA in purified cytomegalovirus virions. *Virus research* **104**, 129-137
74. Terhune, S. S., Schroer, J., and Shenk, T. (2004) RNAs are packaged into human cytomegalovirus virions in proportion to their intracellular concentration. *Journal of virology* **78**, 10390-10398
75. Prosch, S., Priemer, C., Hoflich, C., Liebenthaf, C., Babel, N., Kruger, D. H., and Volk, H. D. (2003) Proteasome inhibitors: a novel tool to suppress human cytomegalovirus replication and virus-induced immune modulation. *Antiviral therapy* **8**, 555-567
76. Tran, K., Mahr, J. A., and Spector, D. H. (2010) Proteasome subunits relocalize during human cytomegalovirus infection, and proteasome activity is necessary for efficient viral gene transcription. *Journal of virology* **84**, 3079-3093
77. Baldick, C. J., Jr., Marchini, A., Patterson, C. E., and Shenk, T. (1997) Human cytomegalovirus tegument protein pp71 (ppUL82) enhances the infectivity of viral DNA and accelerates the infectious cycle. *Journal of virology* **71**, 4400-4408
78. Feinberg, A. P., and Tycko, B. (2004) The history of cancer epigenetics. *Nature reviews. Cancer* **4**, 143-153
79. Cao, R., and Zhang, Y. (2004) The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. *Current opinion in genetics & development* **14**, 155-164
80. Kuzmichev, A., Jenuwein, T., Tempst, P., and Reinberg, D. (2004) Different EZH2-containing complexes target methylation of histone H1 or nucleosomal histone H3. *Molecular cell* **14**, 183-193
81. Raaphorst, F. M., Otte, A. P., and Meijer, C. J. (2001) Polycomb-group genes as regulators of mammalian lymphopoiesis. *Trends in immunology* **22**, 682-690
82. Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumar-Sinha, C., Sanda, M. G., Ghosh, D., Pienta, K. J., Sewalt, R. G., Otte, A. P., Rubin, M. A., and Chinnaiyan, A. M. (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* **419**, 624-629
83. Kleer, C. G., Cao, Q., Varambally, S., Shen, R., Ota, I., Tomlins, S. A., Ghosh, D., Sewalt, R. G., Otte, A. P., Hayes, D. F., Sabel, M. S., Livant, D., Weiss, S. J., Rubin, M. A., and Chinnaiyan, A. M. (2003) EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 11606-11611
84. Soderberg-Naucler, C., Fish, K. N., and Nelson, J. A. (1997) Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell* **91**, 119-126

85. Soderberg-Naucler, C., Streblow, D. N., Fish, K. N., Allan-Yorke, J., Smith, P. P., and Nelson, J. A. (2001) Reactivation of latent human cytomegalovirus in CD14(+) monocytes is differentiation dependent. *Journal of virology* **75**, 7543-7554
86. Kondo, K., Kaneshima, H., and Mocarski, E. S. (1994) Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 11879-11883
87. Cheung, A. K., Abendroth, A., Cunningham, A. L., and Slobedman, B. (2006) Viral gene expression during the establishment of human cytomegalovirus latent infection in myeloid progenitor cells. *Blood* **108**, 3691-3699
88. Fish, K. N., Stenglein, S. G., Ibanez, C., and Nelson, J. A. (1995) Cytomegalovirus persistence in macrophages and endothelial cells. *Scandinavian journal of infectious diseases. Supplementum* **99**, 34-40
89. Goodrum, F., Reeves, M., Sinclair, J., High, K., and Shenk, T. (2007) Human cytomegalovirus sequences expressed in latently infected individuals promote a latent infection in vitro. *Blood* **110**, 937-945
90. Hahn, G., Jores, R., and Mocarski, E. S. (1998) Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 3937-3942
91. Stevenson, E. V., Collins-McMillen, D., Kim, J. H., Cieply, S. J., Bentz, G. L., and Yurochko, A. D. (2014) HCMV reprogramming of infected monocyte survival and differentiation: a Goldilocks phenomenon. *Viruses* **6**, 782-807
92. Goodrum, F. D., Jordan, C. T., High, K., and Shenk, T. (2002) Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: a model for latency. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 16255-16260
93. Slobedman, B., Cao, J. Z., Avdic, S., Webster, B., McAllery, S., Cheung, A. K., Tan, J. C., and Abendroth, A. (2010) Human cytomegalovirus latent infection and associated viral gene expression. *Future microbiology* **5**, 883-900
94. Umashankar, M., and Goodrum, F. (2014) Hematopoietic long-term culture (hLTC) for human cytomegalovirus latency and reactivation. *Methods in molecular biology (Clifton, N.J.)* **1119**, 99-112
95. Goodrum, F., Jordan, C. T., Terhune, S. S., High, K., and Shenk, T. (2004) Differential outcomes of human cytomegalovirus infection in primitive hematopoietic cell subpopulations. *Blood* **104**, 687-695
96. Petrucelli, A., Rak, M., Grainger, L., and Goodrum, F. (2009) Characterization of a novel Golgi apparatus-localized latency determinant encoded by human cytomegalovirus. *Journal of virology* **83**, 5615-5629
97. Umashankar, M., Petrucelli, A., Cicchini, L., Caposio, P., Kreklywich, C. N., Rak, M., Bughio, F., Goldman, D. C., Hamlin, K. L., Nelson, J. A., Fleming, W. H., Streblow, D. N., and Goodrum, F. (2011) A novel human cytomegalovirus locus modulates cell type-specific outcomes of infection. *PLoS pathogens* **7**, e1002444
98. Jackson, S. E., Mason, G. M., and Wills, M. R. (2011) Human cytomegalovirus immunity and immune evasion. *Virus research* **157**, 151-160
99. Engel, P., and Angulo, A. (2012) Viral immunomodulatory proteins: usurping host genes as a survival strategy. *Advances in experimental medicine and biology* **738**, 256-276
100. Miller-Kittrell, M., and Sparer, T. E. (2009) Feeling manipulated: cytomegalovirus immune manipulation. *Virology journal* **6**, 4
101. Benz, C., Reusch, U., Muranyi, W., Brune, W., Atalay, R., and Hengel, H. (2001) Efficient downregulation of major histocompatibility complex class I molecules in human epithelial cells infected with cytomegalovirus. *The Journal of general virology* **82**, 2061-2070

102. Kikkert, M., Hassink, G., Barel, M., Hirsch, C., van der Wal, F. J., and Wiertz, E. (2001) Ubiquitination is essential for human cytomegalovirus US11-mediated dislocation of MHC class I molecules from the endoplasmic reticulum to the cytosol. *The Biochemical journal* **358**, 369-377
103. Hewitt, E. W., Gupta, S. S., and Lehner, P. J. (2001) The human cytomegalovirus gene product US6 inhibits ATP binding by TAP. *The EMBO journal* **20**, 387-396
104. Tomazin, R., Boname, J., Hegde, N. R., Lewinsohn, D. M., Altschuler, Y., Jones, T. R., Cresswell, P., Nelson, J. A., Riddell, S. R., and Johnson, D. C. (1999) Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells. *Nature medicine* **5**, 1039-1043
105. McSharry, B. P., Avdic, S., and Slobedman, B. (2012) Human cytomegalovirus encoded homologs of cytokines, chemokines and their receptors: roles in immunomodulation. *Viruses* **4**, 2448-2470
106. Moutaftsi, M., Mehl, A. M., Borysiewicz, L. K., and Tabi, Z. (2002) Human cytomegalovirus inhibits maturation and impairs function of monocyte-derived dendritic cells. *Blood* **99**, 2913-2921
107. Moutaftsi, M., Brennan, P., Spector, S. A., and Tabi, Z. (2004) Impaired lymphoid chemokine-mediated migration due to a block on the chemokine receptor switch in human cytomegalovirus-infected dendritic cells. *Journal of virology* **78**, 3046-3054
108. Grigoleit, U., Riegler, S., Einsele, H., Laib Sampaio, K., Jahn, G., Hebart, H., Brossart, P., Frank, F., and Sinzger, C. (2002) Human cytomegalovirus induces a direct inhibitory effect on antigen presentation by monocyte-derived immature dendritic cells. *British journal of haematology* **119**, 189-198
109. Tomasec, P., Braud, V. M., Rickards, C., Powell, M. B., McSharry, B. P., Gadola, S., Cerundolo, V., Borysiewicz, L. K., McMichael, A. J., and Wilkinson, G. W. (2000) Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science (New York, N.Y.)* **287**, 1031
110. Stagno, S., Reynolds, D. W., Tsiantos, A., Fuccillo, D. A., Long, W., and Alford, C. A. (1975) Comparative serial virologic and serologic studies of symptomatic and subclinical congenitally and natally acquired cytomegalovirus infections. *The Journal of infectious diseases* **132**, 568-577
111. Schleiss, M. R. (2013) Cytomegalovirus in the neonate: immune correlates of infection and protection. *Clinical & developmental immunology* **2013**, 501801
112. Cope, A. V., Sweny, P., Sabin, C., Rees, L., Griffiths, P. D., and Emery, V. C. (1997) Quantity of cytomegalovirus viruria is a major risk factor for cytomegalovirus disease after renal transplantation. *Journal of medical virology* **52**, 200-205
113. Cope, A. V., Sabin, C., Burroughs, A., Rolles, K., Griffiths, P. D., and Emery, V. C. (1997) Interrelationships among quantity of human cytomegalovirus (HCMV) DNA in blood, donor-recipient serostatus, and administration of methylprednisolone as risk factors for HCMV disease following liver transplantation. *The Journal of infectious diseases* **176**, 1484-1490
114. Emery, V. C., and Griffiths, P. D. (2000) Prediction of cytomegalovirus load and resistance patterns after antiviral chemotherapy. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 8039-8044
115. Emery, V. C., Cope, A. V., Bowen, E. F., Gor, D., and Griffiths, P. D. (1999) The dynamics of human cytomegalovirus replication in vivo. *The Journal of experimental medicine* **190**, 177-182
116. Emery, V. C., Hassan-Walker, A. F., Burroughs, A. K., and Griffiths, P. D. (2002) Human cytomegalovirus (HCMV) replication dynamics in HCMV-naive and -experienced immunocompromised hosts. *The Journal of infectious diseases* **185**, 1723-1728
117. Plotkin, S. A., Starr, S. E., Friedman, H. M., Gonczol, E., and Weibel, R. E. (1989) Protective effects of Towne cytomegalovirus vaccine against low-passage

cytomegalovirus administered as a challenge. *The Journal of infectious diseases* **159**, 860-865

118. Wald, A., Selke, S., Magaret, A., and Boeckh, M. (2013) Impact of human cytomegalovirus (CMV) infection on immune response to pandemic 2009 H1N1 influenza vaccine in healthy adults. *Journal of medical virology* **85**, 1557-1560
119. Gkrania-Klotsas, E., Langenberg, C., Sharp, S. J., Luben, R., Khaw, K. T., and Wareham, N. J. (2013) Seropositivity and higher immunoglobulin g antibody levels against cytomegalovirus are associated with mortality in the population-based European prospective investigation of Cancer-Norfolk cohort. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **56**, 1421-1427
120. Griffiths, P. D. (2012) Burden of disease associated with human cytomegalovirus and prospects for elimination by universal immunisation. *The Lancet. Infectious diseases* **12**, 790-798
121. Bellacosa, A., Testa, J. R., Staal, S. P., and Tsichlis, P. N. (1991) A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science (New York, N.Y.)* **254**, 274-277
122. Marte, B. M., and Downward, J. (1997) PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends in biochemical sciences* **22**, 355-358
123. Hanada, M., Feng, J., and Hemmings, B. A. (2004) Structure, regulation and function of PKB/AKT--a major therapeutic target. *Biochimica et biophysica acta* **1697**, 3-16
124. Masure, S., Haefner, B., Wesselink, J. J., Hoefnagel, E., Mortier, E., Verhasselt, P., Tuytelaars, A., Gordon, R., and Richardson, A. (1999) Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3. *European journal of biochemistry / FEBS* **265**, 353-360
125. Pearce, L. R., Komander, D., and Alessi, D. R. (2010) The nuts and bolts of AGC protein kinases. *Nature reviews. Molecular cell biology* **11**, 9-22
126. Scheid, M. P., and Woodgett, J. R. (2003) Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS letters* **546**, 108-112
127. Bayascas, J. R. (2010) PDK1: the major transducer of PI 3-kinase actions. *Current topics in microbiology and immunology* **346**, 9-29
128. Raimondi, C., and Falasca, M. (2011) Targeting PDK1 in cancer. *Current medicinal chemistry* **18**, 2763-2769
129. Zoncu, R., Efeyan, A., and Sabatini, D. M. (2011) mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews. Molecular cell biology* **12**, 21-35
130. Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A., Sanghera, J., and Dedhar, S. (2000) Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 3207-3212
131. Guertin, D. A., Stevens, D. M., Thoreen, C. C., Burds, A. A., Kalaany, N. Y., Moffat, J., Brown, M., Fitzgerald, K. J., and Sabatini, D. M. (2006) Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Developmental cell* **11**, 859-871
132. Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., Kadowaki, T., and Hay, N. (2001) Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes & development* **15**, 2203-2208
133. Cho, H., Thorvaldsen, J. L., Chu, Q., Feng, F., and Birnbaum, M. J. (2001) Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *The Journal of biological chemistry* **276**, 38349-38352

134. Dummler, B., Tschopp, O., Hynx, D., Yang, Z. Z., Dirnhofer, S., and Hemmings, B. A. (2006) Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. *Molecular and cellular biology* **26**, 8042-8051
135. Iliopoulos, D., Polytarchou, C., Hatziapostolou, M., Kottakis, F., Maroulakou, I. G., Struhl, K., and Tsichlis, P. N. (2009) MicroRNAs differentially regulated by Akt isoforms control EMT and stem cell renewal in cancer cells. *Science signaling* **2**, ra62
136. Polytarchou, C., Iliopoulos, D., Hatziapostolou, M., Kottakis, F., Maroulakou, I., Struhl, K., and Tsichlis, P. N. (2011) Akt2 regulates all Akt isoforms and promotes resistance to hypoxia through induction of miR-21 upon oxygen deprivation. *Cancer research* **71**, 4720-4731
137. Chin, Y. R., and Toker, A. (2010) The actin-bundling protein palladin is an Akt1-specific substrate that regulates breast cancer cell migration. *Molecular cell* **38**, 333-344
138. Gao, D., Inuzuka, H., Tseng, A., Chin, R. Y., Toker, A., and Wei, W. (2009) Phosphorylation by Akt1 promotes cytoplasmic localization of Skp2 and impairs APCCdh1-mediated Skp2 destruction. *Nature cell biology* **11**, 397-408
139. Eichhorn, P. J., Creyghton, M. P., and Bernards, R. (2009) Protein phosphatase 2A regulatory subunits and cancer. *Biochimica et biophysica acta* **1795**, 1-15
140. Warfel, N. A., and Newton, A. C. (2012) Pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP): a new player in cell signaling. *The Journal of biological chemistry* **287**, 3610-3616
141. Brognard, J., Sierecki, E., Gao, T., and Newton, A. C. (2007) PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. *Molecular cell* **25**, 917-931
142. Steelman, L. S., Chappell, W. H., Abrams, S. L., Kempf, R. C., Long, J., Laidler, P., Mijatovic, S., Maksimovic-Ivanic, D., Stivala, F., Mazzarino, M. C., Donia, M., Fagone, P., Malaponte, G., Nicoletti, F., Libra, M., Milella, M., Tafuri, A., Bonati, A., Basecke, J., Cocco, L., Evangelisti, C., Martelli, A. M., Montalto, G., Cervello, M., and McCubrey, J. A. (2011) Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. *Aging* **3**, 192-222
143. Hsieh, A. C., Truitt, M. L., and Ruggero, D. (2011) Oncogenic AKTivation of translation as a therapeutic target. *British journal of cancer* **105**, 329-336
144. Hafsi, S., Pezzino, F. M., Candido, S., Ligresti, G., Spandidos, D. A., Soua, Z., McCubrey, J. A., Travali, S., and Libra, M. (2012) Gene alterations in the PI3K/PTEN/AKT pathway as a mechanism of drug-resistance (review). *International journal of oncology* **40**, 639-644
145. Meuillet, E. J. (2011) Novel inhibitors of AKT: assessment of a different approach targeting the pleckstrin homology domain. *Current medicinal chemistry* **18**, 2727-2742
146. Bartholomeusz, C., and Gonzalez-Angulo, A. M. (2012) Targeting the PI3K signaling pathway in cancer therapy. *Expert opinion on therapeutic targets* **16**, 121-130
147. Arden, K. C., and Biggs, W. H., 3rd. (2002) Regulation of the FoxO family of transcription factors by phosphatidylinositol-3 kinase-activated signaling. *Archives of biochemistry and biophysics* **403**, 292-298
148. Huang, W. C., and Chen, C. C. (2005) Akt phosphorylation of p300 at Ser-1834 is essential for its histone acetyltransferase and transcriptional activity. *Molecular and cellular biology* **25**, 6592-6602
149. Meier, R., Alessi, D. R., Cron, P., Andjelkovic, M., and Hemmings, B. A. (1997) Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bbeta. *The Journal of biological chemistry* **272**, 30491-30497

150. Borgatti, P., Martelli, A. M., Tabellini, G., Bellacosa, A., Capitani, S., and Neri, L. M. (2003) Threonine 308 phosphorylated form of Akt translocates to the nucleus of PC12 cells under nerve growth factor stimulation and associates with the nuclear matrix protein nucleolin. *Journal of cellular physiology* **196**, 79-88
151. Pekarsky, Y., Koval, A., Hallas, C., Bichi, R., Tresini, M., Malstrom, S., Russo, G., Tsichlis, P., and Croce, C. M. (2000) Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 3028-3033
152. Noguchi, M., Ropars, V., Roumestand, C., and Suizu, F. (2007) Proto-oncogene TCL1: more than just a coactivator for Akt. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **21**, 2273-2284
153. Kunstle, G., Laine, J., Pierron, G., Kagami Si, S., Nakajima, H., Hoh, F., Roumestand, C., Stern, M. H., and Noguchi, M. (2002) Identification of Akt association and oligomerization domains of the Akt kinase coactivator TCL1. *Molecular and cellular biology* **22**, 1513-1525
154. Fiorenza, M. T., Torcia, S., Canterini, S., Bevilacqua, A., Narducci, M. G., Ragone, G., Croce, C. M., Russo, G., and Mangia, F. (2008) TCL1 promotes blastomere proliferation through nuclear transfer, but not direct phosphorylation, of AKT/PKB in early mouse embryos. *Cell death and differentiation* **15**, 420-422
155. Badve, S., Collins, N. R., Bhat-Nakshatri, P., Turbin, D., Leung, S., Thorat, M., Dunn, S. E., Geistlinger, T. R., Carroll, J. S., Brown, M., Bose, S., Teitell, M. A., and Nakshatri, H. (2010) Subcellular localization of activated AKT in estrogen receptor- and progesterone receptor-expressing breast cancers: potential clinical implications. *The American journal of pathology* **176**, 2139-2149
156. Teitell, M. A. (2005) The TCL1 family of oncoproteins: co-activators of transformation. *Nature reviews. Cancer* **5**, 640-648
157. Saji, M., Vasko, V., Kada, F., Allbritton, E. H., Burman, K. D., and Ringel, M. D. (2005) Akt1 contains a functional leucine-rich nuclear export sequence. *Biochemical and biophysical research communications* **332**, 167-173
158. Xuan Nguyen, T. L., Choi, J. W., Lee, S. B., Ye, K., Woo, S. D., Lee, K. H., and Ahn, J. Y. (2006) Akt phosphorylation is essential for nuclear translocation and retention in NGF-stimulated PC12 cells. *Biochemical and biophysical research communications* **349**, 789-798
159. Neri, L. M., Martelli, A. M., Borgatti, P., Colamussi, M. L., Marchisio, M., and Capitani, S. (1999) Increase in nuclear phosphatidylinositol 3-kinase activity and phosphatidylinositol (3,4,5) trisphosphate synthesis precede PKC-zeta translocation to the nucleus of NGF-treated PC12 cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **13**, 2299-2310
160. Neri, L. M., Bortul, R., Tabellini, G., Borgatti, P., Baldini, G., Celeghini, C., Capitani, S., and Martelli, A. M. (2002) Erythropoietin-induced erythroid differentiation of K562 cells is accompanied by the nuclear translocation of phosphatidylinositol 3-kinase and intranuclear generation of phosphatidylinositol (3,4,5) trisphosphate. *Cellular signalling* **14**, 21-29
161. Scheid, M. P., Parsons, M., and Woodgett, J. R. (2005) Phosphoinositide-dependent phosphorylation of PDK1 regulates nuclear translocation. *Molecular and cellular biology* **25**, 2347-2363
162. Kikani, C. K., Dong, L. Q., and Liu, F. (2005) "New"-clear functions of PDK1: beyond a master kinase in the cytosol? *Journal of cellular biochemistry* **96**, 1157-1162
163. Liu, J., and Brown, R. E. (2011) Morphoproteomics demonstrates activation of mammalian target of rapamycin pathway in papillary thyroid carcinomas with nuclear translocation of MTOR in aggressive histological variants. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **24**, 1553-1559

164. Rosner, M., and Hengstschlager, M. (2012) Detection of cytoplasmic and nuclear functions of mTOR by fractionation. *Methods in molecular biology (Clifton, N.J.)* **821**, 105-124
165. Vazquez-Martin, A., Cufi, S., Oliveras-Ferraros, C., and Menendez, J. A. (2011) Raptor, a positive regulatory subunit of mTOR complex 1, is a novel phosphoprotein of the rDNA transcription machinery in nucleoli and chromosomal nucleolus organizer regions (NORs). *Cell cycle (Georgetown, Tex.)* **10**, 3140-3152
166. Iadevaia, V., Zhang, Z., Jan, E., and Proud, C. G. (2012) mTOR signaling regulates the processing of pre-rRNA in human cells. *Nucleic acids research* **40**, 2527-2539
167. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) Role of translocation in the activation and function of protein kinase B. *The Journal of biological chemistry* **272**, 31515-31524
168. Ananthanarayanan, B., Ni, Q., and Zhang, J. (2005) Signal propagation from membrane messengers to nuclear effectors revealed by reporters of phosphoinositide dynamics and Akt activity. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 15081-15086
169. Lim, M. A., Kikani, C. K., Wick, M. J., and Dong, L. Q. (2003) Nuclear translocation of 3'-phosphoinositide-dependent protein kinase 1 (PDK-1): a potential regulatory mechanism for PDK-1 function. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 14006-14011
170. Rubio, M., Avitabile, D., Fischer, K., Emmanuel, G., Gude, N., Miyamoto, S., Mishra, S., Schaefer, E. M., Brown, J. H., and Sussman, M. A. (2009) Cardioprotective stimuli mediate phosphoinositide 3-kinase and phosphoinositide dependent kinase 1 nuclear accumulation in cardiomyocytes. *Journal of molecular and cellular cardiology* **47**, 96-103
171. Sale, E. M., and Sale, G. J. (2008) Protein kinase B: signalling roles and therapeutic targeting. *Cellular and molecular life sciences : CMLS* **65**, 113-127
172. Blandino-Rosano, M., Chen, A. Y., Scheys, J. O., Alejandro, E. U., Gould, A. P., Taranukha, T., Elghazi, L., Cras-Meneur, C., and Bernal-Mizrachi, E. (2012) mTORC1 signaling and regulation of pancreatic beta-cell mass. *Cell cycle (Georgetown, Tex.)* **11**, 1892-1902
173. Cheng, G. Z., Zhang, W., and Wang, L. H. (2008) Regulation of cancer cell survival, migration, and invasion by Twist: AKT2 comes to interplay. *Cancer research* **68**, 957-960
174. Buttrick, G. J., and Wakefield, J. G. (2008) PI3-K and GSK-3: Akt-ing together with microtubules. *Cell cycle (Georgetown, Tex.)* **7**, 2621-2625
175. Welsh, G. I., Hers, I., Berwick, D. C., Dell, G., Wherlock, M., Birkin, R., Leney, S., and Tavare, J. M. (2005) Role of protein kinase B in insulin-regulated glucose uptake. *Biochemical Society transactions* **33**, 346-349
176. Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B., and Hay, N. (2001) Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes & development* **15**, 1406-1418
177. Berwick, D. C., Hers, I., Heesom, K. J., Moule, S. K., and Tavare, J. M. (2002) The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes. *The Journal of biological chemistry* **277**, 33895-33900
178. Wu, M., Falasca, M., and Blough, E. R. (2011) Akt/protein kinase B in skeletal muscle physiology and pathology. *Journal of cellular physiology* **226**, 29-36
179. Chaanine, A. H., and Hajjar, R. J. (2011) AKT signalling in the failing heart. *European journal of heart failure* **13**, 825-829
180. Keefe, S. M., Cohen, M. A., and Brose, M. S. (2010) Targeting vascular endothelial growth factor receptor in thyroid cancer: the intracellular and extracellular

implications. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**, 778-783

181. Martelli, A. M., Evangelisti, C., Chiarini, F., Grimaldi, C., Cappellini, A., Ognibene, A., and McCubrey, J. A. (2010) The emerging role of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling network in normal myelopoiesis and leukemogenesis. *Biochimica et biophysica acta* **1803**, 991-1002
182. Martelli, A. M., Evangelisti, C., Follo, M. Y., Ramazzotti, G., Fini, M., Giardino, R., Manzoli, L., McCubrey, J. A., and Cocco, L. (2011) Targeting the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling network in cancer stem cells. *Current medicinal chemistry* **18**, 2715-2726
183. Polak, R., and Buitenhuis, M. (2012) The PI3K/PKB signaling module as key regulator of hematopoiesis: implications for therapeutic strategies in leukemia. *Blood* **119**, 911-923
184. Martelli, A. M., Tazzari, P. L., Evangelisti, C., Chiarini, F., Blalock, W. L., Billi, A. M., Manzoli, L., McCubrey, J. A., and Cocco, L. (2007) Targeting the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin module for acute myelogenous leukemia therapy: from bench to bedside. *Current medicinal chemistry* **14**, 2009-2023
185. Chiarini, F., Fala, F., Tazzari, P. L., Ricci, F., Astolfi, A., Pession, A., Pagliaro, P., McCubrey, J. A., and Martelli, A. M. (2009) Dual inhibition of class IA phosphatidylinositol 3-kinase and mammalian target of rapamycin as a new therapeutic option for T-cell acute lymphoblastic leukemia. *Cancer research* **69**, 3520-3528
186. De Luca, A., Maiello, M. R., D'Alessio, A., Pergameno, M., and Normanno, N. (2012) The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches. *Expert opinion on therapeutic targets* **16 Suppl 2**, S17-27
187. Hers, I., Vincent, E. E., and Tavare, J. M. (2011) Akt signalling in health and disease. *Cellular signalling* **23**, 1515-1527
188. Chappell, W. H., Steelman, L. S., Long, J. M., Kempf, R. C., Abrams, S. L., Franklin, R. A., Basecke, J., Stivala, F., Donia, M., Fagone, P., Malaponte, G., Mazzarino, M. C., Nicoletti, F., Libra, M., Maksimovic-Ivanic, D., Mijatovic, S., Montalto, G., Cervello, M., Laidler, P., Milella, M., Tafuri, A., Bonati, A., Evangelisti, C., Cocco, L., Martelli, A. M., and McCubrey, J. A. (2011) Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. *Oncotarget* **2**, 135-164
189. van Opstal, A., Bijvelt, J., van Donselaar, E., Humbel, B. M., and Boonstra, J. (2012) Inhibition of protein kinase B activity induces cell cycle arrest and apoptosis during early G(1) phase in CHO cells. *Cell biology international* **36**, 357-365
190. Ji, M., Zhang, Q., Ye, J., Wang, X., Yang, W., and Zhu, D. (2008) Myostatin induces p300 degradation to silence cyclin D1 expression through the PI3K/PTEN/Akt pathway. *Cellular signalling* **20**, 1452-1458
191. Mistafa, O., Ghalali, A., Kadekar, S., Hogberg, J., and Stenius, U. (2010) Purinergic receptor-mediated rapid depletion of nuclear phosphorylated Akt depends on pleckstrin homology domain leucine-rich repeat phosphatase, calcineurin, protein phosphatase 2A, and PTEN phosphatases. *The Journal of biological chemistry* **285**, 27900-27910
192. Song, M. S., Salmena, L., and Pandolfi, P. P. (2012) The functions and regulation of the PTEN tumour suppressor. *Nature reviews. Molecular cell biology* **13**, 283-296
193. Cayrol, C., Knibiehler, M., and Ducommun, B. (1998) p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. *Oncogene* **16**, 311-320
194. Ando, T., Kawabe, T., Ohara, H., Ducommun, B., Itoh, M., and Okamoto, T. (2001) Involvement of the interaction between p21 and proliferating cell nuclear antigen

for the maintenance of G2/M arrest after DNA damage. *The Journal of biological chemistry* **276**, 42971-42977
195. Ye, Z. W., Ghalali, A., Hogberg, J., and Stenius, U. (2011) Silencing p110beta prevents rapid depletion of nuclear pAkt. *Biochemical and biophysical research communications* **415**, 613-618
196. Marques, M., Kumar, A., Poveda, A. M., Zuluaga, S., Hernandez, C., Jackson, S., Pasero, P., and Carrera, A. C. (2009) Specific function of phosphoinositide 3-kinase beta in the control of DNA replication. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 7525-7530
197. Valerie, K., Yacoub, A., Hagan, M. P., Curiel, D. T., Fisher, P. B., Grant, S., and Dent, P. (2007) Radiation-induced cell signaling: inside-out and outside-in. *Molecular cancer therapeutics* **6**, 789-801
198. Bozulic, L., and Hemmings, B. A. (2009) PIKKing on PKB: regulation of PKB activity by phosphorylation. *Current opinion in cell biology* **21**, 256-261
199. Xu, Y., and Price, B. D. (2011) Chromatin dynamics and the repair of DNA double strand breaks. *Cell cycle (Georgetown, Tex.)* **10**, 261-267
200. Hill, R., and Lee, P. W. (2010) The DNA-dependent protein kinase (DNA-PK): More than just a case of making ends meet? *Cell cycle (Georgetown, Tex.)* **9**, 3460-3469
201. Bozulic, L., Surucu, B., Hynx, D., and Hemmings, B. A. (2008) PKBalpha/Akt1 acts downstream of DNA-PK in the DNA double-strand break response and promotes survival. *Molecular cell* **30**, 203-213
202. Thompson, L. H. (2012) Recognition, signaling, and repair of DNA double-strand breaks produced by ionizing radiation in mammalian cells: the molecular choreography. *Mutation research* **751**, 158-246
203. Fraser, M., Harding, S. M., Zhao, H., Coackley, C., Durocher, D., and Bristow, R. G. (2011) MRE11 promotes AKT phosphorylation in direct response to DNA double-strand breaks. *Cell cycle (Georgetown, Tex.)* **10**, 2218-2232
204. Stracker, T. H., and Petrini, J. H. (2011) The MRE11 complex: starting from the ends. *Nature reviews. Molecular cell biology* **12**, 90-103
205. Bekker-Jensen, S., and Mailand, N. (2011) The ubiquitin- and SUMO-dependent signaling response to DNA double-strand breaks. *FEBS letters* **585**, 2914-2919
206. Viniegra, J. G., Martinez, N., Modirassari, P., Hernandez Losa, J., Parada Cobo, C., Sanchez-Arevalo Lobo, V. J., Aceves Luquero, C. I., Alvarez-Vallina, L., Ramon y Cajal, S., Rojas, J. M., and Sanchez-Prieto, R. (2005) Full activation of PKB/Akt in response to insulin or ionizing radiation is mediated through ATM. *The Journal of biological chemistry* **280**, 4029-4036
207. Reed, R., and Magni, K. (2001) A new view of mRNA export: separating the wheat from the chaff. *Nature cell biology* **3**, E201-204
208. Zhou, Z., Luo, M. J., Straesser, K., Katahira, J., Hurt, E., and Reed, R. (2000) The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans. *Nature* **407**, 401-405
209. Okada, M., Jang, S. W., and Ye, K. (2008) Akt phosphorylation and nuclear phosphoinositide association mediate mRNA export and cell proliferation activities by ALY. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 8649-8654
210. Valverde, A. M., Benito, M., and Lorenzo, M. (2005) The brown adipose cell: a model for understanding the molecular mechanisms of insulin resistance. *Acta physiologica Scandinavica* **183**, 59-73
211. Maiuri, T., Ho, J., and Stambolic, V. (2010) Regulation of adipocyte differentiation by distinct subcellular pools of protein kinase B (PKB/Akt). *The Journal of biological chemistry* **285**, 15038-15047
212. Lee, S. H., Kim, H. S., Park, W. S., Kim, S. Y., Lee, K. Y., Kim, S. H., Lee, J. Y., and Yoo, N. J. (2002) Non-small cell lung cancers frequently express phosphorylated Akt; an

immunohistochemical study. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* **110**, 587-592
213. Nicholson, K. M., Streuli, C. H., and Anderson, N. G. (2003) Autocrine signalling through erbB receptors promotes constitutive activation of protein kinase B/Akt in breast cancer cell lines. *Breast cancer research and treatment* **81**, 117-128
214. Van de Sande, T., Roskams, T., Lerut, E., Joniau, S., Van Poppel, H., Verhoeven, G., and Swinnen, J. V. (2005) High-level expression of fatty acid synthase in human prostate cancer tissues is linked to activation and nuclear localization of Akt/PKB. *The Journal of pathology* **206**, 214-219
215. Vasko, V., Saji, M., Hardy, E., Kruhlak, M., Larin, A., Savchenko, V., Miyakawa, M., Isozaki, O., Murakami, H., Tsushima, T., Burman, K. D., De Micco, C., and Ringel, M. D. (2004) Akt activation and localisation correlate with tumour invasion and oncogene expression in thyroid cancer. *Journal of medical genetics* **41**, 161-170
216. Brandts, C. H., Sargin, B., Rode, M., Biermann, C., Lindtner, B., Schwable, J., Buerger, H., Muller-Tidow, C., Choudhary, C., McMahon, M., Berdel, W. E., and Serve, H. (2005) Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. *Cancer research* **65**, 9643-9650
217. Trotman, L. C., Alimonti, A., Scaglioni, P. P., Koutcher, J. A., Cordon-Cardo, C., and Pandolfi, P. P. (2006) Identification of a tumour suppressor network opposing nuclear Akt function. *Nature* **441**, 523-527
218. Miraglia, E., Hogberg, J., and Stenius, U. (2012) Statins exhibit anticancer effects through modifications of the pAkt signaling pathway. *International journal of oncology* **40**, 867-875
219. Papadopoulos, G., Delakas, D., Nakopoulou, L., and Kassimatis, T. (2011) Statins and prostate cancer: molecular and clinical aspects. *European journal of cancer (Oxford, England : 1990)* **47**, 819-830
220. Jacobs, R. J., Kodach, L. L., and Hardwick, J. C. (2011) The potential of statins for individualized colorectal cancer chemoprevention. *Current drug targets* **12**, 1903-1908
221. Rahbar, A., Bostrom, L., Lagerstedt, U., Magnusson, I., Soderberg-Naucler, C., and Sundqvist, V. A. (2003) Evidence of active cytomegalovirus infection and increased production of IL-6 in tissue specimens obtained from patients with inflammatory bowel diseases. *Inflammatory bowel diseases* **9**, 154-161
222. Cobbs, C. S., Harkins, L., Samanta, M., Gillespie, G. Y., Bharara, S., King, P. H., Nabors, L. B., Cobbs, C. G., and Britt, W. J. (2002) Human cytomegalovirus infection and expression in human malignant glioma. *Cancer research* **62**, 3347-3350
223. Soderberg-Naucler, C. (2006) Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer? *Journal of internal medicine* **259**, 219-246
224. Soderberg-Naucler, C. (2006) Human cytomegalovirus persists in its host and attacks and avoids elimination by the immune system. *Critical reviews in immunology* **26**, 231-264
225. Cha, T. L., Zhou, B. P., Xia, W., Wu, Y., Yang, C. C., Chen, C. T., Ping, B., Otte, A. P., and Hung, M. C. (2005) Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. *Science (New York, N.Y.)* **310**, 306-310
226. Sourvinos, G., Morou, A., Sanidas, I., Codruta, I., Ezell, S. A., Doxaki, C., Kampranis, S. C., Kottakis, F., and Tsichlis, P. N. (2014) The downregulation of GFI1 by the EZH2-NDY1/KDM2B-JARID2 axis and by human cytomegalovirus (HCMV) associated factors allows the activation of the HCMV major IE promoter and the transition to productive infection. *PLoS pathogens* **10**, e1004136

227. Wentworth, B. B., and French, L. (1970) Plaque assay of cytomegalovirus strains of human origin. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)* **135**, 253-258