THE ABSENCE OF THE CELLULAR PROTEIN P53 INHIBITED EFFICIENT TRAFFICKING OF HCMV VIRAL CAPSIDS FROM THE NUCLEUS

A Dissertation

Presented in Partial Fulfillment of the Requirements for the

Degree of Doctorate of Philosophy

with a

Major in Microbiology, Molecular Biology and Biochemistry

in the

College of Graduate Studies

University of Idaho

by

Man I Kuan

March 2015

Major Professor: Elizabeth A. Fortunato, Ph.D.

Authorization to Submit Dissertation

This dissertation of Man I Kuan, submitted for the degree of Doctor of Philosophy with a Major in Microbiology, Molecular Biology, and Biochemistry and titled "The Absence of the Cellular Protein p53 Inhibited Efficient Trafficking of HCMV Viral Capsids from the Nucleus," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____ Date: _____
Elizabeth Fortunato, Ph.D.

Committee Members: _____ Date: _____
John Alderete, Ph.D.

_____ Date: _____
Tanya Miura, Ph.D.

_____ Date: _____
Deborah Stenkamp, Ph.D.

Department Administrator: _____ Date: _____
James Nagler, Ph.D.

Discipline's College Dean: _____ Date: _____
Paul Joyce, Ph.D.

Final Approval and Acceptance

Dean of the College of Graduate Studies: _____ Date: _____
Jie Chen, Ph.D.

Abstract

Human Cytomegalovirus (HCMV) infection is compromised by the absence of cellular p53. p53's activation leads to cell cycle arrest and/or apoptosis. HCMV-infection stabilizes p53 protein, however normal p53-mediated responses are inhibited. Fibroblasts lacking p53 (p53KOs) were used to study this protein's role during HCMV infection. Earlier studies found p53KOs produced 25-fold lower viral titers compared to parental wild type (wt) Lox cells. In this study we have investigated the source of the decreased functional virion production in p53KOs. Infectious center assays found most p53KOs released functional virions. Electron micrograph analysis revealed modestly decreased capsid production in p53KOs compared to wt. Significantly fewer p53KOs displayed HCMV-induced infoldings of the inner nuclear membrane (IINMs). The IINMs present in p53KOs were smaller and fewer. Reduced numbers of capsids were found in the cytoplasm and a disproportionately smaller number present were enveloped. Negatively stained infected p53KO cell supernatant found vastly fewer viral particles. Reintroduction of p53 into the KO cells substantially recovered these deficits. The absence of p53 inhibited the primary HCMV nuclear capsid egress portal and re-envelopment of the reduced number of particles able to reach the cytoplasm.

Having identified the structural deficit by electron microscopic analysis, we extended the study to the effect of p53's absence on HCMV's nuclear egress machinery. Normal egress requires nuclear lamina phosphorylation and remodeling. KOs expressed functional lamina phosphorylation-linked viral kinase UL97, however this did not lead to remodeling. A failure to remodel suggested malfunctioning of the

nuclear egress complex (NEC). A key NEC protein, UL50, was expressed in nearly 100% of all cell types. It re-localized to the nucleus in ~90% of wt cells, but only ~40% of KOs nuclei. Re-introduction of p53 recovered UL50 nuclear re-localization to ~75%. All cells containing UL50 nuclear signal always displayed nuclear rim staining, co-localized with its binding partner, UL53. UL50/53 was seen as “threads” extending from the INM. These structures were smaller or absent in infected KOs. We believe these structures were tubular IINMs. The absence of p53 inhibited the wt behavior of UL50, preventing formation of IINMs, drastically reducing capsid nuclear egress.

Acknowledgements

I sincerely thank my advisor Dr. Elizabeth Fortunato for her guidance, advice, mentoring, and support throughout my graduate study. I would also like to thank the current and the past members in her lab including John O'Dowd, Anamaria G. Zavala, and Amit Kulkarni. All of whom, including Dr. Fortunato, have helped me to grow to be an independent scientist and thinker.

I would also like to thank my committee members, Dr. John Alderete from Washington State University (WSU), Dr. Deborah Stenkamp and Dr. Tanya Miura from the University of Idaho (UI), for their time and efforts in making every meeting, as well as, their invaluable suggestions for my experiments.

I would like to thank our collaborator Dr. Christine Davitt from WSU for her time instructing me in the preparation of samples for transmission electron microscopy. I would like to thank Dr. Celeste Brown from the UI for her time and effort in assisting with statistical analyses. I would also like to thank Mrs. Ann Norton from the UI Optical Imaging Center for her instruction in the usage of the confocal microscope.

This work was supported by NIH grants RO1 AIO51563, and COBRE program P20 RR015587. I also thank Dr. John Sedivy (Brown University, USA) for providing the p53KO fibroblasts, Dr. James C. Alwine (University of Pennsylvania, USA) for providing the UL50 antibody, Dr. Laura Hertel (Children's Hospital of Oakland Research Institute, USA) for providing UL50-HA and UL53-FLAG constructs, Dr. Mark N. Prichard (University of Alabama at Birmingham, USA) for

providing UL97 antibody, Dr. Stipan Jonjic (University of Rijeka, Croatia) for providing the UL53 antibody.

Last, but not least, I would like to thank my beloved mother for all the support, without her I would never have made it this far.

Table of Contents

List of Figures

Chapter 1

Literature Review

1.1 Introduction of Human Herpesviruses

The family Herpesviridae includes more than 130 characterized herpesviruses. They are grouped together based on their similarities of architecture and basic genomic structure. All herpesviruses are composed of an icosadeltahedral capsid encasing a large, linear, double stranded DNA genome ranging from ~125 to 240k base pairs in length depending on the species. This capsid is surrounded by different layers of tegument proteins that contain viral proteins and enzymes that facilitate viral replication. These are all enveloped in a glycoprotein-coated lipid membrane (see Figure 1.1; Roizman 2001; Davison 2007).

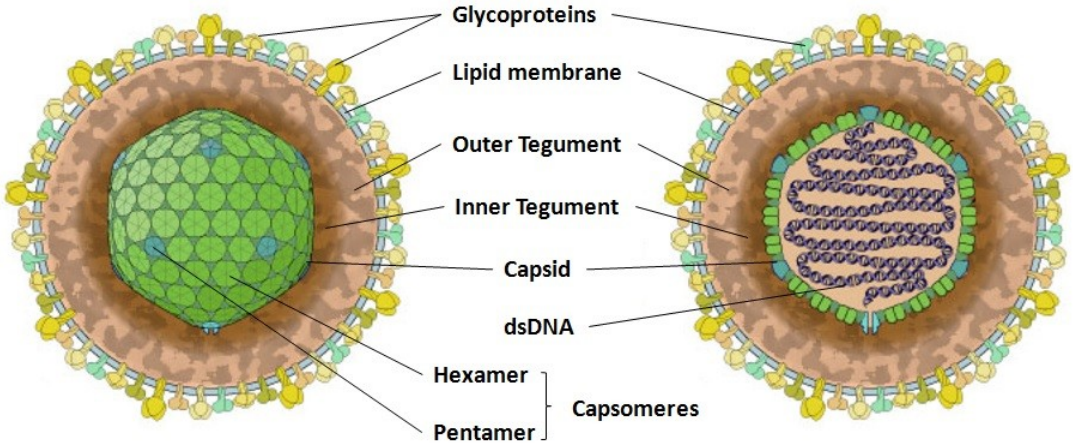


Figure 1.1 General structure of the family Herpesviridae. Image from http://viralzone.expasy.org/all_by_species/176.html (ViralZone, SIB Swiss Institute of Bioinformatics) has been modified.

Herpesviruses infect a wide range of hosts with a high degree of species specificity (Brown and Newcomb 2011). These hosts include mammals, birds, fish, reptiles, amphibians, and even some invertebrates, such as oysters (Roizman 2001). The herpesviruses are unique. They carry enzymes into cells in their teguments, and also encode a high number of proteins that facilitate viral replication, establish virus-host interactions, as well as manipulate the host cell cycle and immune response. Viral DNA synthesis and capsid assembly occurs in the nucleus. After exiting the nucleus, capsids acquire their envelope from the host's golgi membrane, followed by the release from the cells by exocytosis, or by lysis. Last but not least, herpesviruses are able to establish latent/recurrent infections within specific tissues, meaning that they remain in hosts for life and can be reactivated (Roizman 2001; Brown and Newcomb 2011).

The herpesviruses are grouped into three subfamilies (alpha-, beta-, and gamma-herpesviruses) based on the differences in viral characteristics, including tissue tropism, genome structure, and other biological characteristics (Karlin, Mocarski et al. 1994; Roizman 2001). The alpha-herpesviruses are neurotropic, indicating that they actively infect nervous tissue. This group of viruses usually has a short reproductive cycle (~24 hours). Their latent infections primarily establish in sensory ganglia (Roizman 2001). The beta- and gamma-herpesviruses are lymphotropic, indicating that they infect tissues within the lymphatic system. Their reproductive cycles are relatively long (72-96 hours). These two groups are divided based on the specifics of their genome structure, cytopathologic effect, and the site of latency (Karlin, Mocarski et al. 1994; Roizman 2001). The beta-herpesviruses

have the ability to form enlarged cells. Their latent infections establish in secretory glands, T cells, B cells, kidneys, and other tissues. The gamma-herpesvirues specifically target either T or B cells, and their latent infections are established in lymphoid tissue (Roizman 2001).

Among all the herpesviruses, eight of them specifically infect and cause characteristic diseases in humans (Roizman 2001; Brown and Newcomb 2011). There are three human alpha-herpesviruses that include herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), as well as Varicella Zoster virus (VZV) (Arvin 2001; Cohen 2001; Roizman 2001; Roizman 2001; Whitley 2001). HSV-1 and HSV-2 cause recurrent facial and genital herpetic lesions, respectively (Whitley 2001). VZV causes chicken pox and shingles (Arvin 2001). There are three human beta-herpesviruses that include human cytomegalovirus (HCMV), human herpesviruses-6 and -7 (HHV-6 and HHV-7) (Mocarski 2001; Pellett 2001; Roizman 2001). HCMV causes congenital infection of the nervous system in newborns. It can also cause severe disease in immunocompromised individuals, such as AIDS patients and transplant recipients (Mocarski 2001; Pass 2001). HHV-6 and HHV-7 cause mild early-childhood diseases (Yamanishi 2001). Two gamma-herpesviruses from humans, Epstein Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8), are convincingly linked to human cancers (Kieff 2001; Moore 2001; Rickinson 2001).

Human herpesviruses are ubiquitous, and their infections are common and widespread over the world's population. Most individuals contact these viruses by early adulthood. In fact, by age 50, 90% of adults are infected with at least one type

of human herpesvirus (Braun, Dominguez et al. 1997; Black and Pellett 1999; Blauvelt 2001; Whitley 2001; Yamanishi 2001; Macsween and Crawford 2003; Selinsky, Luke et al. 2005; Chayavichitsilp, Buckwalter et al. 2009; Pergam and Limaye 2009). These viruses usually cause mild or asymptomatic diseases in healthy individuals, however, they can also cause significant morbidity and mortality, especially in children and immunosuppressed individuals (Arvin 2001; Moore 2001; Pass 2001; Rickinson 2001; Whitley 2001; Yamanishi 2001; Steininger 2007). Therefore, vaccination against these viruses is still a desirable goal.

1.2 Significance of Human Cytomegalovirus Study

Human cytomegalovirus (HCMV) is a ubiquitous human pathogen. It can cause severe disease in immunocompromised individuals, such as AIDS patients and transplant recipients (Zhang, Hanff et al. 1995; Fornara, Lilleri et al. 2011; Shi, Lu et al. 2011). It can also cause severe congenital birth defects in infected fetuses when women have a primary infection of HCMV during pregnancy. HCMV is a major viral cause of congenital birth defects in the United States (Grosse, Ross et al. 2008). Each year approximately 35,000 infants are born with HCMV infection. Of those infants, approximately 8,000 show severe neurological defects including blindness, deafness, mental retardation, microencephaly, cerebral calcification and, in extreme cases, death (Poland, Costello et al. 1990; Fowler, Stagno et al. 1992; Boppana and Britt 1995; Cinque 1997; Fowler, McCollister et al. 1997; Boppana, Fowler et al. 1999; Gaytant, Steegers et al. 2002; Shan, Wang et al. 2009). Some

studies also show that HCMV is a causative agent for the development of some cancers and inflammatory diseases (Soderberg-Naucler 2006).

Elimination of HCMV from an infected individual is impossible due to its establishment of latency. Current anti-HCMV treatments including ganciclovir, foscarnet, and cidofovir can alleviate the symptoms in patients with an impaired immune system, but they have limitations and cause serious side effects such as hematologic abnormalities (primarily neutropenia, anemia, and thrombocytopenia), potential long-term reproductive toxicity, and renal toxicity (Biron 2006). Developing new effective treatments for HCMV-affected patients and an effective vaccine are highly desirable and necessary.

1.3 HCMV Structure

All herpesviruses have a similar basic structure. HCMV is composed of a 130-nm icosahedral capsid encasing a linear 235 kbp double-stranded DNA genome, surrounded by layers of tegument proteins and enveloped in a glycoprotein-coated lipid membrane (see Figure 1.1; Mocarski 2001). The entire particle, approximately 230 nm in diameter, is called a virion.

The HCMV genome contains over 200 predicted open reading frames. Mass spectrometry based analysis reveals that the HCMV virion consists of 71 virally encoded proteins as well as 70 cellular proteins (Varnum, Streblow et al. 2004). In terms of relative abundance of virion proteins, 50% are tegument proteins, 30% are

capsid proteins, 13% are envelope proteins, and 7% are undefined (Varnum, Streblow et al. 2004).

1.3.1 HCMV genome

The 235 kbp HCMV genome contains both inverted and directly repeated sequences (see Figure 1.2; a, b and c sequences). The HCMV genome contains unique long (U_L) and unique short (U_S) segments, which are flanked by two large inverted repeated sequences, terminal repeats (TR) and internal repeats (IR) (see Figure 1.2; reviewed in reference Gibson and Bogner 2013). In addition, multiple *a* sequences are located within TR and IR in the genome. The end of U_S segment contains a single copy of the *a* sequence, while the end of U_L segment and the L/S junction contain variable copies of the *a* sequence (Tamashiro, Filpula et al. 1984; Mocarski, Liu et al. 1987).

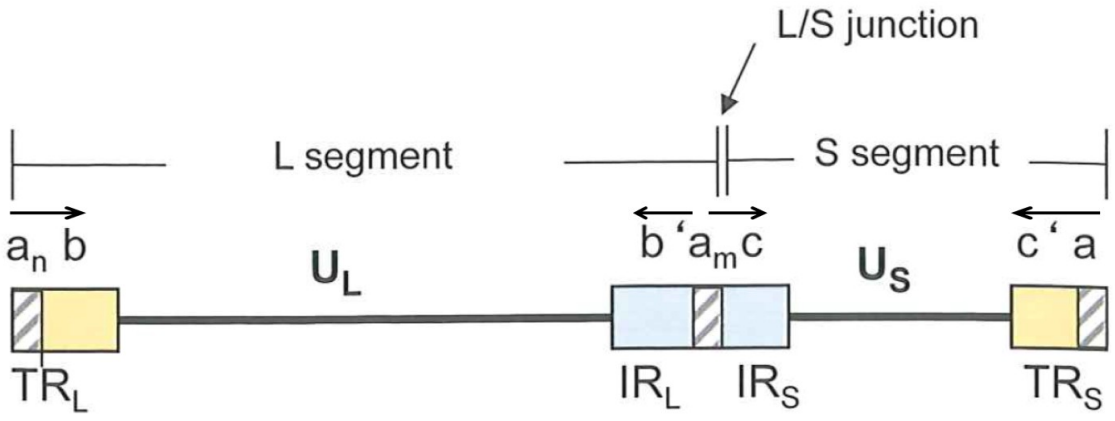


Figure 1.2 Structure of HCMV genome. See the text for more details. Image has been modified from its original source (Gibson and Bogner, 2013, p. 236).

During DNA replication, the terminal inverted repeated sequences allow for circularization and recombination within the genome. This is important due to the rolling circle method of replicating carried out by the herpesviruses (Jacob, Morse et al. 1979).The recombination among the inverted repeats allows for inversion of the orientation of U_L and U_S gene segments, resulting in four genomic isomers in equimolar concentrations (reviewed in reference Gibson and Bogner 2013). A recent study suggested that this genome isomerization was not required for efficient DNA replication (Sauer, Wang et al. 2010) but may provide an evolutionary advantage, such as maintaining genome length or genome stability during replication (Cui, McGregor et al. 2009).

1.3.2 Capsid proteins

The size of the capsid is 130 nm, which is 5% larger than HSV (Mocarski 2001). The capsid by itself is composed of four integral proteins including major capsid protein (MCP) encoded by UL86, minor capsid protein (mCP) encoded by UL85, minor capsid binding protein (mC-BP) encoded by UL46, and the smallest capsid protein (SCP) encoded by UL48.5 (or UL48/49). These proteins make up the outer shell of the HCMV capsid.

The MCP is the most highly conserved capsid protein among herpesviruses. Multimerization of 5 or 6 MCP monomers forms the major structural capsid elements, pentamers and hexamers, respectively. These elements are called capsomeres (see Figure 1.1). The HCMV capsid contains 162 capsomeres (12

pentamers plus 150 hexamers). One of the 12 pentamers is replaced with a portal complex that is composed of 12 copies of the portal protein (UL104, 79 kDa). This portal complex allows viral DNA entry and exit of the capsid shell (Newcomb, Cockrell et al. 2009). The mCP and mC-BP that form triplexes in a 2:1 ratio (mCP_2/mC-BP) interlock pentamers and hexamers on capsid surface (reviewed in references Gibson 1996; Mocarski 2001; Gibson 2008). The SCP, tightly bound to the external face of their capsomeres, is associated with the tegument protein pp150 to stabilize the DNA-containing capsids (Dai, Yu et al. 2013).

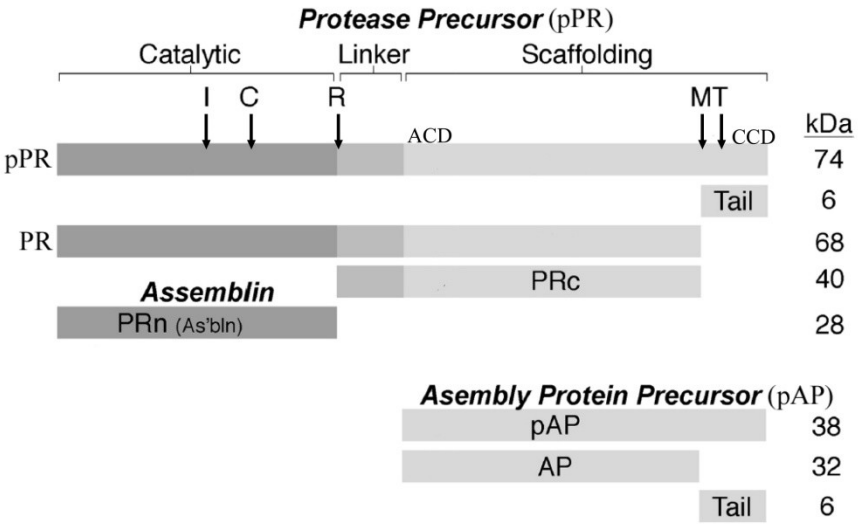


Figure 1.3 Schematic representation of pPR and pAP proteins. pPR self-cleaves at five sites indicated at the top: I (internal), C (cryptic), R (release), M (maturational), and T (tail). ACD, amino conserved domain; CCD, carboxyl conserved domain. The assembly protein precursor (pAP) and its M-site cleavage products (AP and tail) are also shown. Image has been modified from its original source (Fernandes et al. 2011, J Virol. 85(7): 3526-3534).

In addition, there are two internal capsid proteins, protease precursor (pPR, 74 kDa) encoded by UL80a, and assembly protein precursor (pAP, 38 kDa) encoded by UL80.5, which have a transient role in capsid formation and assembly and are believed to be absent in the infectious virions (see Figure 1.3; Mocarski 2001; Gibson 2008).

pAP has 2 functions: 1) guiding MCP into the nucleus, and 2) acting as a scaffold for conducting capsid shell formation (Wood, Baxter et al. 1997; Plafker and Gibson 1998; Nguyen, Loveland et al. 2008). MCP does not contain a nuclear localization signal (NLS). During capsid assembly, MCP is translocated into the nucleus via nuclear pores through the interaction with pAP that contains two NLSs (Wood, Baxter et al. 1997; Plafker and Gibson 1998; Loveland, Nguyen et al. 2007; Nguyen, Loveland et al. 2008). pAP interacts with MCP through the carboxyl conserved domain (CCD) at its carboxyl terminus. pAP also promote self interaction through the amino conserved domain (ACD) sequence. The ACD-mediated multimerization of the pAP stabilizes pAP-MCP complexes and scaffolds MCP into larger structural elements (pentamers and hexamers) required for capsid formation (see Figure 1.4; Wood, Baxter et al. 1997).

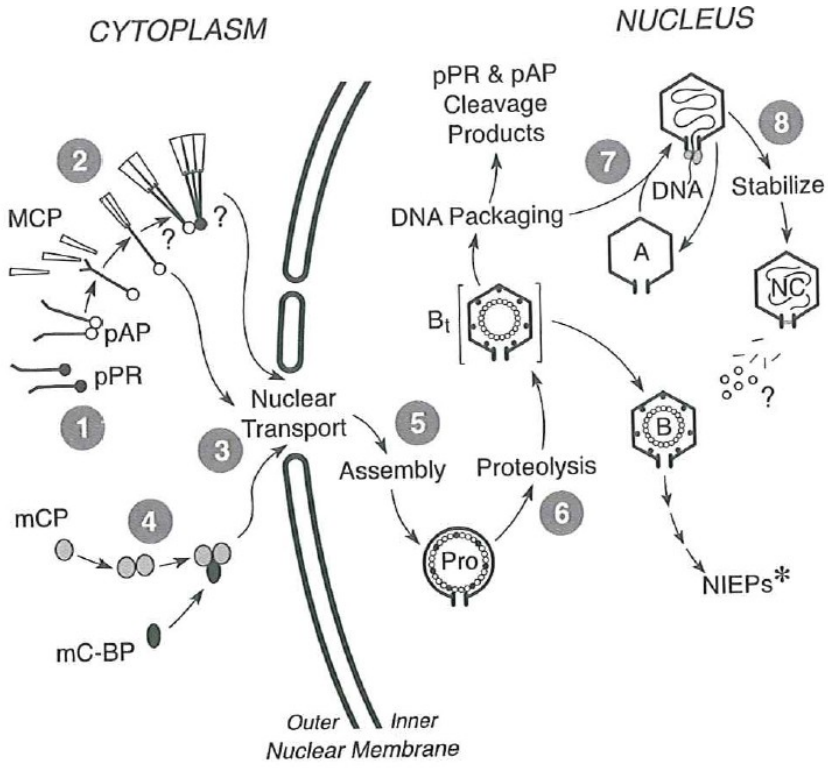


Figure 1.4 Capsid assembly model. See text for details. Image has been modified from its original source (Gibson and Bogner, 2013, p. 234).

During capsid assembly, it is believed that scaffolding proteins are eliminated from the internal capsid shell to make additional space for the accommodation of the large HCMV genome (Chan, Brignole et al. 2002; Loveland, Chan et al. 2005). Elimination of the scaffolding proteins from the internal capsid shell is done by activated pPR through autocatalytic proteolysis. pPR has 5 self cleavage sites within its protein sequence (see Figure 1.3; Welch, Woods et al. 1991; Baum, Bebernitz et al. 1993; DiIanni, Drier et al. 1993; Welch, McNally et al. 1993; Burck, Berg et al. 1994; Jones, Sun et al. 1994; Brignole and Gibson 2007). Maturational (M) and release (R) sites are conserved in all herpesviruses (Welch, Woods et al.

1991; Baum, Bebernitz et al. 1993; DiIanni, Drier et al. 1993). Additional internal (I), cryptic (C), and tail (T) sites are recognized only in cytomegaloviruses (Baum, Bebernitz et al. 1993; Welch, McNally et al. 1993; Burck, Berg et al. 1994; Jones, Sun et al. 1994; Brignole and Gibson 2007).

pPR autocatalytic cleavage at the R site releases proteolytic assemblin (28 kDa) at the amino end of pPR from the scaffolding portion at the carboxyl end (see Figure 1.3; Welch, Woods et al. 1991; Baum, Bebernitz et al. 1993; DiIanni, Drier et al. 1993; Robertson, McCann et al. 1996; Wood, Baxter et al. 1997; Brignole and Gibson 2007). The scaffolding portion of pPR has the same protein sequence as pAP. Therefore, pPR can interact with itself or with pAP through its ACD, and with MCP through its CCD (Wood, Baxter et al. 1997). Cleavage at the M site releases pPR and pAP from MCP in the capsid shell, leading to capsid maturation and formation (Preston, Rixon et al. 1992; DiIanni, Drier et al. 1993; Kennard, Rixon et al. 1995; Brignole and Gibson 2007). The I and C sites are located within the assemblin, and their cleavages are involved in the size reduction and the inactivation of assemblin (Jones, Sun et al. 1994; Chan, Brignole et al. 2002; Loveland, Chan et al. 2005). The significance of the cleavage at T site is undetermined (Brignole and Gibson 2007). Interestingly, a recent study revealed that full length pPR cleaves scaffolding proteins more efficiently than assemblin (Fernandes, Brignole et al. 2011). Both pPR and pAP are essential for infectious virion production (Matusick-Kumar, McCann et al. 1995). They can be potential targets for the development of therapeutic anti-HCMV agents.

Assembly proteins (AP) and protease (PR) are believed to be cleaved and eliminated from the internal capsids before or during DNA insertion (Chan, Brignole et al. 2002; Loveland, Nguyen et al. 2007; Gibson 2008), although assembly proteins (AP) were detected in mature HCMV virions by mass spectrometry (Varnum, Streblow et al. 2004). Whether AP is contained in a mature HCMV virion is still controversial.

During capsid assembly, there are three types of capsids produced within the nucleus, termed A, B, and C capsids (see Figure 1.5; reviewed in reference Mocarski 2001). Type A capsids contain the four integral proteins that compose the capsid shell but lack viral DNA. Type B capsids also contain the integral components of the capsid shell as well as the internal scaffolding proteins, but also lack viral DNA (Lee, Irmiere et al. 1988; Butcher, Aitken et al. 1998). Both A and B capsids fail to package viral DNA during assembly. They are dead end products of HCMV packaging. Type C capsids contain the viral DNA genome in capsid shells. C capsids are fully mature nucleocapsids that potentially become functional and infectious virion (reviewed in reference Mocarski 2001).

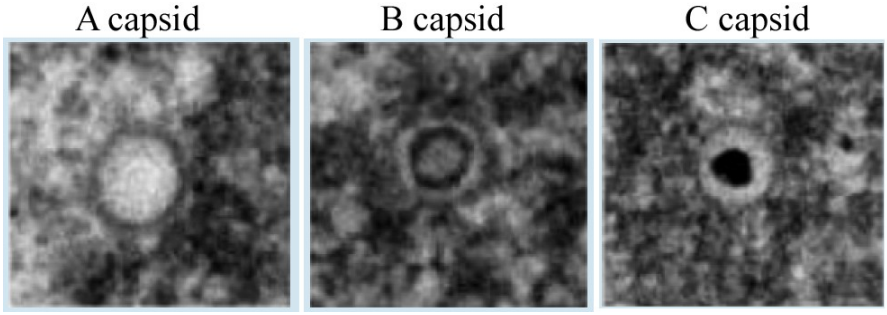


Figure 1.5 Capsid A, B, and C. Original source is Tandon et al. 2012, Trends Microbiol. 20(8): 392-401.

1.3.3 Tegument proteins

The layer between the capsid and envelope membrane is a matrix of tegument proteins that contain 40% of the virion protein mass (Irmiere and Gibson 1983). Most of them are phosphorylated. However, the significance of this and other post-translational modifications remains unclear (Kalejta 2008). There are 71 viral proteins found within virions, over half of them are located in the tegument layer (Varnum, Streblow et al. 2004). A few predominant tegument proteins, including UL48, UL47, pp65, pp71, pp150, and pp28, play significant roles in viral entry, gene expression, immune evasion, assembly and egress.

UL48 (high molecular-weight protein or HMWP, 250 kDa) is bound by UL47 (HMWP-binding protein or hmw-BP, 115 kDa). Their roles are not clear yet during HCMV infection. In alphaherpesviruses, UL37 and UL36 (VP1/2) are the homologs of HCMV UL47 and UL48, respectively. Evidence shows that UL37/UL36 complexes associated with capsids help transport capsids along microtubules to the nucleus (Luxton, Haverlock et al. 2005; Wolfstein, Nagel et al. 2006; Zaichick, Bohannon et al. 2013). Presumably, HCMV UL47/UL48 complexes may have similar activity (reviewed in reference Kalejta 2008).

pp65 (lower matrix protein, 65 kDa), encoded by UL83, is involved in evasion of innate and adaptive immune response during HCMV infection. The mechanisms in immune evasion include 1) disruption of antigen (Immediate Early protein) presentation by major histocompatibility complex class I (MHC I) molecules, 2) disruption of MHC II expression, 3) inhibition of natural killer cell cytotoxicity, and 4)

attenuating the interferon response (reviewed in reference Kalejta 2008; Tomtishen 2012). pp65 is the most abundant tegument protein found in virions. At the early stage of infection, pp65 is delivered to the nucleus independent of the capsid via its nuclear localization signal (NLS) (Revello, Percivalle et al. 1992; Schmolke, Drescher et al. 1995; Arcangeletti, Rodighiero et al. 2011). Starting from 48 hour post infection, pp65 migrates into the cytoplasm (Sanchez, Greis et al. 2000). pp65 is the primary target for phosphorylation *in vitro* by virion associated protein kinase (Somogyi, Michelson et al. 1990; Oien, Thomsen et al. 1997). Surprisingly, this phosphoprotein pp65, which constitutes ~18% of the virion protein mass, is not essential for viral replication and the production of new infectious HCMV progeny (Schmolke, Kern et al. 1995; Chevillotte, Landwehr et al. 2009). Interestingly, large amounts of pp65 is produced by laboratory strains of HCMV, which results in increased formation of dense bodies that are composed of predominantly pp65. However, pp65 production is largely reduced in the infection with clinical isolates of HCMV (Klages, Ruger et al. 1989).

pp71 (upper matrix protein, 71 kDa), encoded by UL82, is a transactivator of gene expression (Liu and Stinski 1992) for efficient viral replication. Its activities include 1) activation of IE gene expression through interfering with the normal activity of the cellular protein Daxx that promotes transcriptional repression (Cantrell and Bresnahan 2005). 2) Stimulation of cell cycle progression toward DNA replicating S phase by blocking Rb function. Rb normally binds to the E2F transcription factors and represses transcription from the promoter. During HCMV infection, pp71 binds to the Rb, stimulating E2F activity, which in turn, facilitates

efficient viral DNA replication (Kalejta, Bechtel et al. 2003; Kalejta and Shenk 2003). Furthermore, a study shows that pp71 is involved in immune evasion, like pp65, by disrupting MHC I cell surface expression (Trgovcich, Cebulla et al. 2006).

pp150 (basic phosphoprotein or BPP, 150 kDa), encoded by UL32, is required for HCMV cytoplasmic maturation from the cells (AuCoin, Smith et al. 2006). This protein is the second most abundant tegument protein found in virions and is highly immunogenic (Landini, Re et al. 1985; Jahn, Scholl et al. 1987). Recent study shows that pp150 recruited by SCP stabilizes viral genome-containing capsids for the production of infectious HCMV virions (Dai, Yu et al. 2013).

pp28 (28 kDa), encoded by UL99, is also highly immunogenic (Meyer, Bankier et al. 1988). This phosphoprotein is required for the final envelopment of the infectious virions in the cytoplasm (Silva, Yu et al. 2003). Both pp150 and pp28 are essential during HCMV infection and play roles in viral cytoplasmic egress, but there are some distinctions between them. The current speculation is that pp150 stabilizes the DNA containing capsids and likely directs them after trafficking from the nucleus to the viral assembly compartment (VAC) in the cytoplasm where pp28 likely helps enclosing these tegument-associated capsids into their final envelopes (reviewed in reference Kalejta 2008).

Viral tegument proteins apparently play significant roles during HCMV infection. Other than these predominant tegument proteins, several tegument proteins including UL35, UL69, pIRS1/pTRS1, ppUL97, UL36, UL45, UL76, UL77,

UL88, UL94, and US24, have also been characterized and play either essential or nonessential roles during HCMV infection (reviewed in reference Kalejta 2008).

In addition to viral components, 70 cellular proteins also found in HCMV virions (Varnum, Streblow et al. 2004), are presumably packaged in the tegument layer. More interestingly, some cellular and viral RNAs found within virions, non-infectious enveloped particles (NIEPs), and dense bodies (Terhune, Schroer et al. 2004), are also presumably packaged in the tegument layer.

1.3.4 Glycoproteins

A group of the abundant structural glycoproteins in HCMV, glycoprotein B (gB), H (gH), L (gL), O (gO), M (gM), and N (gN), are homologous to that in other herpesviruses and serve conserved functions in entry. These proteins, except gO, are essential for HCMV productivity. Several other identified HCMV glycoproteins that are non-essential for HCMV growth do not have known function in entry (Dunn, Chou et al. 2003).

To date, at least four glycoprotein complexes on the viral envelope are defined, designated as gCI, gCII, gCIII, as well as a recently defined pentamer. The gCI is composed of a homodimeric molecule of gB (Britt 1984; Britt and Auger 1986). The gCII is composed of a heterodimeric complex of gM and gN (Kari and Gehrz 1992; Karlin, Mocarski et al. 1994; Mach, Kropff et al. 2000). The gCIII is composed of a heterotrimer of gH, gL, and gO (Huber and Compton 1997; Li, Nelson et al. 1997; Huber and Compton 1998). Recent studies show an additional

pentameric glycoprotein complex, gH/gL/pUL128-pUL130-pUL131A (gH/gL/UL128-131), is required for entry into epithelial cells, endothelial cells, as well as leukocytes (Wang and Shenk 2005; Ryckman, Jarvis et al. 2006; Ryckman, Chase et al. 2008; Ryckman, Rainish et al. 2008).

Glycoprotein B is the primary receptor binding protein. One of the binding partners is the cellular protein heparin sulfate proteoglycans (HSPGs) (Kari and Gehrz 1992; Compton, Nowlin et al. 1993; Kari and Gehrz 1993; Carlson, Britt et al. 1997; Boyle and Compton 1998; Compton 2004). Binding to HSPGs is a conserved feature in entry in all herpesviruses. A second binding partner is an integrin (Feire, Koss et al. 2004). The gB-integrin interaction mediates entry into fibroblasts, endothelial and epithelial cells (Feire, Koss et al. 2004; Feire, Roy et al. 2010). In some HCMV permissive cell types, gB also interacts with epidermal growth factor receptor (EGFR) (Wang, Huong et al. 2003). However, that EGFR is one of the receptors for HCMV entry is still controversial (Wang, Huong et al. 2003; Isaacson, Feire et al. 2007; Chan, Nogalski et al. 2009). The complex gM/gN plays a role during viral entry (Mach, Kropff et al. 2000; Shimamura, Mach et al. 2006). However, their interaction partners and its contribution are still unclear.

Evidence shows that the combination of both gB and gH/gL is required for the membrane fusion process in fibroblasts, epithelial cells and endothelial cells (Keay and Baldwin 1991; Netterwald, Jones et al. 2004; Vanarsdall, Ryckman et al. 2008; Isaacson and Compton 2009; Potzsch, Spindler et al. 2011). gH/gL complexes play a role in recognition of cellular receptors. The accessory proteins within gH/gL complexes determine target cell tropism. Epstein-Barr virus (EBV) and

HHV-6 have a similar feature. For EBV, presence or absence of the accessory protein gp42 within the gH/gL complexes determine cell tropism. Unmodified gH/gL complexes alone bind to specific integrins on the cell surface and mediate the entry into epithelial cells (Chesnokova, Nishimura et al. 2009; Chesnokova and Hutt-Fletcher 2011). With the accessory protein gp42 within the gH/gL complexes, gp42 bind to MHC-II of B cells and shifts the tropism from epithelial to B cells (Miller and Hutt-Fletcher 1992; Spriggs, Armitage et al. 1996; Borza, Morgan et al. 2004; Hutt-Fletcher 2007; Matsuura, Kirschner et al. 2010).

Like EBV, HCMV entry into different cell types is mediated by the different accessory proteins (either gO or UL128-131) incorporated within the gH/gL complex on the viral envelope (reviewed in reference Adler and Sinzger 2013). For instance, the laboratory HCMV strains, such as Towne and AD169, contain only gH/gL/gO complexes (Huber and Compton 1998; Ryckman, Chase et al. 2010) on their envelope for the entry of fibroblasts, however, these strains with these trimeric complexes are not sufficient to enter endothelial and epithelial cells. The clinical strain TR contains additional pentameric complexes gH/gL/UL128-131 on their viral envelope for the entry of endothelial and epithelial cells (Wang and Shenk 2005; Adler, Scrivano et al. 2006; Schuessler, Sampaio et al. 2008; Straschewski, Patrone et al. 2011).

However, new recent evidence (Jiang, Adler et al. 2008; Scrivano, Esterlechner et al. 2010; Wille, Knoche et al. 2010; Scrivano, Sinzger et al. 2011; Vanarsdall, Chase et al. 2011) suggests a different point of view. The pentameric complex is sufficient for entry into a broad range of cells including endothelial cells,

epithelial cells, and even fibroblasts, while the trimeric complex is an additional variant that mediates the entry of a restricted set of cells, ie, fibroblasts only but not endothelial and epithelial cells (reviewed in reference Adler and Sinzger 2013).

The most recent study reveals that all strains, including the clinical strains, TR and Merlin, and the laboratory strains, Towne and AD169, contain both gH/gL/gO trimers and gH/gL/UL128-131 pentamers. However, the ratio between these complexes in the virion envelope is strain dependent (Zhou, Yu et al. 2013). For instance, TR contains more trimers than the pentamers in the viral envelope, while Merlin contains mostly pentamers (Zhou, Yu et al. 2013).

Other cellular receptors, including β2-microglobulin, annexin II, and CD13, were also reported to be potential HCMV receptors (Feire and Compton 2013). These putative receptors are still under debate (reviewed in reference Adler and Sinzger 2013).

1.4 HCMV Replication Cycle

Cytomegalovirus is a host species specific pathogen, and HCMV only infects humans. In general, HCMV infects and completes a full replication cycle in fibroblasts, epithelial cells, endothelial cells, and smooth muscle cells. These cells support highly productive HCMV infection (Sinzger, Grefte et al. 1995). HCMV can also infect neurons, macrophages, neutrophils, hepatocytes, and dendritic cells but with dramatically less production of infectious virions (Myerson, Hackman et al. 1984; Ibanez, Schrier et al. 1991; Plachter, Sinzger et al. 1996; Sinzger, Kahl et al.

2000). This wide range of cell tropism is the root of HCMV systemic infection in immunocompromised hosts. Its replication steps are described below (see Figure 1.6).

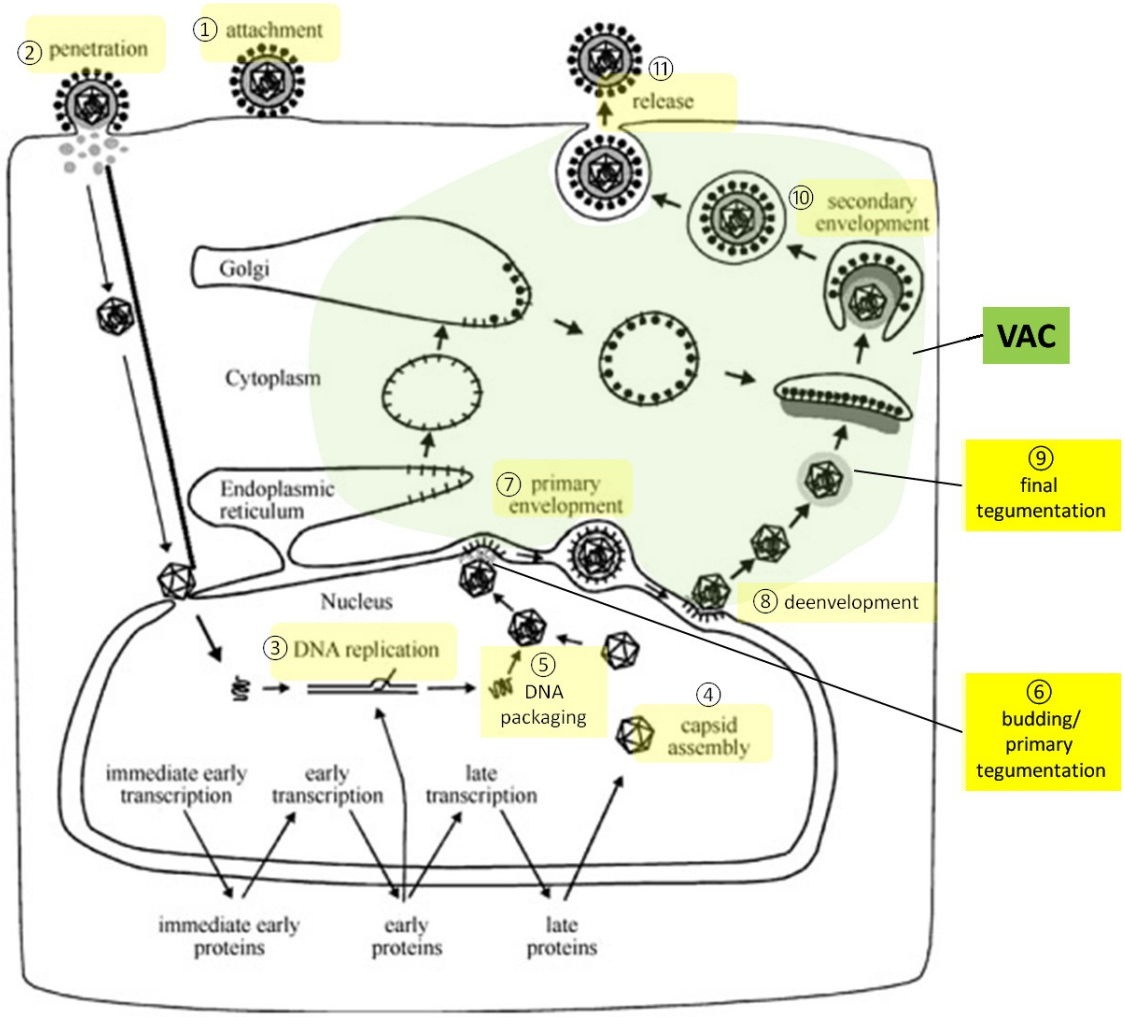


Figure 1.6 HCMV replication cycle. Entry process: ① attachment, ② penetration/fusion. Nuclear stage of maturation: ③ DNA replication, ④ capsid assembly, ⑤ DNA packaging, ⑥ budding/primary tegumentation, ⑦ primary envelopment, ⑧ deenvelopment (step ⑥, ⑦, and ⑧ are nuclear egress process). Cytoplasmic stages of maturation: ⑨ final tegumentation, ⑩ final envelopment, ⑪ release. Image from its original source (Halwachs-Baumann, 2011, p. 15) has been modified.

1.4.1 Attachment, Fusion, and Entry

HCMV entry into a host cell is a complicated multi-step process starting from binding through the specific interactions between glycoproteins and cellular receptors, leading to conformational changes in glycoproteins that induce fusion of the viral envelope with the cellular membrane (reviewed in reference Connolly, Jackson et al. 2011).

In the current working model for entry into cells, HCMV initially attaches to the host cell surface receptor heparin sulfate proteoglycans (HSPGs) via the glycoprotein gB and/or gM/gN (Kari and Gehrz 1992; Compton, Nowlin et al. 1993; Kari and Gehrz 1993; Carlson, Britt et al. 1997; Boyle and Compton 1998; Compton 2004). After attachment, other interactions between HCMV envelope glycoproteins (including gH/gL trimer or pentamer complexes) and cellular integrins as co-receptors promote receptor clustering on the cell surface. These interactions may activate conformational change in the glycoproteins that trigger fusion of viral envelope into cellular membrane, resulting in deposition of virion components into the cytoplasm (Lopper and Compton 2004). Currently the membrane fusion machinery and all the involved components still remain unclear due to the lack of a reliable fusion assay (Feire and Compton 2013).

Upon entry, the DNA-containing capsids are delivered to the nucleus along microtubules. This transport is assisted by the capsid-associated tegument proteins UL47, UL48, and perhaps pp150, as well as the cellular motor proteins such as dynein. The capsids deposit their viral genome into the nucleus through the nuclear

pore complexes. Other tegument proteins, including pp65 and pp71, are transported independently into the nucleus (Reviewed in references Isaacson, Juckem et al. 2008; Kalejta 2008; Kalejta 2008). Upon genome deposition into the nucleus, viral immediate early (IE) genes are expressed, and these proteins modulate the host cell environment and stimulate the expression of viral early genes. The early proteins stimulate the viral genomic DNA replication. After DNA replication, the expression of late genes, whose products are mainly structural components of the virions (tegument, glycoproteins, etc) that are required for assembly and maturation, is turned on (reviewed in reference Kalejta 2008).

1.4.2 Nuclear Stage of HCMV Maturation: HCMV DNA replication, capsid assembly, DNA encapsidation, tegumentation, primary envelopment and nuclear egress

1.4.2.1 HCMV DNA replication

Replication of the double stranded DNA genome takes place in discrete sites (also termed viral replication centers) within the nucleus. These centers concentrate both viral and cellular proteins/factors required for efficient viral genome replication. Cellular DNA is excluded from these sites. During infection, HCMV induces a cell cycle arrest at the G1/S transition for the purpose of enhancing viral DNA replication while inhibiting cellular DNA replication. This feature is essentially conserved in other herpesviruses (reviewed in reference Adler and Sinzger 2013; Spector 2013).

During DNA replication, linear DNA is circularized and is replicated via a mechanism like rolling circle replication, which produces long linear concatamers that contain multiple copies of the same genomic sequences linked in series (reviewed in reference Roizman 2001). These concatamers are then cleaved into unit-length genomes and packaged into preassembled capsids via a cleavage/packaging process catalyzed by terminase.

1.4.2.2 Capsid formation and assembly

Capsid assembly also takes place within the replication centers. During capsid assembly, pAP interacts with MCP in the cytoplasm and migrates to the nucleus (Wood, Baxter et al. 1997; Plafker and Gibson 1998; Loveland, Nguyen et al. 2007; Nguyen, Loveland et al. 2008). The oligomerization of pAP assembles MCP into capsomeres required for capsid shell formation (Wood, Baxter et al. 1997; Plafker and Gibson 1998; Nguyen, Loveland et al. 2008). The interaction of all the integral capsid proteins including pAP-MCP complexes (forming capsomeres), mCP/mC-BP triplexes in a 2:1 ratio, SCP, and the portal complex results in the formation of procapsids, short-lived unstable capsid precursors. Activated pPR cleaves the scaffolding proteins from the internal capsid shell of the procapsid, resulting in transition B capsids (Bt) that either become stable B capsids, or turn into C capsids by elimination of the scaffolding proteins from the capsid shells coupled with incorporation of viral DNA into the capsids (see Figure 1.4). Empty A capsids result from complete elimination of the scaffolding proteins but unsuccessful DNA

packaging (reviewed in references Gibson 1996; Mocarski 2001; Gibson 2008; Gibson and Bogner 2013).

1.4.2.3 DNA encapsidation

Like other herpesviruses, the HCMV DNA cleavage/packaging process is mediated by a terminase. The terminase is an enzymatic protein complex that is composed of a homodimer of UL56 and a monomer of UL89. It interacts with both DNA and the portal protein UL104 to initiate genome encapsidation. Both UL56 and UL89 are essential for HCMV replication. They are highly conserved proteins among the herpesvirus family (reviewed in reference Gibson and Bogner 2013). Twelve copies of the portal protein UL104 form a channel for DNA entry into the capsid shell (Holzenburg, Dittmer et al. 2009; Newcomb, Cockrell et al. 2009). In addition, UL51, UL52, UL77, UL93 may also be involved in the DNA/packaging process (Bogner 2002; Borst, Kleine-Albers et al. 2013).

During DNA packaging/encapsidation, the UL56 dimer of the terminase binds to the viral DNA at specific nucleotide sequences termed packing signals (*pac1* and *pac2* sequences) (Bogner, Radsak et al. 1998; Scheffczik, Savva et al. 2002). The terminase-DNA complex then interacts with the portal complex of a newly assembled capsid shell (Komazin, Townsend et al. 2004; Dittmer and Bogner 2005; Dittmer, Drach et al. 2005). Using ATP hydrolysis (ATPase activity), viral DNA is inserted into the capsid shell (Hwang and Bogner 2002; Scholz, Rechter et al. 2003).

After one genome length is packaged, concatameric DNA is cleaved by UL89 of the terminase (Scheffczik, Savva et al. 2002).

1.4.2.4 Primary tegumentation, primary envelopment and nuclear egress

During the translocation of nuclear capsids into the cytoplasm, some tegument proteins are believed to be transiently or permanently associated with the capsids in the nucleus. This has been demonstrated in HSV that some tegument proteins are associated with capsids within their primary envelope (Naldinho-Souto, Browne et al. 2006; Padula, Sydnor et al. 2009). In HCMV, both pp65 and pp150 are strongly associated with the nuclear membrane (Hensel, Meyer et al. 1995). However, little direct evidence shows that primary tegumentation occurs at the nuclear membrane. Interestingly, pp150 is also associated with the capsids within the replication center in the nucleus during the capsid assembly process (Baxter and Gibson 2001; Sampaio, Cavignac et al. 2005; Tandon and Mocarski 2011). pp150 associated with capsids is later localized in the cytoplasm at late times of infection (Hensel, Meyer et al. 1995). This demonstrates that initial tegumentation occurs within the nucleus, but the majority of pp150 and other tegument proteins are added to the capsids after nuclear trafficking and before final envelopment in the cytoplasm. Several studies suggest that the tegumentation of the capsids before nuclear trafficking is to provide structural stability to the capsids during the translocation from the nucleus to the cytoplasm (Baxter and Gibson 2001; AuCoin, Smith et al. 2006; Tandon and Mocarski 2011; Dai, Yu et al. 2013).

After initial tegumentation within the nucleus, the capsids then translocate from the nucleus into the cytoplasm. The capsid is about 130 nm in diameter, however, only molecules with 40 nm can be transported through the nuclear pore complexes (Pante and Kann 2002). Obviously, a nuclear egress mechanism is required for HCMV.

Nuclear egress is an envelopment/de-envelopment process used to cross the double nuclear membrane. Our understanding of the precise mechanism of HCMV egress from the nucleus is limited. In recent years the Nuclear Egress Complex (NEC) has been suggested to facilitate capsid egress from the nucleus by destabilization of the nuclear lamina (Marschall, Marzi et al. 2005; Camozzi, Pignatelli et al. 2008; Hamirally, Kamil et al. 2009; Milbradt, Webel et al. 2010; Sharma, Kamil et al. 2014). The nuclear lamina is a dense meshwork composed of lamins and lamin-associated membrane proteins. It mechanically supports the shape of the nucleus (reviewed in references Goldman, Gruenbaum et al. 2002; Dechat, Pfleghaar et al. 2008). In order to exit the nucleus, the lamina must be dissociated.

The NEC is made of both viral and cellular proteins (see Figure 1.7). Viral proteins include UL50, UL53, UL97, nuclear Rim-ASsociated CytomegalovirAL protein (RASCAL), and perhaps some yet undefined proteins. Cellular proteins include the Lamin B receptor (LBR), protein kinase C (PKC), p32, peptidyl-prolyl *cis-trans* isomerase (Pin1), and emerin (Krosky, Baek et al. 2003; Marschall, Marzi et al. 2005; Camozzi, Pignatelli et al. 2008; Hamirally, Kamil et al. 2009; Milbradt, Auerochs et al. 2009; Sam, Evans et al. 2009; Milbradt, Webel et al. 2010; Miller,

Furlong et al. 2010; Milbradt, Kraut et al. 2014; Sharma, Kamil et al. 2014). UL50 is a key component required to recruit UL53, RASCAL, and PKC to the nuclear lamina (Muranyi, Haas et al. 2002; Camozzi, Pignatelli et al. 2008; Milbradt, Auerochs et al. 2009; Sam, Evans et al. 2009; Miller, Furlong et al. 2010). p32 is recruited to the lamina through the interaction with the lamin B receptor (Milbradt, Auerochs et al. 2009). UL97 is recruited to the lamina via binding to p32 (Marschall, Marzi et al. 2005). PKC and UL97 play roles in dissociation of the nuclear lamins by phosphorylating them (Muranyi, Haas et al. 2002; Krosky, Baek et al. 2003; Hamirally, Kamil et al. 2009; Milbradt, Auerochs et al. 2009; Milbradt, Webel et al. 2010). Phosphorylated lamins generate a binding site for Pin1. The recruitment of Pin1 may promote conformational changes of lamins, and thus, leading to localized depletion of the nuclear lamina (see Figure 1.7; Milbradt, Webel et al. 2010).

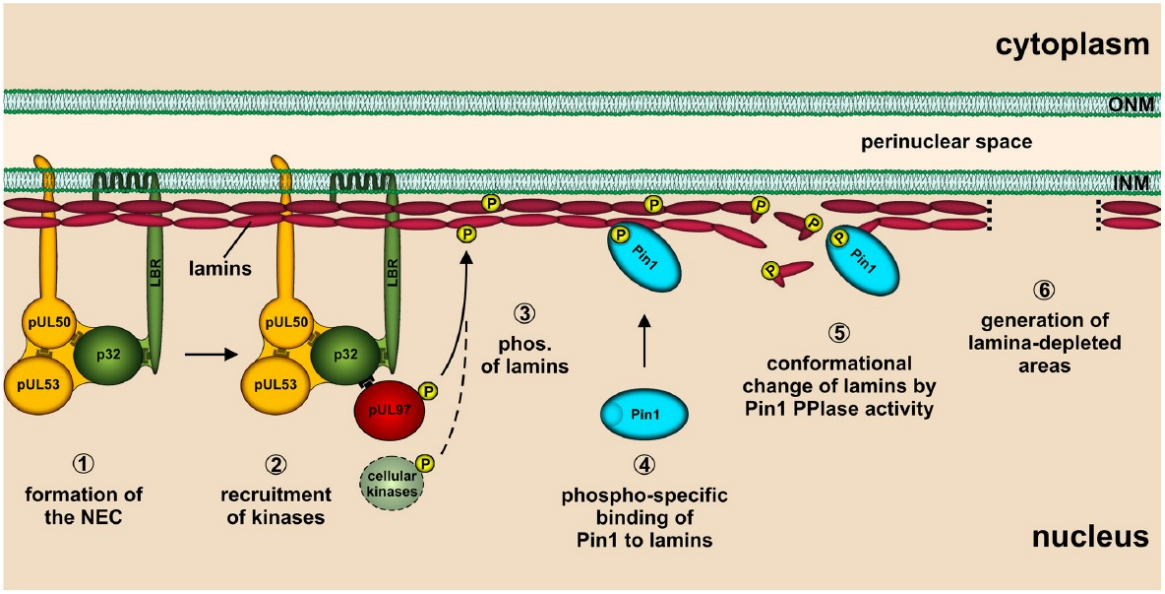


Figure 1.7 NEC formation and a working model of the course of events leading to lamina dissociation. Original source is Milbradt et al. 2010, J Biol Chem. 285(18): 13979-13989. See the texts for more details.

In HCMV, nuclear egress mediated by the NEC, particularly UL50 and UL53, is currently the most widely accepted mechanism. Other egress pathways have also been described (Buser, Walther et al. 2007; Klupp, Granzow et al. 2011). Many studies have documented the tubule-like infoldings of the inner nuclear membrane (IINM) as a site where nuclear capsids undergo the envelopment/de-envelopment process when crossing the double nuclear membrane (Ruebner, Miyai et al. 1964; Papadimitriou, Shellam et al. 1984; Severi, Landini et al. 1988; Gilloteaux and Nassiri 2000; Dal Monte, Pignatelli et al. 2002; Buser, Walther et al. 2007). One study reports that these structures account for only 4.8% of the total INM surface, but that the majority of capsids (86%) preferentially bud at these sites (see Figure 1.8), suggesting that the IINMs are the principle sites for nuclear egress (Buser, Walther et al. 2007). More interestingly, a recent study in HSV-1 showed that UL34 and UL31 (homologs of UL50 and UL53) alone were sufficient to induce membrane deformation in vitro without additional cellular or viral factors (Bigalke, Heuser et al. 2014). This indicates that UL50-UL53 may induce the IINM formation in HCMV.

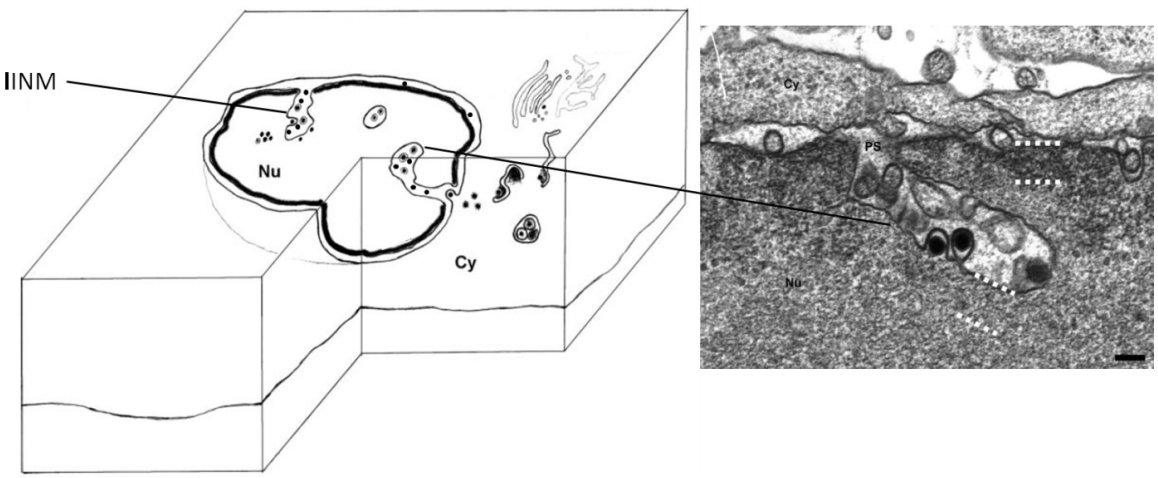


Figure 1.8 Model of HCMV nuclear egress predominantly at the infoldings of the inner nuclear membrane (IINM). Image has been modified from its original source (Buser et al. 2007, J Virol. 81(6): 3042-3048).

In addition, other cytoplasmic cellular proteins, such as BiP/GRP78 and dynein, may also influence nuclear egress into the cytoplasm (Buchkovich, Maguire et al. 2010). BiP is an ER chaperone that contributes to and maintains the integrity of the VAC during HCMV infection (Buchkovich, Maguire et al. 2009). UL50 interacts with BiP. Therefore, nuclear egress may also depend on the UL50-BiP interaction that mediates signaling between the VAC and the nucleus (Buchkovich, Maguire et al. 2010).

After disruption of the lamina, capsids bud through the inner nuclear membrane (INM), acquiring their first envelope. These enveloped capsids within the perinuclear space or IINM then bud through the outer nuclear membrane (ONM), losing their primary envelope and entering into the VAC within the cytoplasm as non-enveloped capsids (reviewed in references Mettenleiter, Klupp et al. 2009; Johnson and Baines 2011). In pseudorabies virus (PRV), only C capsids are

translocated into the cytoplasm but not A and B capsids (Leelawong, Guo et al. 2011). However, HSV and HCMV do not have this filter during nuclear egress.

1.4.3 Cytoplasmic Stage of HCMV Maturation: final tegumentation, final envelopment, and release

After the translocation from the nucleus, capsids are deposited into the viral assembly compartment (VAC) where they acquire the remaining tegument proteins in the ER and derive their final envelope from the golgi in the cytoplasm (Sanchez, Greis et al. 2000; Sanchez, Sztul et al. 2000). The VAC is formed by rearrangement of the vesicles derived from Golgi bodies, the trans-Golgi network, and early endosomes as nested cylinders (Das, Vasanji et al. 2007; Das and Pellett 2011). The cellular proteins BiP/GRP78 and dynein also contribute to the VAC formation and integrity (Buchkovich, Maguire et al. 2009; Buchkovich, Maguire et al. 2010; Indran, Ballestas et al. 2010).

Proteins accumulated within the VAC contain both cellular and viral proteins that play roles in the final assembly of capsids in the cytoplasm. Cellular proteins include the endosomal sorting complex required for transport III (ESCRT III) and several Rab GTPase (Rab6, Rab27a, Rab11) (Krzyzaniak, Mach et al. 2009; Tandon, AuCoin et al. 2009; Indran and Britt 2011). Viral proteins include the tegument proteins pp65, pp150, pp28, UL94, UL96, and UL103 , as well as the glycoproteins gB, gM/gN, gH/gL/gO, and gH/gL/UL128-131 (reviewed in reference Tandon and Mocarski 2012).

The remaining tegument proteins are added to the capsids within the VAC before final envelopment. They also mediate the interaction required for the envelopment. The envelope is a major determinant of HCMV infectivity. It is derived from cellular membranes, including trans-Golgi network (TGN) and endosomal membranes that contribute to HCMV envelopment (Tooze, Hollinshead et al. 1993; Das, Vasanji et al. 2007; Cepeda, Esteban et al. 2010). These membranes are modified by insertion of HCMV glycoproteins. Cytoplasmic capsids bud through the glycosylated endosomal membranes to acquire their envelope (Severi, Landini et al. 1988; Tooze, Hollinshead et al. 1993; Silva, Yu et al. 2003; Liu, Sharon-Friling et al. 2011). The tegument protein pp28 plays an important role in enclosing these capsids into their final envelopes (Silva, Yu et al. 2003). Ultimately, these vesicles containing enveloped virions are fused with the plasma membrane, resulting in the release of the fully matured and infectious virions (reviewed in reference Kalejta 2008).

In addition to pp28, HCMV envelopment and release are also influenced by UL71 and UL103. UL71 promotes efficient formation of enveloped virions and facilitates virion release from the cells (Womack and Shenk 2010; Schauflinger, Fischer et al. 2011; Meissner, Suffner et al. 2012). UL103 promotes exocytic release of virions and dense bodies (Ahlqvist and Mocarski 2011).

In addition to infectious virions, two other enveloped HCMV viral particles, non-infectious enveloped particles (NIEPs) and dense bodies (DBs), are also released from the cells (Irmiere and Gibson 1983). Studies show that NIEPs come from B capsids and lack viral DNA (Irmiere and Gibson 1983; Irmiere and Gibson

1985). DBs are solid spherical aggregates predominately pp65 tegument protein (Sarov and Abady 1975; Gibson and Irmiere 1984). Neither NIEPs nor DBs show infectivity due to their lack of DNA within their envelope (Irmiere and Gibson 1983). The significance of their existence (NIEPs and DBs) is not well understood.

1.5 p53 Structure and Functions

HCMV infection can cause significant stress on host cells. The cellular responses to stress are mediated by the cell cycle protein p53. During HCMV infection, p53 is hijacked into viral replication centers within the nucleus (Fortunato and Spector 1998). It has been suggested that p53 plays roles in HCMV infection (Casavant, Luo et al. 2006; Rosenke, Samuel et al. 2006; Hannemann, Rosenke et al. 2009). Before we explore the roles of p53 during HCMV infection, it is important to review the structure and functions of p53.

p53 is a multifunctional tumor suppressor protein encoded by the TP53 gene located on the short arm of chromosome 17 in humans (Freed-Pastor and Prives 2012). p53 is widely known as 'the guardian of the genome' since it has an important role in prevention of genome mutation (Lane 1992). p53 mutations are present in over 50% of all cancers, therefore, p53 is considered to be a critical player in cancer biology (Levine and Oren 2009; Freed-Pastor and Prives 2012).

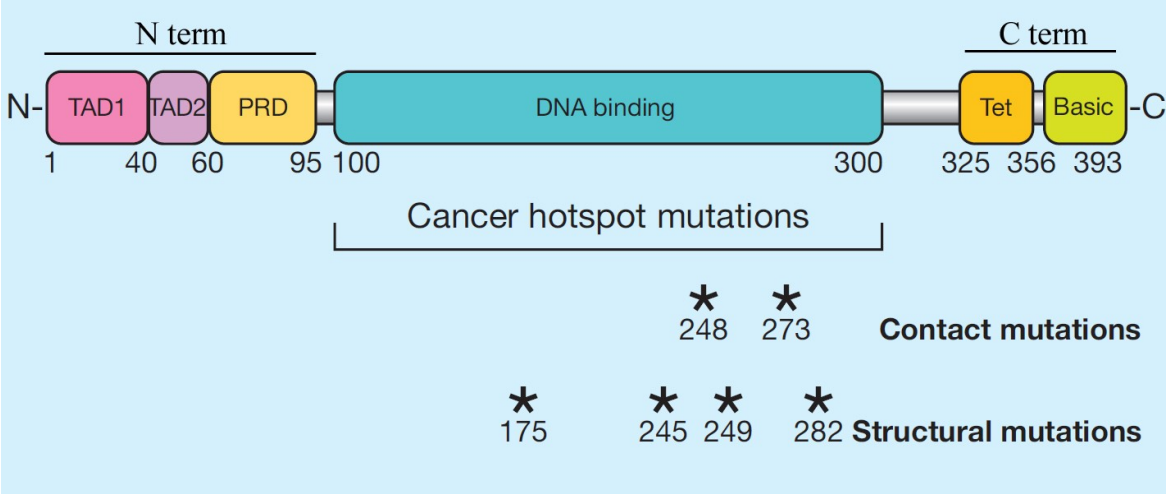


Figure 1.9 The regulatory domains of p53. TAD = transactivation domain, PRD = proline-rich domain, Tet = tetramerization domain, * = residues that are often mutated. See texts for more details. Image is cropped from Brady et al. 2010, J Cell Sci. 123(15): 2527-2532.

1.5.1 p53 structure

p53 is a specific transcription factor. It contains five domains (see Figure 1.9). The N-terminus of p53 contains a transactivation domain (TAD), which is further divided into two subdomains, TAD1 and TAD2. These domains are where p53 interacts with the components of basal transcriptional machinery, histone-modifying enzymes, and co-activator complexes, such as TATA box-binding protein (TBP)(Liu, Miller et al. 1993), transcription factor II H (TFIIH) (Xiao, Pearson et al. 1994), replication protein A (RPA) (Dutta, Ruppert et al. 1993; Li and Botchan 1993), and STAGA (Gamper and Roeder 2008), to promote and enhance transcription of p53 target genes (Brady and Attardi 2010). The N terminus also contains a prolin-rich domain that plays a role in structural function of p53 (Brady and Attardi 2010). The center region of p53 is a sequence-specific DNA-binding domain. This region is

often prone to mutation, which can lead to developing cancer. The mutation hotspots, including G154, R175, G245, R248, R249, R273 and R280, are the most common residues changed in cancer (Raycroft, Wu et al. 1990; Brady and Attardi 2010; Freed-Pastor and Prives 2012). p53 binds to its response elements (REs) in target genes. These REs are composed of two identical sequences separated by 0-13 nucleotides (5'-Pu Pu Pu C A/T A/T G Py Py Py n_{0-13} Pu Pu Pu C A/T A/T G Py Py Py-3'. Pu = purine, Py = pyrimidine, n = any nucleotides). These REs are frequently found either in the promoters or the first introns of p53 target genes (el-Deiry, Kern et al. 1992; Freed-Pastor and Prives 2012). The C terminus of p53 contains a tetramerization (or oligomerization) domain, as well as a regulatory basic domain that allows for non-specific DNA binding and interaction with DNA repair proteins (May and May 1999). p53 functions as a homotetramer for strong transactivation. The nuclear localization signal of p53 is located between the DNA binding and tetramerization domains (residues 303-323) (May and May 1999).

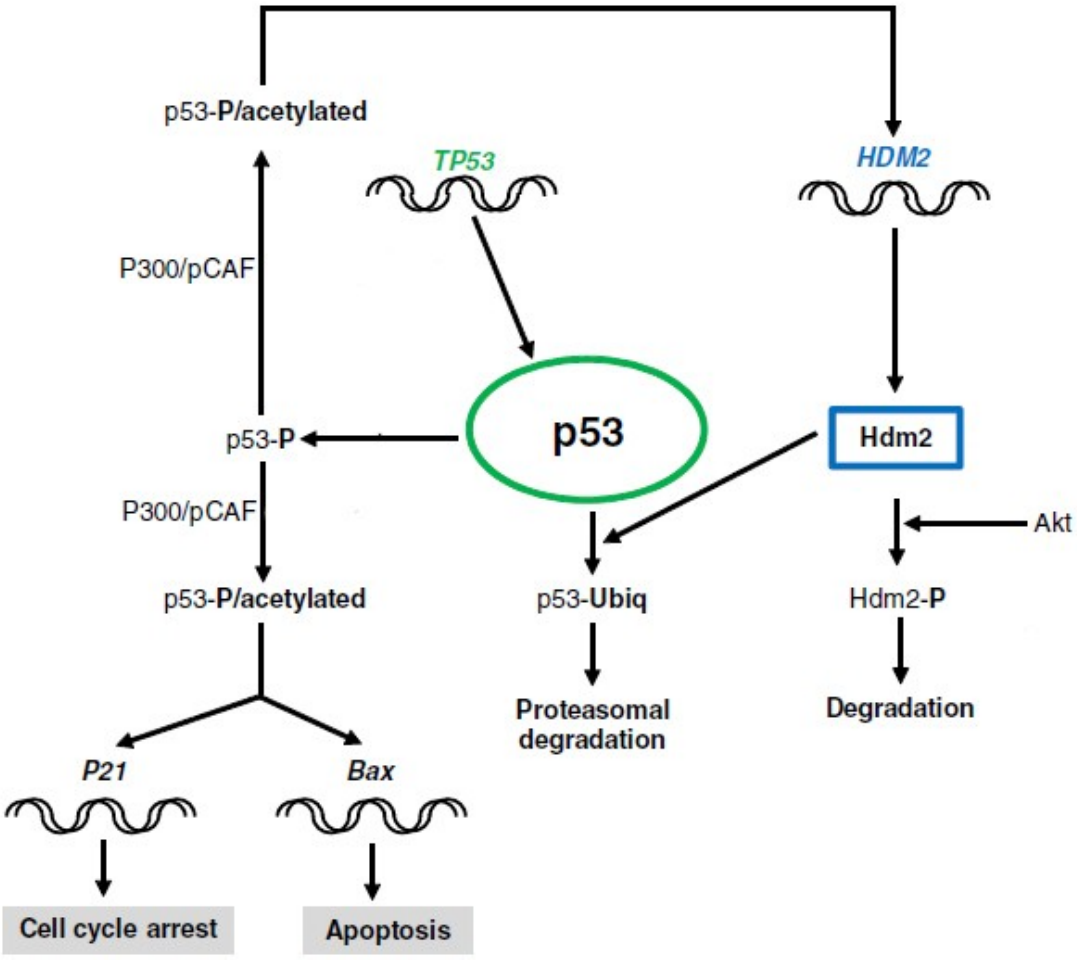


Figure 1.10 p53 signaling pathway and its mediated responses. Image has been modified from its original source (Lazo et al. 2011, Rev Med Virol. 21(5):285-300)**.** See texts for more details.

1.5.2 p53 functions

p53 is involved in extremely diverse mechanisms in cells. Mainly, it serves as a sensor of many different types of cellular stress including DNA damage, UV and X-ray irradiation, chemicals, heat shock, hypoxia, cytokines, oncogene expression, nutrient deprivation, etc (Vousden and Prives 2009; Brady and Attardi 2010; Lazo and Santos 2011). p53 mediates the cellular responses to these stresses, and subsequently, it induces DNA repair, cell cycle arrest, apoptosis, or autophagy (see Figure 1.10; Schwartz and Rotter 1998; Chipuk and Green 2006; Brady and Attardi 2010). Mechanistically, p53 functions as a specific transcription regulator of its target genes, including p21 (cell cycle inhibitor), BAX and PUMA (cell death inductors), to mediate the downstream cellular effects from p53 activation (Prives and Hall 1999; Vousden and Lu 2002; Vousden and Prives 2009; Freed-Pastor and Prives 2012).

p53 is a short-lived transcription factor. Normally without stress in cells, the basal levels of p53 are very low. In response to stress, p53 is rapidly elevated and activated by post-translational phosphorylation which is mediated by different cellular kinases such as ataxia telangiectasia mutated (ATM), ATM related (ATR), casein kinase 1 (CK1) and several other kinases. The TAD1 at the N-terminus of p53 is always heavily modified by phosphorylation. It contains seven different residues targeted by kinases. p53 phosphorylation by different kinases with different phosphorylation patterns determine its interaction partners, either with transcriptional co-activators, such as p300/CRP and pCAF, or with its negative

regulators, such as Mdm2 (also known as Hdm2), PIRH2, COP1, and ARF-BP1 (Meek and Anderson 2009; Brady and Attardi 2010; Lazo and Santos 2011).

In response to DNA damage, activated p53 interacts with its transcriptional co-activators such as p300/CRP or pCAF. These transcriptional co-activators acetylate several lysine residues including K320, K373 and K382 at the C terminus of p53 (Gu and Roeder 1997; Sakaguchi, Herrera et al. 1998; Liu, Scolnick et al. 1999; Lazo and Santos 2011). The acetylation of p53 protein increases its affinity for DNA binding and results in activation of its target genes (Luo, Li et al. 2004). Also, these transcriptional co-activators acetylate histones on p53-dependent promoters, inducing DNA binding of p53 and thus up-regulating p53-dependent gene expression (Bannister and Kouzarides 1996; Ogryzko, Schiltz et al. 1996; Brown, Lechner et al. 2000; Hsu, Chang et al. 2004). The resulting gene products mediate cell cycle arrest (presumably for DNA repair) or apoptosis (in response of severe cellular stress).

In addition to stimulating transcription of its target genes, p53 can also repress gene expression either by associating with co-repressors such as Sin3a and histone deacetylases on the promoters of its target genes, or by blocking the binding sites of other transcriptional co-activators from binding to the target promoters (Brady and Attardi 2010).

High levels of p53 are deleterious for cells due to downstream outcomes. When the stress is gone, p53 induces the expression of Mdm2, a p53 negative regulator. Mdm2 is an E3 ubiquitin ligase that ubiquitylates the tetramerization

domain of p53 and delivers it to the proteosome for degradation. This p53-Mdm2 autoregulatory feedback loop turns off p53 and prevents it from further transactivation of its target genes that induce downstream cellular effects (Brooks and Gu 2006; Murray-Zmijewski, Slee et al. 2008; Lazo and Santos 2011).

To prevent degradation, p53 can be phosphorylated on residues Ser15, Thr18, or Ser20, which change the alpha helix of p53, preventing the recognition by Mdm2 and interaction with it, thus resulting in p53 accumulation in the nucleus (Kussie, Gorina et al. 1996; Lazo and Santos 2011). However, dephosphorylation of p53 promotes the interaction with Mdm2, subsequently resulting in p53 degradation through the ubiquitin-proteosome proteolytic pathway. This maintains p53 at a low level in cells (Brooks and Gu 2006; Lazo and Santos 2011).

The interaction between p53 and Mdm2 can also be changed by phosphorylation of Mdm2. However, the varied outcomes depend on the residues that are phosphorylated in Mdm2, which either facilitates the degradation of p53, or degradation of its own. Mdm2 phosphorylation is mediated by several cellular kinases. Some kinases, along with PI3K/Akt cell survival signaling, phosphorylate Mdm2 on residues Ser166 and Ser186 to facilitate p53 degradation within the nucleus. Other kinases, such as ATM and c-Abl which phosphorylate Mdm2 on Ser395 and Tyr394, respectively, induce Mdm2 degradation. This allows p53 elevation and accumulation to mediate the downstream outcomes (Khosravi, Maya et al. 1999; Goldberg, Vogt Sionov et al. 2002; Meek and Knippschild 2003; Laptenko and Prives 2006; Lazo and Santos 2011).

In response to severe stress, activated p53 promotes cell death by transcriptional activation of several pro-apoptotic genes, whose products include BAX and PUMA. PUMA is a p53 upregulated modulator of apoptosis. It is a member of the Bcl-2 family of apoptosis regulators, also known as Bcl-2 homology 3 (BH3)-only proteins. Activated PUMA interacts with anti-apoptotic Bcl-2 family members including Bcl-2, Bcl-X_L, and Mcl-1that bind to the pro-apoptotic effectors BAX and BAK, also members of Bcl-2 family (Yu and Zhang 2008). The interaction of PUMA and anti-apoptotic effectors releases the pro-apoptotic effectors. BAX and BAK then oligomerize to create pores in the mitochondrial outer membrane, promoting permeabilization of the membrane and inducing apoptosis (Vaseva and Moll 2009; Brady and Attardi 2010).

Under lower levels of stress such as repairable DNA damage, p53 induces cell cycle arrest for DNA repair, rather than apoptosis, by transcriptional activation of several target genes such as p21, PAI1, PML, cyclin G1, and GADD45 (Murray-Zmijewski, Slee et al. 2008; Riley, Sontag et al. 2008). p21 is an inhibitor of most cyclin dependent kinases (cdks) that complex with cyclins to regulate the progression of cell cycle in G0, G1, S, G2, and M phase. The members of the retinoblastoma (Rb) protein family, including Rb, p107, and p130, are involved in the regulation of the G1/S checkpoint. In their hypo-phosphorylated forms, Rb proteins physically bind to the transcription factor E2F, preventing it from transcription of its target genes whose products are normally involved in DNA synthesis. However, hyper-phosphorylation of Rb by cyclin D in combination of cdk4 or cdk6 (cyclin D/cdk4/6) releases E2F, activating the transcription of target genes,

including cyclin E that complexes with cdk2 to promote the transition into S phase. Cdk2/cyclin E is also able to phosphorylate Rb (Harbour, Luo et al. 1999) as a consequence of activation of E2F dependent-gene transcription and transition into S phase. Cdk2/cyclin A activates the firing of DNA origins of replication and initiation of DNA replication. To arrest the cell cycle, p53 activates the expression of p21. p21 effectively binds and inhibits the activity of cdk2 by blocking the interaction between the cdk2/cyclin E complex and its substrate Rb, thus, inhibiting the transition into S phase and arresting the cell cycle at G1/S phase (Abbas and Dutta 2009). p21 also inhibits cdk1, leading to cell cycle arrest at G2 phase (Abbas and Dutta 2009). The temporary pause of cell cycle progression mediated by p53 allows for DNA repair. In this aspect, p53 plays an important role in maintaining genomic stability and integrity.

1.6 Viral Mechanisms for Manipulation of p53-Mediated Responses

The cellular responses to stress are mediated by p53, either by cell cycle arrest or apoptosis. One of these cellular stresses is viral infection. For successful completion of their life cycles, viruses have developed strategies or mechanisms to bypass these p53-mediated responses. These viral mechanisms of interference with p53 mainly include direct viral protein interaction with p53 and degradation of p53 either by ubiquitination of p53 or activation of its negative regulatory proteins Hdm2 or Mdm2. Below I will introduce the mechanisms of p53 interference by multiple viruses.

Direct viral protein interaction with p53 is the simplest mechanism to inhibit p53 downstream activity. For instance, Large T antigen of Simian virus 40 (SV40) directly interacts with the p53 DNA binding domain, blocking p53 normal function and inhibiting p53-mediated responses (Reich and Levine 1982; Jiang, Srinivasan et al. 1993).

Adenoviruses encode a protein called E1B-55K. This protein binds to p53 through interaction with another adenoviral protein E4-ORF6. This leads to p53 ubiquitination and thus degradation (Steegenga, Riteco et al. 1998; Wienzek, Roth et al. 2000). E1B-55K can also directly interact with p53 in its N-terminus to block its transcriptional activation ability (Martin and Berk 1998; Martin and Berk 1999). It can also cause p53 to be sequestered into the cytoplasm (Zhao and Liao 2003). Moreover, E1B-55K directly blocks the interaction between p53 and the acetyl transferase pCAF, thus preventing p53 acetylation by pCAF (Liu, Colosimo et al. 2000). This leads to the reduction of Bax gene expression mediated by acetylated p53, and thus inhibition of apoptosis (Debbas and White 1993; Han, Sabbatini et al. 1996). More recently, a new adenoviral protein, E4-ORF3, was found to inactivate p53 independently of E1B-55K by forming a nuclear structure that induces de novo H3K9me3 (histone-3 methylation) in heterochromatin at the promoters of p53 transcriptional targets. This prevents p53 dependent transcription of target genes (Soria, Estermann et al. 2010).

The early viral protein E6 from human papillomavirus (HPV) strains 16 and 18 is an E3 ubiquitin ligase (Thomas, Matlashewski et al. 1996). E6 directly interacts with the transactivation domain of p53, followed by binding with the cellular

protein E6AP that functions as an E2 ligase, together leading to p53 ubiquitination and subsequent degradation by the proteasome (Scheffner, Werness et al. 1990; Thomas, Matlashewski et al. 1996; Cooper, Schneider et al. 2003). The loss of p53 results in loss of p53-mediated responses such as cell cycle arrest and apoptosis. Another viral protein E7 also plays a role in deregulation of the cell cycle by interaction with the retinoblastoma (Rb) tumor suppressor protein that inhibits cell cycle progression (Dyson, Howley et al. 1989; White, Livanos et al. 1994). The deregulation of the cell cycle by E6 is independent of E7 activity (Thomas and Laimins 1998).

Hepatitis B virus (HBV) protein X (HBV X) can strongly repress p53 gene expression (Lee and Rho 2000). This protein also directly interacts with p53 and inhibits p53-dependent transcriptional activity (Wang, Forrester et al. 1994). Interestingly, HBV X removes p53 from the nucleus and retains it in the cytoplasm, inhibiting p53 function in the nucleus where p53 induces cell cycle arrest (Takada, Kaneniwa et al. 1997; Murakami 2001). This action allows HBV to enter S phase for its genome replication, but it also abrogates the cellular response to DNA repair and thus contributes to HBV-induced oncogenesis (Doitsh and Shaul 1999).

The immediate early 2 (IE2) protein of human cytomegalovirus (HCMV) can physically bind to the C-terminus of p53 in vitro and in vivo transient expression assays (Speir, Modali et al. 1994; Tsai, Kou et al. 1996; Bonin and McDougall 1997; Wang, Marker et al. 2000; Song and Stinski 2005), which inhibits the binding of p53 to target promoters. IE2 also binds to the histone acetyltransferase (HAT) domain of p53 coactivators, p300 and CREB-binding protein (CBP), which blocks p53

acetylation and local histone acetylation on p53-dependent promoters, reducing DNA binding of p53 and thus down-regulating p53-dependent gene expression (Hsu, Chang et al. 2004).

IE2 also interacts with Mdm2 and facilitates its degradation (Zhang, Evers et al. 2006). This results in prevention of ubiquitination and subsequent degradation of p53 (Zhang, Evers et al. 2006; Chen, Knutson et al. 2007). p53 levels are elevated during HCMV infection (Muganda, Mendoza et al. 1994; Fortunato and Spector 1998; Muganda, Carrasco et al. 1998), but the protein does not activate the expression of its target genes, such as the cyclin/cdk inhibitor p21 (Muganda, Mendoza et al. 1994; Bresnahan, Boldogh et al. 1996; Fortunato and Spector 1998; Chen, Knutson et al. 2001).

The elevated and stabilized p53 is sequestered in viral replication centers within the nucleus (Fortunato and Spector 1998). The sequestration of p53 not only inhibits the p53-dependent downstream cellular effects, but it also benefits the activation of some viral genes. Several viral genes carrying p53 binding sites are specifically bound by p53 during HCMV infection. This implies that viral transcription may be activated by p53 (Rosenke, Samuel et al. 2006). In addition, p53 influences viral gene expression at early times of infection, either by direct or indirect interaction of p53 with the HCMV genome (Hannemann, Rosenke et al. 2009). Absence of p53 decreases the production of infectious viral particles by 25-fold at the late stages of infection. Also, the expression of both early and late viral proteins is delayed. Re-introduction of p53 recovers the viral production to near normal levels. p53 appears to enhance HCMV productivity (Casavant, Luo et al. 2006).

1.7 Research Objectives

The overall objective of this dissertation was to determine what caused the 25-fold reduction of infectious HCMV production at the late stages of infection in the absence of p53.

1.8 Research Methods

In order to study the role of p53 during HCMV infection, we used cells in which p53 was completely absent. By electron microscopy, we investigated several key events in the HCMV life cycle, including capsid production in the nucleus, DNA packaging into capsids, capsid egress from the nucleus, and capsid final envelopment in the cytoplasm. These are discussed in Chapter 2. One of the major issues for the decreased HCMV production was caused by impaired nuclear egress of HCMV. In Chapter 3, we further investigated several of the players involved in the nuclear trafficking, including UL97, UL50, and UL53, by examining their expression, localization, or functionality in cells without p53. Finally, we re-introduced p53 back into those cells without p53 and examined the recovery levels of any defective parameters. This determined whether the impaired events were caused by the absence of p53.

1.9 Research Significance

The significance of this research will not only lead to a greater insight into the role of p53 during herpesvirus development but also give us a greater understanding of p53's functions inside the cell.

References

Abbas, T. and A. Dutta (2009). "p21 in cancer: intricate networks and multiple activities." Nature reviews. Cancer **9**(6): 400-414.

Adler, B., L. Scrivano, et al. (2006). "Role of human cytomegalovirus UL131A in cell type-specific virus entry and release." The Journal of general virology **87**(Pt 9): 2451-2460.

Adler, B. and C. Sinzger (2013). Cytomegalovirus Interstrain Variance in Cell Type Tropism. Cytomegaloviruses: From Molecular Pathogenesis to Intervention. M. J. Reddehase and N. A. W. Lemmermann. Norfolk, UK, Caister Academic Press. **1:** 297-321.

Ahlqvist, J. and E. Mocarski (2011). "Cytomegalovirus UL103 controls virion and dense body egress." Journal of virology **85**(10): 5125-5135.

Arcangeletti, M. C., I. Rodighiero, et al. (2011). "Cell-cycle-dependent localization of human cytomegalovirus UL83 phosphoprotein in the nucleolus and modulation of viral gene expression in human embryo fibroblasts in vitro." Journal of cellular biochemistry **112**(1): 307-317.

Arvin, A. M. (2001). Varicella-Zoster Virus. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2731-2767.

AuCoin, D. P., G. B. Smith, et al. (2006). "Betaherpesvirus-conserved cytomegalovirus tegument protein ppUL32 (pp150) controls cytoplasmic events during virion maturation." Journal of virology **80**(16): 8199-8210.

Bannister, A. J. and T. Kouzarides (1996). "The CBP co-activator is a histone acetyltransferase." Nature **384**(6610): 641-643.

Baum, E. Z., G. A. Bebernitz, et al. (1993). "Expression and analysis of the human cytomegalovirus UL80-encoded protease: identification of autoproteolytic sites." Journal of virology **67**(1): 497-506.

Baxter, M. K. and W. Gibson (2001). "Cytomegalovirus basic phosphoprotein (pUL32) binds to capsids in vitro through its amino one-third." Journal of virology **75**(15): 6865-6873.

Bigalke, J. M., T. Heuser, et al. (2014). "Membrane deformation and scission by the HSV-1 nuclear egress complex." Nature communications **5**: 4131.

Biron, K. K. (2006). "Antiviral drugs for cytomegalovirus diseases." Antiviral research **71**(2-3): 154-163.

Black, J. B. and P. E. Pellett (1999). "Human herpesvirus 7." Reviews in medical virology **9**(4): 245-262.

Blauvelt, A. (2001). "Skin diseases associated with human herpesvirus 6, 7, and 8 infection." The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research **6**(3): 197-202.

Bogner, E. (2002). "Human cytomegalovirus terminase as a target for antiviral chemotherapy." Reviews in medical virology **12**(2): 115-127.

Bogner, E., K. Radsak, et al. (1998). "The gene product of human cytomegalovirus open reading frame UL56 binds the pac motif and has specific nuclease activity." J Virol **72**(3): 2259-2264.

Bonin, L. R. and J. K. McDougall (1997). "Human cytomegalovirus IE2 86-kilodalton protein binds p53 but does not abrogate G1 checkpoint function." Journal of virology **71**(8): 5861-5870.

Boppana, S. B. and W. J. Britt (1995). "Antiviral antibody responses and intrauterine transmission after primary maternal cytomegalovirus infection." The Journal of infectious diseases **171**(5): 1115-1121.

Boppana, S. B., K. B. Fowler, et al. (1999). "Symptomatic congenital cytomegalovirus infection in infants born to mothers with preexisting immunity to cytomegalovirus." Pediatrics **104**(1 Pt 1): 55-60.

Borst, E. M., J. Kleine-Albers, et al. (2013). "The human cytomegalovirus UL51 protein is essential for viral genome cleavage-packaging and interacts with the terminase subunits pUL56 and pUL89." Journal of virology **87**(3): 1720-1732.

Borza, C. M., A. J. Morgan, et al. (2004). "Use of gHgL for attachment of Epstein-Barr virus to epithelial cells compromises infection." Journal of virology **78**(10): 5007-5014.

Boyle, K. A. and T. Compton (1998). "Receptor-binding properties of a soluble form of human cytomegalovirus glycoprotein B." Journal of virology **72**(3): 1826-1833.

Brady, C. A. and L. D. Attardi (2010). "p53 at a glance." Journal of cell science **123**(Pt 15): 2527-2532.

Braun, D. K., G. Dominguez, et al. (1997). "Human herpesvirus 6." Clinical microbiology reviews **10**(3): 521-567.

Bresnahan, W. A., I. Boldogh, et al. (1996). "Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1." Virology **224**(1): 150-160.

Brignole, E. J. and W. Gibson (2007). "Enzymatic activities of human cytomegalovirus maturational protease assemblin and its precursor (pPR, pUL80a) are comparable: [corrected] maximal activity of pPR requires self-interaction through its scaffolding domain." Journal of virology **81**(8): 4091-4103.

Britt, W. J. (1984). "Neutralizing antibodies detect a disulfide-linked glycoprotein complex within the envelope of human cytomegalovirus." Virology **135**(2): 369-378.

Britt, W. J. and D. Auger (1986). "Human cytomegalovirus virion-associated protein with kinase activity." Journal of virology **59**(1): 185-188.

Brooks, C. L. and W. Gu (2006). "p53 ubiquitination: Mdm2 and beyond." Molecular cell **21**(3): 307-315.

Brown, C. E., T. Lechner, et al. (2000). "The many HATs of transcription coactivators." Trends in biochemical sciences **25**(1): 15-19.

Brown, J. C. and W. W. Newcomb (2011). "Herpesvirus capsid assembly: insights from structural analysis." Current opinion in virology **1**(2): 142-149.

Buchkovich, N. J., T. G. Maguire, et al. (2010). "Role of the endoplasmic reticulum chaperone BiP, SUN domain proteins, and dynein in altering nuclear morphology during human cytomegalovirus infection." Journal of virology **84**(14): 7005-7017.

Buchkovich, N. J., T. G. Maguire, et al. (2009). "The endoplasmic reticulum chaperone BiP/GRP78 is important in the structure and function of the human cytomegalovirus assembly compartment." Journal of virology **83**(22): 11421-11428.

Burck, P. J., D. H. Berg, et al. (1994). "Human cytomegalovirus maturational proteinase: expression in Escherichia coli, purification, and enzymatic characterization by using peptide substrate mimics of natural cleavage sites." Journal of virology **68**(5): 2937-2946.

Buser, C., P. Walther, et al. (2007). "Cytomegalovirus primary envelopment occurs at large infoldings of the inner nuclear membrane." Journal of virology **81**(6): 3042-3048.

Butcher, S. J., J. Aitken, et al. (1998). "Structure of the human cytomegalovirus B capsid by electron cryomicroscopy and image reconstruction." Journal of structural biology **124**(1): 70-76.

Camozzi, D., S. Pignatelli, et al. (2008). "Remodelling of the nuclear lamina during human cytomegalovirus infection: role of the viral proteins pUL50 and pUL53." The Journal of general virology **89**(Pt 3): 731-740.

Cantrell, S. R. and W. A. Bresnahan (2005). "Interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early gene expression and viral replication." Journal of virology **79**(12): 7792-7802.

Carlson, C., W. J. Britt, et al. (1997). "Expression, purification, and characterization of a soluble form of human cytomegalovirus glycoprotein B." Virology **239**(1): 198-205.

Casavant, N. C., M. H. Luo, et al. (2006). "Potential role for p53 in the permissive life cycle of human cytomegalovirus." Journal of virology **80**(17): 8390-8401.

Cepeda, V., M. Esteban, et al. (2010). "Human cytomegalovirus final envelopment on membranes containing both trans-Golgi network and endosomal markers." Cellular microbiology **12**(3): 386-404.

Chan, C. K., E. J. Brignole, et al. (2002). "Cytomegalovirus assemblin (pUL80a): cleavage at internal site not essential for virus growth; proteinase absent from virions." Journal of virology **76**(17): 8667-8674.

Chan, G., M. T. Nogalski, et al. (2009). "Activation of EGFR on monocytes is required for human cytomegalovirus entry and mediates cellular motility." Proc Natl Acad Sci U S A **106**(52): 22369-22374.

Chayavichitsilp, P., J. V. Buckwalter, et al. (2009). "Herpes simplex." Pediatrics in review / American Academy of Pediatrics **30**(4): 119-129; quiz 130.

Chen, Z., E. Knutson, et al. (2001). "Degradation of p21cip1 in cells productively infected with human cytomegalovirus." Journal of virology **75**(8): 3613-3625.

Chen, Z., E. Knutson, et al. (2007). "Stabilization of p53 in human cytomegalovirus-initiated cells is associated with sequestration of HDM2 and decreased p53 ubiquitination." The Journal of biological chemistry **282**(40): 29284-29295.

Chesnokova, L. S. and L. M. Hutt-Fletcher (2011). "Fusion of Epstein-Barr virus with epithelial cells can be triggered by alphavbeta5 in addition to alphavbeta6 and alphavbeta8, and integrin binding triggers a conformational change in glycoproteins gHgL." Journal of virology **85**(24): 13214-13223.

Chesnokova, L. S., S. L. Nishimura, et al. (2009). "Fusion of epithelial cells by Epstein-Barr virus proteins is triggered by binding of viral glycoproteins gHgL to integrins alphavbeta6 or alphavbeta8." Proceedings of the National Academy of Sciences of the United States of America **106**(48): 20464-20469.

Chevillotte, M., S. Landwehr, et al. (2009). "Major tegument protein pp65 of human cytomegalovirus is required for the incorporation of pUL69 and pUL97 into the virus particle and for viral growth in macrophages." Journal of virology **83**(6): 2480-2490.

Chipuk, J. E. and D. R. Green (2006). "Dissecting p53-dependent apoptosis." Cell death and differentiation **13**(6): 994-1002.

Cinque, P., R. Marenzi, and D. Ceresa (1997). "Cytomegalovirus infections of the nervous system." Intervirology **40**: 85-97.

Cohen, J. I., and S. E. Straus (2001). Varicella-Zoster Virus and Its Replication. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2707-2730.

Compton, T. (2004). "Receptors and immune sensors: the complex entry path of human cytomegalovirus." Trends in cell biology **14**(1): 5-8.

Compton, T., D. M. Nowlin, et al. (1993). "Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate." Virology **193**(2): 834-841.

Connolly, S. A., J. O. Jackson, et al. (2011). "Fusing structure and function: a structural view of the herpesvirus entry machinery." Nature reviews. Microbiology **9**(5): 369-381.

Cooper, B., S. Schneider, et al. (2003). "Requirement of E6AP and the features of human papillomavirus E6 necessary to support degradation of p53." Virology **306**(1): 87-99.

Cui, X., A. McGregor, et al. (2009). "The impact of genome length on replication and genome stability of the herpesvirus guinea pig cytomegalovirus." Virology **386**(1): 132-138.

Dai, X., X. Yu, et al. (2013). "The smallest capsid protein mediates binding of the essential tegument protein pp150 to stabilize DNA-containing capsids in human cytomegalovirus." PLoS pathogens **9**(8): e1003525.

Dal Monte, P., S. Pignatelli, et al. (2002). "Analysis of intracellular and intraviral localization of the human cytomegalovirus UL53 protein." The Journal of general virology **83**(Pt 5): 1005-1012.

Das, S. and P. E. Pellett (2011). "Spatial relationships between markers for secretory and endosomal machinery in human cytomegalovirus-infected cells versus those in uninfected cells." Journal of virology **85**(12): 5864-5879.

Das, S., A. Vasanji, et al. (2007). "Three-dimensional structure of the human cytomegalovirus cytoplasmic virion assembly complex includes a reoriented secretory apparatus." Journal of virology **81**(21): 11861-11869.

Davison, A. J. (2007). Comparative analysis of the genomes. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. C.-F. G. Arvin A., Mocarski E., Moore, P. S., Roizman, B., Whitley, R., and Yamanishi, K Cambridge, Cambridge University Press**:** Chapter 2.

Debbas, M. and E. White (1993). "Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B." Genes & development **7**(4): 546-554.

Dechat, T., K. Pfleghaar, et al. (2008). "Nuclear lamins: major factors in the structural organization and function of the nucleus and chromatin." Genes Dev **22**(7): 832-853.

Dilanni, C. L., D. A. Drier, et al. (1993). "Identification of the herpes simplex virus-1 protease cleavage sites by direct sequence analysis of autoproteolytic cleavage products." The Journal of biological chemistry **268**(3): 2048-2051.

Dittmer, A. and E. Bogner (2005). "Analysis of the quaternary structure of the putative HCMV portal protein PUL104." Biochemistry **44**(2): 759-765.

Dittmer, A., J. C. Drach, et al. (2005). "Interaction of the putative human cytomegalovirus portal protein pUL104 with the large terminase subunit pUL56 and its inhibition by benzimidazole-D-ribonucleosides." J Virol **79**(23): 14660-14667.

Doitsh, G. and Y. Shaul (1999). "HBV transcription repression in response to genotoxic stress is p53-dependent and abrogated by pX." Oncogene **18**(52): 7506-7513.

Dunn, W., C. Chou, et al. (2003). "Functional profiling of a human cytomegalovirus genome." Proceedings of the National Academy of Sciences of the United States of America **100**(24): 14223-14228.

Dutta, A., J. M. Ruppert, et al. (1993). "Inhibition of DNA replication factor RPA by p53." Nature **365**(6441): 79-82.

Dyson, N., P. M. Howley, et al. (1989). "The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product." Science **243**(4893): 934-937.

el-Deiry, W. S., S. E. Kern, et al. (1992). "Definition of a consensus binding site for p53." Nat Genet **1**(1): 45-49.

Feire, A. L. and T. Compton (2013). Virus Entry and Activation of Innate Defence. Cytomegaloviruses: From Molecular Pathogenesis to Intervention. M. J. Reddehase and N. A. W. Lemmermann. Norfolk, UK, Caister Academic Press. **1:** 125-140.

Feire, A. L., H. Koss, et al. (2004). "Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain." Proceedings of the National Academy of Sciences of the United States of America **101**(43): 15470-15475.

Feire, A. L., R. M. Roy, et al. (2010). "The glycoprotein B disintegrin-like domain binds beta 1 integrin to mediate cytomegalovirus entry." Journal of virology **84**(19): 10026-10037.

Fernandes, S. M., E. J. Brignole, et al. (2011). "Cytomegalovirus capsid protease: biological substrates are cleaved more efficiently by full-length enzyme (pUL80a) than by the catalytic domain (assemblin)." Journal of virology **85**(7): 3526-3534.

Fornara, C., D. Lilleri, et al. (2011). "Kinetics of effector functions and phenotype of virus-specific and gammadelta T lymphocytes in primary human cytomegalovirus infection during pregnancy." Journal of clinical immunology **31**(6): 1054-1064.

Fortunato, E. A. and D. H. Spector (1998). "p53 and RPA are sequestered in viral replication centers in the nuclei of cells infected with human cytomegalovirus." Journal of virology **72**(3): 2033-2039.

Fowler, K. B., F. P. McCollister, et al. (1997). "Progressive and fluctuating sensorineural hearing loss in children with asymptomatic congenital cytomegalovirus infection." The Journal of pediatrics **130**(4): 624-630.

Fowler, K. B., S. Stagno, et al. (1992). "The outcome of congenital cytomegalovirus infection in relation to maternal antibody status." The New England journal of medicine **326**(10): 663-667.

Freed-Pastor, W. A. and C. Prives (2012). "Mutant p53: one name, many proteins." Genes & development **26**(12): 1268-1286.

Gamper, A. M. and R. G. Roeder (2008). "Multivalent binding of p53 to the STAGA complex mediates coactivator recruitment after UV damage." Molecular and cellular biology **28**(8): 2517-2527.

Gaytant, M. A., E. A. Steegers, et al. (2002). "Congenital cytomegalovirus infection: review of the epidemiology and outcome." Obstetrical & gynecological survey **57**(4): 245-256.

Gibson, W. (1996). "Structure and assembly of the virion." Intervirology **39**(5-6): 389-400.

Gibson, W. (2008). "Structure and formation of the cytomegalovirus virion." Current topics in microbiology and immunology **325**: 187-204.

Gibson, W. and E. Bogner (2013). Morphogenesis of the Cytomegalovirus Virion and Subviral Particles. Cytomegaloviruses: From Molecular Pathogenesis to Intervention. M. J. Reddehase and N. A. W. Lemmermann. Norfolk, UK, Caister Academic Press. **1:** 230-246.

Gibson, W. and A. Irmiere (1984). "Selection of particles and proteins for use as human cytomegalovirus subunit vaccines." Birth defects original article series **20**(1): 305-324.

Gilloteaux, J. and M. R. Nassiri (2000). "Human bone marrow fibroblasts infected by cytomegalovirus: ultrastructural observations." Journal of submicroscopic cytology and pathology **32**(1): 17-45.

Goldberg, Z., R. Vogt Sionov, et al. (2002). "Tyrosine phosphorylation of Mdm2 by c-Abl: implications for p53 regulation." The EMBO journal **21**(14): 3715-3727.

Goldman, R. D., Y. Gruenbaum, et al. (2002). "Nuclear lamins: building blocks of nuclear architecture." Genes Dev **16**(5): 533-547.

Grosse, S. D., D. S. Ross, et al. (2008). "Congenital cytomegalovirus (CMV) infection as a cause of permanent bilateral hearing loss: a quantitative assessment." Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology **41**(2): 57-62.

Gu, W. and R. G. Roeder (1997). "Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain." Cell **90**(4): 595-606.

Halwachs-Baumann, G. (2011). Virus-host interaction for defence and transmission. Congenital Cytomegalovirus Infection: Epidemiology, Diagnosis, Therapy. G. Halwachs-Baumann, SpringerWienNewYork**:** 15.

Hamirally, S., J. P. Kamil, et al. (2009). "Viral mimicry of Cdc2/cyclin-dependent kinase 1 mediates disruption of nuclear lamina during human cytomegalovirus nuclear egress." PLoS pathogens **5**(1): e1000275.

Han, J., P. Sabbatini, et al. (1996). "The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein." Genes & development **10**(4): 461-477.

Hannemann, H., K. Rosenke, et al. (2009). "The presence of p53 influences the expression of multiple human cytomegalovirus genes at early times postinfection." Journal of virology **83**(9): 4316-4325.

Harbour, J. W., R. X. Luo, et al. (1999). "Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1." Cell **98**(6): 859-869.

Hensel, G., H. Meyer, et al. (1995). "Nuclear localization of the human cytomegalovirus tegument protein pp150 (ppUL32)." The Journal of general virology **76 (Pt 7)**: 1591-1601.

Holzenburg, A., A. Dittmer, et al. (2009). "Assembly of monomeric human cytomegalovirus pUL104 into portal structures." J Gen Virol **90**(Pt 10): 2381-2385.

Hsu, C. H., M. D. Chang, et al. (2004). "HCMV IE2-mediated inhibition of HAT activity downregulates p53 function." The EMBO journal **23**(11): 2269-2280.

Huber, M. T. and T. Compton (1997). "Characterization of a novel third member of the human cytomegalovirus glycoprotein H-glycoprotein L complex." Journal of virology **71**(7): 5391-5398.

Huber, M. T. and T. Compton (1998). "The human cytomegalovirus UL74 gene encodes the third component of the glycoprotein H-glycoprotein L-containing envelope complex." Journal of virology **72**(10): 8191-8197.

Hutt-Fletcher, L. M. (2007). "Epstein-Barr virus entry." Journal of virology **81**(15): 7825-7832.

Hwang, J. S. and E. Bogner (2002). "ATPase activity of the terminase subunit pUL56 of human cytomegalovirus." J Biol Chem **277**(9): 6943-6948.

Ibanez, C. E., R. Schrier, et al. (1991). "Human cytomegalovirus productively infects primary differentiated macrophages." Journal of virology **65**(12): 6581-6588.

Indran, S. V., M. E. Ballestas, et al. (2010). "Bicaudal D1-dependent trafficking of human cytomegalovirus tegument protein pp150 in virus-infected cells." Journal of virology **84**(7): 3162-3177.

Indran, S. V. and W. J. Britt (2011). "A role for the small GTPase Rab6 in assembly of human cytomegalovirus." Journal of virology **85**(10): 5213-5219.

Irmiere, A. and W. Gibson (1983). "Isolation and characterization of a noninfectious virion-like particle released from cells infected with human strains of cytomegalovirus." Virology **130**(1): 118-133.

Irmiere, A. and W. Gibson (1985). "Isolation of human cytomegalovirus intranuclear capsids, characterization of their protein constituents, and demonstration that the B-capsid assembly protein is also abundant in noninfectious enveloped particles." Journal of virology **56**(1): 277-283.

Isaacson, M. K. and T. Compton (2009). "Human cytomegalovirus glycoprotein B is required for virus entry and cell-to-cell spread but not for virion attachment, assembly, or egress." Journal of virology **83**(8): 3891-3903.

Isaacson, M. K., A. L. Feire, et al. (2007). "Epidermal growth factor receptor is not required for human cytomegalovirus entry or signaling." Journal of virology **81**(12): 6241-6247.

Isaacson, M. K., L. K. Juckem, et al. (2008). "Virus entry and innate immune activation." Current topics in microbiology and immunology **325**: 85-100.

Jacob, R. J., L. S. Morse, et al. (1979). "Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA." Journal of virology **29**(2): 448-457.

Jahn, G., B. C. Scholl, et al. (1987). "The two major structural phosphoproteins (pp65 and pp150) of human cytomegalovirus and their antigenic properties." The Journal of general virology **68 (Pt 5)**: 1327-1337.

Jiang, D., A. Srinivasan, et al. (1993). "SV40 T antigen abrogates p53-mediated transcriptional activity." Oncogene **8**(10): 2805-2812.

Jiang, X. J., B. Adler, et al. (2008). "UL74 of human cytomegalovirus contributes to virus release by promoting secondary envelopment of virions." Journal of virology **82**(6): 2802-2812.

Johnson, D. C. and J. D. Baines (2011). "Herpesviruses remodel host membranes for virus egress." Nature reviews. Microbiology **9**(5): 382-394.

Jones, T. R., L. Sun, et al. (1994). "Proteolytic activity of human cytomegalovirus UL80 protease cleavage site mutants." Journal of virology **68**(6): 3742-3752.

Kalejta, R. F. (2008). "Functions of human cytomegalovirus tegument proteins prior to immediate early gene expression." Current topics in microbiology and immunology **325**: 101-115.

Kalejta, R. F. (2008). "Tegument proteins of human cytomegalovirus." Microbiology and molecular biology reviews : MMBR **72**(2): 249-265, table of contents.

Kalejta, R. F., J. T. Bechtel, et al. (2003). "Human cytomegalovirus pp71 stimulates cell cycle progression by inducing the proteasome-dependent degradation of the retinoblastoma family of tumor suppressors." Molecular and cellular biology **23**(6): 1885-1895.

Kalejta, R. F. and T. Shenk (2003). "Proteasome-dependent, ubiquitin-independent degradation of the Rb family of tumor suppressors by the human

cytomegalovirus pp71 protein." Proceedings of the National Academy of Sciences of the United States of America **100**(6): 3263-3268.

Kari, B. and R. Gehrz (1992). "A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope." Journal of virology **66**(3): 1761-1764.

Kari, B. and R. Gehrz (1993). "Structure, composition and heparin binding properties of a human cytomegalovirus glycoprotein complex designated gC-II." The Journal of general virology **74 (Pt 2)**: 255-264.

Karlin, S., E. S. Mocarski, et al. (1994). "Molecular evolution of herpesviruses: genomic and protein sequence comparisons." Journal of virology **68**(3): 1886-1902.

Keay, S. and B. Baldwin (1991). "Anti-idiotype antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment." Journal of virology **65**(9): 5124-5128.

Kennard, J., F. J. Rixon, et al. (1995). "The 25 amino acid residues at the carboxy terminus of the herpes simplex virus type 1 UL26.5 protein are required for the formation of the capsid shell around the scaffold." The Journal of general virology **76 (Pt 7)**: 1611-1621.

Khosravi, R., R. Maya, et al. (1999). "Rapid ATM-dependent phosphorylation of MDM2 precedes p53 accumulation in response to DNA damage." Proceedings of the National Academy of Sciences of the United States of America **96**(26): 14973-14977.

Kieff, E., and A. B. Rickinson (2001). Epstein-Barr Virus and Its Replication. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2511-2573.

Klages, S., B. Ruger, et al. (1989). "Multiplicity dependent expression of the predominant phosphoprotein pp65 of human cytomegalovirus." Virus Res **12**(2): 159-168.

Klupp, B. G., H. Granzow, et al. (2011). "Nuclear envelope breakdown can substitute for primary envelopment-mediated nuclear egress of herpesviruses." Journal of virology **85**(16): 8285-8292.

Komazin, G., L. B. Townsend, et al. (2004). "Role of a mutation in human cytomegalovirus gene UL104 in resistance to benzimidazole ribonucleosides." J Virol **78**(2): 710-715.

Krosky, P. M., M. C. Baek, et al. (2003). "The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress." Journal of virology **77**(2): 905-914.

Krzyzaniak, M. A., M. Mach, et al. (2009). "HCMV-encoded glycoprotein M (UL100) interacts with Rab11 effector protein FIP4." Traffic **10**(10): 1439-1457.

Kussie, P. H., S. Gorina, et al. (1996). "Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain." Science **274**(5289): 948-953.

Landini, M. P., M. C. Re, et al. (1985). "Human immune response to cytomegalovirus structural polypeptides studied by immunoblotting." J Med Virol **17**(4): 303-311.

Lane, D. P. (1992). "Cancer. p53, guardian of the genome." Nature **358**(6381): 15-16.

Laptenko, O. and C. Prives (2006). "Transcriptional regulation by p53: one protein, many possibilities." Cell Death Differ **13**(6): 951-961.

Lazo, P. A. and C. R. Santos (2011). "Interference with p53 functions in human viral infections, a target for novel antiviral strategies?" Reviews in medical virology.

Lee, J. Y., A. Irmiere, et al. (1988). "Primate cytomegalovirus assembly: evidence that DNA packaging occurs subsequent to B capsid assembly." Virology **167**(1): 87-96.

Lee, S. G. and H. M. Rho (2000). "Transcriptional repression of the human p53 gene by hepatitis B viral X protein." Oncogene **19**(3): 468-471.

Leelawong, M., D. Guo, et al. (2011). "A physical link between the pseudorabies virus capsid and the nuclear egress complex." Journal of virology **85**(22): 11675-11684.

Levine, A. J. and M. Oren (2009). "The first 30 years of p53: growing ever more complex." Nature reviews. Cancer **9**(10): 749-758.

Li, L., J. A. Nelson, et al. (1997). "Glycoprotein H-related complexes of human cytomegalovirus: identification of a third protein in the gCIII complex." Journal of virology **71**(4): 3090-3097.

Li, R. and M. R. Botchan (1993). "The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein A and stimulate in vitro BPV-1 DNA replication." Cell **73**(6): 1207-1221.

Liu, B. and M. F. Stinski (1992). "Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 cis-acting elements." Journal of virology **66**(7): 4434-4444.

Liu, L., D. M. Scolnick, et al. (1999). "p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage." Molecular and cellular biology **19**(2): 1202-1209.

Liu, S. T., R. Sharon-Friling, et al. (2011). "Synaptic vesicle-like lipidome of human cytomegalovirus virions reveals a role for SNARE machinery in virion egress." Proceedings of the National Academy of Sciences of the United States of America **108**(31): 12869-12874.

Liu, X., C. W. Miller, et al. (1993). "The p53 activation domain binds the TATA box-binding polypeptide in Holo-TFIID, and a neighboring p53 domain inhibits transcription." Molecular and cellular biology **13**(6): 3291-3300.

Liu, Y., A. L. Colosimo, et al. (2000). "Adenovirus E1B 55-kilodalton oncoprotein inhibits p53 acetylation by PCAF." Molecular and cellular biology **20**(15): 5540-5553.

Lopper, M. and T. Compton (2004). "Coiled-coil domains in glycoproteins B and H are involved in human cytomegalovirus membrane fusion." Journal of virology **78**(15): 8333-8341.

Loveland, A. N., C. K. Chan, et al. (2005). "Cleavage of human cytomegalovirus protease pUL80a at internal and cryptic sites is not essential but enhances infectivity." Journal of virology **79**(20): 12961-12968.

Loveland, A. N., N. L. Nguyen, et al. (2007). "The amino-conserved domain of human cytomegalovirus UL80a proteins is required for key interactions during early stages of capsid formation and virus production." Journal of virology **81**(2): 620-628.

Luo, J., M. Li, et al. (2004). "Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo." Proceedings of the National Academy of Sciences of the United States of America **101**(8): 2259-2264.

Luxton, G. W., S. Haverlock, et al. (2005). "Targeting of herpesvirus capsid transport in axons is coupled to association with specific sets of tegument proteins." Proceedings of the National Academy of Sciences of the United States of America **102**(16): 5832-5837.

Mach, M., B. Kropff, et al. (2000). "Complex formation by human cytomegalovirus glycoproteins M (gpUL100) and N (gpUL73)." Journal of virology **74**(24): 11881-11892.

Macsween, K. F. and D. H. Crawford (2003). "Epstein-Barr virus-recent advances." The Lancet infectious diseases **3**(3): 131-140.

Marschall, M., A. Marzi, et al. (2005). "Cellular p32 recruits cytomegalovirus kinase pUL97 to redistribute the nuclear lamina." The Journal of biological chemistry **280**(39): 33357-33367.

Martin, M. E. and A. J. Berk (1998). "Adenovirus E1B 55K represses p53 activation in vitro." Journal of virology **72**(4): 3146-3154.

Martin, M. E. and A. J. Berk (1999). "Corepressor required for adenovirus E1B 55,000-molecular-weight protein repression of basal transcription." Molecular and cellular biology **19**(5): 3403-3414.

Matsuura, H., A. N. Kirschner, et al. (2010). "Crystal structure of the Epstein-Barr virus (EBV) glycoprotein H/glycoprotein L (gH/gL) complex." Proceedings of the National Academy of Sciences of the United States of America **107**(52): 22641-22646.

Matusick-Kumar, L., P. J. McCann, 3rd, et al. (1995). "Release of the catalytic domain N(o) from the herpes simplex virus type 1 protease is required for viral growth." Journal of virology **69**(11): 7113-7121.

May, P. and E. May (1999). "Twenty years of p53 research: structural and functional aspects of the p53 protein." Oncogene **18**(53): 7621-7636.

Meek, D. W. and C. W. Anderson (2009). "Posttranslational modification of p53: cooperative integrators of function." Cold Spring Harbor perspectives in biology **1**(6): a000950.

Meek, D. W. and U. Knippschild (2003). "Posttranslational modification of MDM2." Molecular cancer research : MCR **1**(14): 1017-1026.

Meissner, C. S., S. Suffner, et al. (2012). "A leucine zipper motif of a tegument protein triggers final envelopment of human cytomegalovirus." Journal of virology **86**(6): 3370-3382.

Mettenleiter, T. C., B. G. Klupp, et al. (2009). "Herpesvirus assembly: an update." Virus research **143**(2): 222-234.

Meyer, H., A. T. Bankier, et al. (1988). "Identification and procaryotic expression of the gene coding for the highly immunogenic 28-kilodalton structural phosphoprotein (pp28) of human cytomegalovirus." Journal of virology **62**(7): 2243-2250.

Milbradt, J., S. Auerochs, et al. (2009). "Cytomegaloviral proteins that associate with the nuclear lamina: components of a postulated nuclear egress complex." The Journal of general virology **90**(Pt 3): 579-590.

Milbradt, J., A. Kraut, et al. (2014). "Proteomic analysis of the multimeric nuclear egress complex of human cytomegalovirus." Molecular & cellular proteomics : MCP **13**(8): 2132-2146.

Milbradt, J., R. Webel, et al. (2010). "Novel mode of phosphorylation-triggered reorganization of the nuclear lamina during nuclear egress of human cytomegalovirus." The Journal of biological chemistry **285**(18): 13979-13989.

Miller, M. S., W. E. Furlong, et al. (2010). "RASCAL is a new human cytomegalovirus-encoded protein that localizes to the nuclear lamina and in cytoplasmic vesicles at late times postinfection." Journal of virology **84**(13): 6483-6496.

Miller, N. and L. M. Hutt-Fletcher (1992). "Epstein-Barr virus enters B cells and epithelial cells by different routes." Journal of virology **66**(6): 3409-3414.

Mocarski, E. S., and C.T. Courcelle (2001). Cytomegaloviruses and Their Replication. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2629-2673.

Mocarski, E. S., A. C. Liu, et al. (1987). "Structure and variability of the a sequence in the genome of human cytomegalovirus (Towne strain)." J Gen Virol **68 (Pt 8)**: 2223-2230.

Moore, P. S., and Y. Chang (2001). Kaposi's Sarcoma-Associated Herpesvirus. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2803-2833.

Muganda, P., R. Carrasco, et al. (1998). "The human cytomegalovirus IE2 86 kDa protein elevates p53 levels and transactivates the p53 promoter in human fibroblasts." Cellular and molecular biology **44**(2): 321-331.

Muganda, P., O. Mendoza, et al. (1994). "Human cytomegalovirus elevates levels of the cellular protein p53 in infected fibroblasts." Journal of virology **68**(12): 8028-8034.

Murakami, S. (2001). "Hepatitis B virus X protein: a multifunctional viral regulator." Journal of gastroenterology **36**(10): 651-660.

Muranyi, W., J. Haas, et al. (2002). "Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina." Science **297**(5582): 854-857.

Murray-Zmijewski, F., E. A. Slee, et al. (2008). "A complex barcode underlies the heterogeneous response of p53 to stress." Nature reviews. Molecular cell biology **9**(9): 702-712.

Myerson, D., R. C. Hackman, et al. (1984). "Widespread presence of histologically occult cytomegalovirus." Human pathology **15**(5): 430-439.

Naldinho-Souto, R., H. Browne, et al. (2006). "Herpes simplex virus tegument protein VP16 is a component of primary enveloped virions." Journal of virology **80**(5): 2582-2584.

Netterwald, J. R., T. R. Jones, et al. (2004). "Postattachment events associated with viral entry are necessary for induction of interferon-stimulated genes by human cytomegalovirus." Journal of virology **78**(12): 6688-6691.

Newcomb, W. W., S. K. Cockrell, et al. (2009). "Polarized DNA ejection from the herpesvirus capsid." Journal of molecular biology **392**(4): 885-894.

Nguyen, N. L., A. N. Loveland, et al. (2008). "Nuclear localization sequences in cytomegalovirus capsid assembly proteins (UL80 proteins) are required for virus production: inactivating NLS1, NLS2, or both affects replication to strikingly different extents." Journal of virology **82**(11): 5381-5389.

Ogryzko, V. V., R. L. Schiltz, et al. (1996). "The transcriptional coactivators p300 and CBP are histone acetyltransferases." Cell **87**(5): 953-959.

Oien, N. L., D. R. Thomsen, et al. (1997). "Assembly of herpes simplex virus capsids using the human cytomegalovirus scaffold protein: critical role of the C terminus." J Virol **71**(2): 1281-1291.

Padula, M. E., M. L. Sydnor, et al. (2009). "Isolation and preliminary characterization of herpes simplex virus 1 primary enveloped virions from the perinuclear space." Journal of virology **83**(10): 4757-4765.

Pante, N. and M. Kann (2002). "Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm." Molecular biology of the cell **13**(2): 425-434.

Papadimitriou, J. M., G. R. Shellam, et al. (1984). "An ultrastructural investigation of cytomegalovirus replication in murine hepatocytes." The Journal of general virology **65 (Pt 11)**: 1979-1990.

Pass, R. F. (2001). Cytomegalovirus. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2675-2705.

Pellett, P. E., and G. Dominguez (2001). Human Herpesviruses 6A, 6B, and 7 and Their Replication. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2769-2784.

Pergam, S. A. and A. P. Limaye (2009). "Varicella zoster virus (VZV) in solid organ transplant recipients." American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons **9 Suppl 4**: S108-115.

Plachter, B., C. Sinzger, et al. (1996). "Cell types involved in replication and distribution of human cytomegalovirus." Advances in virus research **46**: 195-261.

Plafker, S. M. and W. Gibson (1998). "Cytomegalovirus assembly protein precursor and proteinase precursor contain two nuclear localization signals that mediate their own nuclear translocation and that of the major capsid protein." Journal of virology **72**(10): 7722-7732.

Poland, S. D., P. Costello, et al. (1990). "Cytomegalovirus in the brain: in vitro infection of human brain-derived cells." The Journal of infectious diseases **162**(6): 1252-1262.

Potzsch, S., N. Spindler, et al. (2011). "B cell repertoire analysis identifies new antigenic domains on glycoprotein B of human cytomegalovirus which are target of neutralizing antibodies." PLoS pathogens **7**(8): e1002172.

Preston, V. G., F. J. Rixon, et al. (1992). "Processing of the herpes simplex virus assembly protein ICP35 near its carboxy terminal end requires the product of the whole of the UL26 reading frame." Virology **186**(1): 87-98.

Prives, C. and P. A. Hall (1999). "The p53 pathway." J Pathol **187**(1): 112-126.

Raycroft, L., H. Y. Wu, et al. (1990). "Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene." Science **249**(4972): 1049-1051.

Reich, N. C. and A. J. Levine (1982). "Specific interaction of the SV40 T antigen-cellular p53 protein complex with SV40 DNA." Virology **117**(1): 286-290.

Revello, M. G., E. Percivalle, et al. (1992). "Nuclear expression of the lower matrix protein of human cytomegalovirus in peripheral blood leukocytes of immunocompromised viraemic patients." The Journal of general virology **73 (Pt 2)**: 437-442.

Rickinson, A. B., and E. Kieff (2001). Epstein-Barr Virus. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2575-2627.

Riley, T., E. Sontag, et al. (2008). "Transcriptional control of human p53-regulated genes." Nat Rev Mol Cell Biol **9**(5): 402-412.

Robertson, B. J., P. J. McCann, 3rd, et al. (1996). "Separate functional domains of the herpes simplex virus type 1 protease: evidence for cleavage inside capsids." Journal of virology **70**(7): 4317-4328.

Roizman, B., and D. M. Knipe (2001). Herpes Simplex Viruses and Their Replication. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2399-2459.

Roizman, B., and P. E. Pellet (2001). The Family Herpesviridae: A Brief Introduction. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2**.

Rosenke, K., M. A. Samuel, et al. (2006). "An intact sequence-specific DNA-binding domain is required for human cytomegalovirus-mediated sequestration of

p53 and may promote in vivo binding to the viral genome during infection." Virology **348**(1): 19-34.

Ruebner, B. H., K. Miyai, et al. (1964). "Mouse Cytomegalovirus Infection. An Electron Microscopic Study of Hepatic Parenchymal Cells." The American journal of pathology **44**: 799-821.

Ryckman, B. J., M. C. Chase, et al. (2008). "HCMV gH/gL/UL128-131 interferes with virus entry into epithelial cells: evidence for cell type-specific receptors." Proceedings of the National Academy of Sciences of the United States of America **105**(37): 14118-14123.

Ryckman, B. J., M. C. Chase, et al. (2010). "Human cytomegalovirus TR strain glycoprotein O acts as a chaperone promoting gH/gL incorporation into virions but is not present in virions." Journal of virology **84**(5): 2597-2609.

Ryckman, B. J., M. A. Jarvis, et al. (2006). "Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion." Journal of virology **80**(2): 710-722.

Ryckman, B. J., B. L. Rainish, et al. (2008). "Characterization of the human cytomegalovirus gH/gL/UL128-131 complex that mediates entry into epithelial and endothelial cells." Journal of virology **82**(1): 60-70.

Sakaguchi, K., J. E. Herrera, et al. (1998). "DNA damage activates p53 through a phosphorylation-acetylation cascade." Genes & development **12**(18): 2831-2841.

Sam, M. D., B. T. Evans, et al. (2009). "Biochemical, biophysical, and mutational analyses of subunit interactions of the human cytomegalovirus nuclear egress complex." Journal of virology **83**(7): 2996-3006.

Sampaio, K. L., Y. Cavignac, et al. (2005). "Human cytomegalovirus labeled with green fluorescent protein for live analysis of intracellular particle movements." Journal of virology **79**(5): 2754-2767.

Sanchez, V., K. D. Greis, et al. (2000). "Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly." Journal of virology **74**(2): 975-986.

Sanchez, V., E. Sztul, et al. (2000). "Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment." Journal of virology **74**(8): 3842-3851.

Sarov, I. and I. Abady (1975). "The morphogenesis of human cytomegalovirus. Isolation and polypeptide characterization of cytomegalovirions and dense bodies." Virology **66**(2): 464-473.

Sauer, A., J. B. Wang, et al. (2010). "A human cytomegalovirus deleted of internal repeats replicates with near wild type efficiency but fails to undergo genome isomerization." Virology **401**(1): 90-95.

Schauflinger, M., D. Fischer, et al. (2011). "The tegument protein UL71 of human cytomegalovirus is involved in late envelopment and affects multivesicular bodies." Journal of virology **85**(8): 3821-3832.

Scheffczik, H., C. G. Savva, et al. (2002). "The terminase subunits pUL56 and pUL89 of human cytomegalovirus are DNA-metabolizing proteins with toroidal structure." Nucleic Acids Res **30**(7): 1695-1703.

Scheffner, M., B. A. Werness, et al. (1990). "The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53." Cell **63**(6): 1129-1136.

Schmolke, S., P. Drescher, et al. (1995). "Nuclear targeting of the tegument protein pp65 (UL83) of human cytomegalovirus: an unusual bipartite nuclear localization signal functions with other portions of the protein to mediate its efficient nuclear transport." Journal of virology **69**(2): 1071-1078.

Schmolke, S., H. F. Kern, et al. (1995). "The dominant phosphoprotein pp65 (UL83) of human cytomegalovirus is dispensable for growth in cell culture." Journal of virology **69**(10): 5959-5968.

Scholz, B., S. Rechter, et al. (2003). "Identification of the ATP-binding site in the terminase subunit pUL56 of human cytomegalovirus." Nucleic Acids Res **31**(5): 1426-1433.

Schuessler, A., K. L. Sampaio, et al. (2008). "Charge cluster-to-alanine scanning of UL128 for fine tuning of the endothelial cell tropism of human cytomegalovirus." Journal of virology **82**(22): 11239-11246.

Schwartz, D. and V. Rotter (1998). "p53-dependent cell cycle control: response to genotoxic stress." Seminars in cancer biology **8**(5): 325-336.

Scrivano, L., J. Esterlechner, et al. (2010). "The m74 gene product of murine cytomegalovirus (MCMV) is a functional homolog of human CMV gO and determines the entry pathway of MCMV." Journal of virology **84**(9): 4469-4480.

Scrivano, L., C. Sinzger, et al. (2011). "HCMV spread and cell tropism are determined by distinct virus populations." PLoS pathogens **7**(1): e1001256.

Selinsky, C., C. Luke, et al. (2005). "A DNA-based vaccine for the prevention of human cytomegalovirus-associated diseases." Human vaccines **1**(1): 16-23.

Severi, B., M. P. Landini, et al. (1988). "Human cytomegalovirus morphogenesis: an ultrastructural study of the late cytoplasmic phases." Archives of virology **98**(1-2): 51-64.

Shan, R., X. Wang, et al. (2009). "Growth and development of infants with asymptomatic congenital cytomegalovirus infection." Yonsei medical journal **50**(5): 667-671.

Sharma, M., J. P. Kamil, et al. (2014). "Human cytomegalovirus UL50 and UL53 recruit viral protein kinase UL97, not protein kinase C, for disruption of nuclear lamina and nuclear egress in infected cells." Journal of virology **88**(1): 249-262.

Shi, Y., H. Lu, et al. (2011). "Prevalence and clinical management of cytomegalovirus retinitis in AIDS patients in Shanghai, China." BMC infectious diseases **11**: 326.

Shimamura, M., M. Mach, et al. (2006). "Human cytomegalovirus infection elicits a glycoprotein M (gM)/gN-specific virus-neutralizing antibody response." Journal of virology **80**(9): 4591-4600.

Silva, M. C., Q. C. Yu, et al. (2003). "Human cytomegalovirus UL99-encoded pp28 is required for the cytoplasmic envelopment of tegument-associated capsids." Journal of virology **77**(19): 10594-10605.

Sinzger, C., A. Grefte, et al. (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.

Sinzger, C., M. Kahl, et al. (2000). "Tropism of human cytomegalovirus for endothelial cells is determined by a post-entry step dependent on efficient

translocation to the nucleus." The Journal of general virology **81**(Pt 12): 3021-3035.

Soderberg-Naucler, C. (2006). "Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer?" Journal of internal medicine **259**(3): 219-246.

Somogyi, T., S. Michelson, et al. (1990). "Genomic location of a human cytomegalovirus protein with protein kinase activity (PK68)." Virology **174**(1): 276-285.

Song, Y. J. and M. F. Stinski (2005). "Inhibition of cell division by the human cytomegalovirus IE86 protein: role of the p53 pathway or cyclin-dependent kinase 1/cyclin B1." Journal of virology **79**(4): 2597-2603.

Soria, C., F. E. Estermann, et al. (2010). "Heterochromatin silencing of p53 target genes by a small viral protein." Nature **466**(7310): 1076-1081.

Spector, D. H. (2013). Exploitation of Host Cell Cycle Regulatory Pathways by HCMV. Cytomegaloviruses: From Molecular Pathogenesis to Intervention. M. J. Reddehase and N. A. W. Lemmermann. Norfolk, UK, Caister Academic Press. **1:** 246-263.

Speir, E., R. Modali, et al. (1994). "Potential role of human cytomegalovirus and p53 interaction in coronary restenosis." Science **265**(5170): 391-394.

Spriggs, M. K., R. J. Armitage, et al. (1996). "The extracellular domain of the Epstein-Barr virus BZLF2 protein binds the HLA-DR beta chain and inhibits antigen presentation." Journal of virology **70**(8): 5557-5563.

Steegenga, W. T., N. Riteco, et al. (1998). "The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells." Oncogene **16**(3): 349-357.

Steininger, C. (2007). "Clinical relevance of cytomegalovirus infection in patients with disorders of the immune system." Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases **13**(10): 953-963.

Straschewski, S., M. Patrone, et al. (2011). "Protein pUL128 of human cytomegalovirus is necessary for monocyte infection and blocking of migration." Journal of virology **85**(10): 5150-5158.

Takada, S., N. Kaneniwa, et al. (1997). "Cytoplasmic retention of the p53 tumor suppressor gene product is observed in the hepatitis B virus X gene-transfected cells." Oncogene **15**(16): 1895-1901.

Tamashiro, J. C., D. Filpula, et al. (1984). "Structure of the heterogeneous L-S junction region of human cytomegalovirus strain AD169 DNA." J Virol **52**(2): 541-548.

Tandon, R., D. P. AuCoin, et al. (2009). "Human cytomegalovirus exploits ESCRT machinery in the process of virion maturation." Journal of virology **83**(20): 10797-10807.

Tandon, R. and E. S. Mocarski (2011). "Cytomegalovirus pUL96 is critical for the stability of pp150-associated nucleocapsids." Journal of virology **85**(14): 7129-7141.

Tandon, R. and E. S. Mocarski (2012). "Viral and host control of cytomegalovirus maturation." Trends in microbiology **20**(8): 392-401.

Terhune, S. S., J. Schroer, et al. (2004). "RNAs are packaged into human cytomegalovirus virions in proportion to their intracellular concentration." Journal of virology **78**(19): 10390-10398.

Thomas, J. T. and L. A. Laimins (1998). "Human papillomavirus oncoproteins E6 and E7 independently abrogate the mitotic spindle checkpoint." Journal of virology **72**(2): 1131-1137.

Thomas, M., G. Matlashewski, et al. (1996). "Induction of apoptosis by p53 is independent of its oligomeric state and can be abolished by HPV-18 E6 through ubiquitin mediated degradation." Oncogene **13**(2): 265-273.

Tomtishen, J. P., 3rd (2012). "Human cytomegalovirus tegument proteins (pp65, pp71, pp150, pp28)." Virology journal **9**: 22.

Tooze, J., M. Hollinshead, et al. (1993). "Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes." European journal of cell biology **60**(1): 163-178.

Trgovcich, J., C. Cebulla, et al. (2006). "Human cytomegalovirus protein pp71 disrupts major histocompatibility complex class I cell surface expression." Journal of virology **80**(2): 951-963.

Tsai, H. L., G. H. Kou, et al. (1996). "Human cytomegalovirus immediate-early protein IE2 tethers a transcriptional repression domain to p53." The Journal of biological chemistry **271**(7): 3534-3540.

Vanarsdall, A. L., M. C. Chase, et al. (2011). "Human cytomegalovirus glycoprotein gO complexes with gH/gL, promoting interference with viral entry into human fibroblasts but not entry into epithelial cells." Journal of virology **85**(22): 11638-11645.

Vanarsdall, A. L., B. J. Ryckman, et al. (2008). "Human cytomegalovirus glycoproteins gB and gH/gL mediate epithelial cell-cell fusion when expressed either in cis or in trans." J Virol **82**(23): 11837-11850.

Varnum, S. M., D. N. Streblow, et al. (2004). "Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome." Journal of virology **78**(20): 10960-10966.

Vaseva, A. V. and U. M. Moll (2009). "The mitochondrial p53 pathway." Biochimica et biophysica acta **1787**(5): 414-420.

Vousden, K. H. and X. Lu (2002). "Live or let die: the cell's response to p53." Nat Rev Cancer **2**(8): 594-604.

Vousden, K. H. and C. Prives (2009). "Blinded by the Light: The Growing Complexity of p53." Cell **137**(3): 413-431.

Wang, D. and T. Shenk (2005). "Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism." Journal of virology **79**(16): 10330-10338.

Wang, D. and T. Shenk (2005). "Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism." Proceedings of the National Academy of Sciences of the United States of America **102**(50): 18153-18158.

Wang, J., P. H. Marker, et al. (2000). "Human cytomegalovirus immediate early proteins upregulate endothelial p53 function." FEBS letters **474**(2-3): 213-216.

Wang, X., S. M. Huong, et al. (2003). "Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus." Nature **424**(6947): 456-461.

Wang, X. W., K. Forrester, et al. (1994). "Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3." Proceedings of the National Academy of Sciences of the United States of America **91**(6): 2230-2234.

Welch, A. R., L. M. McNally, et al. (1993). "Herpesvirus proteinase: site-directed mutagenesis used to study maturational, release, and inactivation cleavage sites of precursor and to identify a possible catalytic site serine and histidine." Journal of virology **67**(12): 7360-7372.

Welch, A. R., A. S. Woods, et al. (1991). "A herpesvirus maturational proteinase, assemblin: identification of its gene, putative active site domain, and cleavage site." Proceedings of the National Academy of Sciences of the United States of America **88**(23): 10792-10796.

White, A. E., E. M. Livanos, et al. (1994). "Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoproteins." Genes & development **8**(6): 666-677.

Whitley, R. J. (2001). Herpes Simplex Viruses. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2461-2509.

Wienzek, S., J. Roth, et al. (2000). "E1B 55-kilodalton oncoproteins of adenovirus types 5 and 12 inactivate and relocalize p53, but not p51 or p73, and cooperate with E4orf6 proteins to destabilize p53." Journal of virology **74**(1): 193-202.

Wille, P. T., A. J. Knoche, et al. (2010). "A human cytomegalovirus gO-null mutant fails to incorporate gH/gL into the virion envelope and is unable to enter fibroblasts and epithelial and endothelial cells." Journal of virology **84**(5): 2585-2596.

Wolfstein, A., C. H. Nagel, et al. (2006). "The inner tegument promotes herpes simplex virus capsid motility along microtubules in vitro." Traffic **7**(2): 227-237.

Womack, A. and T. Shenk (2010). "Human cytomegalovirus tegument protein pUL71 is required for efficient virion egress." mBio **1**(5).

Wood, L. J., M. K. Baxter, et al. (1997). "Human cytomegalovirus capsid assembly protein precursor (pUL80.5) interacts with itself and with the major capsid protein (pUL86) through two different domains." Journal of virology **71**(1): 179-190.

Xiao, H., A. Pearson, et al. (1994). "Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53." Molecular and cellular biology **14**(10): 7013-7024.

Yamanishi, K. (2001). Human Herpesvirus 6 and Human Herpesvirus 7. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2785-2801.

Yu, J. and L. Zhang (2008). "PUMA, a potent killer with or without p53." Oncogene **27 Suppl 1**: S71-83.

Zaichick, S. V., K. P. Bohannon, et al. (2013). "The herpesvirus VP1/2 protein is an effector of dynein-mediated capsid transport and neuroinvasion." Cell host & microbe **13**(2): 193-203.

Zhang, L. J., P. Hanff, et al. (1995). "Detection of human cytomegalovirus DNA, RNA, and antibody in normal donor blood." The Journal of infectious diseases **171**(4): 1002-1006.

Zhang, Z., D. L. Evers, et al. (2006). "Evidence that the human cytomegalovirus IE2-86 protein binds mdm2 and facilitates mdm2 degradation." Journal of virology **80**(8): 3833-3843.

Zhao, L. Y. and D. Liao (2003). "Sequestration of p53 in the cytoplasm by adenovirus type 12 E1B 55-kilodalton oncoprotein is required for inhibition of p53-mediated apoptosis." Journal of virology **77**(24): 13171-13181.

Zhou, M., Q. Yu, et al. (2013). "Comparative analysis of gO isoforms reveals that strains of human cytomegalovirus differ in the ratio of gH/gL/gO and gH/gL/UL128-131 in the virion envelope." Journal of virology **87**(17): 9680-9690.

Chapter 2

Human cytomegalovirus nuclear egress and secondary envelopment is negatively affected in the absence of cellular p53

2.1 Abstract

Human Cytomegalovirus (HCMV) infection is compromised by the absence of cellular p53, a specific transcription factor that mediates cellular stress responses. p53's activation leads to cell cycle arrest and/or apoptosis. HCMV-infection stabilizes p53, however normal p53-mediated responses are inhibited. We have used fibroblasts that lack p53 (p53KOs) to study this protein's role during HCMV infection. Our earlier studies showed functional virion production in p53KOs was reduced 25-fold compared to their parental wild type (wt) Lox cells at 120 h post-infection (pi). Immediate Early viral proteins were expressed normally in p53KOs, while Early and Late viral protein expression was delayed. In this study we have further investigated this compromised functional virion production in p53KOs. Infectious center assays found most p53KOs released functional virions. Analysis of electron micrographs revealed modestly decreased capsid production in infected p53KOs compared to wt. Significantly fewer p53KOs displayed HCMV-induced infoldings of the inner nuclear membrane (IINMs). The IINMs present in p53KOs were reduced in number and size. The deficit in virus-induced membrane production within the nucleus was also reflected in the membranes of the cytoplasm. Reduced numbers of capsids were found there and a disproportionately smaller

number of those found were enveloped. Negative staining of infected p53KO cell supernatant found vastly fewer viral particles released from the cells. Reintroduction of p53 substantially recovered these deficits. Overall, the absence of p53 inhibited the formation and function of the primary HCMV nuclear capsid egress portal and re-envelopment of the reduced number of particles able to reach the cytoplasm.

2.2 Introduction

Human Cytomegalovirus (HCMV) is a ubiquitous human pathogen. It can cause severe disease in immunocompromised individuals and AIDS patients. In addition, it is a major cause of central nervous system birth defects. Each year 1% of all newborns are congenitally infected by HCMV. Of these infected newborns, 5 to 10% show signs of serious neurological defects at birth. These defects include hearing loss, vision loss, mental retardation, microencephaly, and cerebral calcification (Poland, Costello et al. 1990; Fowler, Stagno et al. 1992; Boppana and Britt 1995; Britt 1996; Cinque 1997; Fowler, McCollister et al. 1997; Boppana, Fowler et al. 1999; Gaytant, Steegers et al. 2002; Shan, Wang et al. 2009). This virus has also been linked to certain types of cancers including malignant gliomas, prostate carcinomas, and colorectal cancers (Soderberg-Naucler 2006). There are relatively few antiviral drugs available to interrupt the course of infection. The most commonly used drugs are ganciclovir and maribavir, both of which show high levels of toxicity (Biron 2006). There is currently no vaccine to prevent HCMV infection.

The HCMV lifecycle commences with the virion's entrance into the cell via fusion of the viral envelope with the cellular membrane. Binding is initiated by the interaction of viral envelope glycoprotreins B (gB) and the gH/gL trimeric or pentameric complex (Wang and Shenk 2005; Ryckman, Jarvis et al. 2006; Ryckman, Chase et al. 2008; Ryckman, Rainish et al. 2008; Vanarsdall, Ryckman et al. 2008; Isaacson and Compton 2009) with the cellular surface receptor heparin sulfate proteoglycans (HSPGs) (Kari and Gehrz 1992; Compton, Nowlin et al. 1993; Kari and Gehrz 1993; Boyle and Compton 1998; Compton 2004). This may or may not be followed by interactions with additional receptors to trigger a fusion event between the viral envelope and the plasma membrane (Adler and Sinzger 2013; Feire and Compton 2013). Upon entry, the capsid is transported along microtubules toward the nuclear pores, through which the 235 kb double stranded DNA viral genome is deposited into the nucleus (reviewed in reference Kalejta 2008). The viral lifecycle is defined by several stages, which can be loosely defined by immediate early (IE), early (E) and late (L) protein expression. Various physiological and morphological effects have been noted concomitant with these stages, including p53 protein stabilization and strong association of this tumor suppressor protein with the viral replication centers at early times pi (Muganda, Mendoza et al. 1994; Fortunato and Spector 1998; Muganda, Carrasco et al. 1998), as well as the swelling of an infected cell starting at 72 hpi.

The reproductive component of the virus lifecycle begins with replication and encapsidation of its genome in discrete viral replication centers within the nucleus. This is followed by initial tegumentation of the capsid and transit of the

encapsidated particles across the nuclear membrane (which includes primary envelopment and de-envelopment). Completion of tegumentation and re-envelopment of these particles then occurs in the cytoplasm and is ultimately followed by the shedding of these particles via fusion of secretory vesicles containing these enveloped particles with the plasma membrane (reviewed in reference Colberg-Poley and Williamson 2013). Ultra-structural analysis of the nucleus of infected cells has identified three capsid types, A, B and C. These are characteristic of herpesvirus replication and represent stages in virion morphogenesis. The A capsid is empty, containing no scaffolding or viral genome. The B capsid has a translucent core composed of the scaffolding assembly protein (AP) not yet removed by proteolytic digestion and lacking a viral genome. C capsids have a dark electron dense core composed of the packed viral genome (reviewed in reference Mocarski 2001).

All three types of capsids can be tegumented. The first layers of tegument protein are acquired inside the nucleus. pp150, the tegument layer closest to the capsid proper, is thought to provide stability to the capsid structure during packaging and passage through the nuclear envelope's layers (Baxter and Gibson 2001; AuCoin, Smith et al. 2006; Tandon and Mocarski 2011; Dai, Yu et al. 2013). Tegumented capsids bud through the inner nuclear membrane into the perinuclear space, in the process acquiring a primary envelope. Infoldings of the inner nuclear membrane (IINMs), structures that have been observed by several groups to contain enveloped capsids (Ruebner, Miyai et al. 1964; Papadimitriou, Shellam et al. 1984; Severi, Landini et al. 1988; Gilloteaux and Nassiri 2000; Dal Monte, Pignatelli

et al. 2002; Buser, Walther et al. 2007), have been proposed as the principal site of transit through the nuclear membrane (Buser, Walther et al. 2007). The enveloped capsids fuse with the outer nuclear membrane, in the process becoming de-enveloped, and are deposited into the cytoplasm (reviewed in references Mettenleiter, Klupp et al. 2009; Johnson and Baines 2011).

The viral assembly complex (VAC) begins to appear within the cytoplasm of infected cells by approximately 48 hours post infection (h pi). This cytoplasmic inclusion can be observed by light microscopy and is composed of the early endosomes and vesicles derived from the Golgi apparatus (Das, Vasanji et al. 2007; Das and Pellett 2011). Formation of the VAC distorts the nucleus into its characteristic kidney-bean shape (Buchkovich, Maguire et al. 2009; Buchkovich, Maguire et al. 2010; Indran, Ballestas et al. 2010). Within the VAC capsids become fully tegumented and acquire a second and final envelope (re-envelopment) (Sanchez, Greis et al. 2000; Sanchez, Sztul et al. 2000). Another viral particle is also enveloped in the VAC, the dense body (DB). DBs are enveloped “bags” of viral tegument proteins, largely composed of pp65 (Sarov and Abady 1975; Gibson and Irmiere 1984). DBs constitute a large proportion of the Golgi-derived vesicles containing viral particles within the cytoplasm of cells infected with laboratory-adapted virus strains, e.g. Towne and AD169. These vesicles are transported to the plasma membrane and released by fusion with the membrane (reviewed in reference Kalejta 2008). The entire array of virus-derived particles including DBs, non-infectious enveloped particles (NIEPs), and functional/non-functional virions, are released from the infected cell and compromise the viral components of infected

supernatant. Once viral maturation starts, at approximately 72 hpi, infected cells continue to produce virus for several days until they are ultimately lysed.

Transcription of the tumor suppressor protein p53 is not increased in HCMV-infected fibroblasts, however by 4h pi steady state levels of the protein are increased via stabilization of the protein present within the cell (Muganda, Mendoza et al. 1994; Fortunato and Spector 1998; Muganda, Carrasco et al. 1998). Despite the increased steady-state levels of p53 in an infected cell, its cellular targets, including p21, are not activated and the infected cell does not undergo apoptosis, nor does p53 trigger a successful DNA damage response (Muganda, Mendoza et al. 1994; Speir, Modali et al. 1994; Zhu, Shen et al. 1995; Bresnahan, Boldogh et al. 1996; Tsai, Kou et al. 1996; Fortunato and Spector 1998; Castillo, Yurochko et al. 2000; Murphy, Streblow et al. 2000; Chen, Knutson et al. 2001; Hsu, Chang et al. 2004). p53 is tightly associated with the viral replication centers as soon as they are established (Fortunato and Spector 1998). p53's normal function as a transcription factor suggest it may also be activating or inhibiting the expression of viral genes. In support of this supposition, earlier work in our laboratory found 21 p53-binding sites in the HCMV genome (Rosenke, Samuel et al. 2006) and, more recently, that the viral genome was selectively repaired in favor of the cellular genome (O'Dowd, Zavala et al. 2012). These findings indicate that p53 plays an important role in the dynamics of HCMV infection.

The availability of p53 knockout cells (p53KO), in which the protein is totally absent, have facilitated study of p53's role during HCMV infection (Casavant, Luo et al. 2006). Immediate Early gene expression was near normal. The accumulation of

both early and late viral proteins was delayed by 24-48h (Casavant, Luo et al. 2006). However, the most abundant of the tegument proteins, pp65, did not relocate from the nucleus to the cytoplasm in the majority of cells, even at late infection times (Casavant, Luo et al. 2006). By 120h pi expression of the majority of viral proteins in the p53KO cells had reached near wild type (wt) levels, however functional virion production remained reduced by 25-fold in the absence of wt p53. This indicated these cells were inherently inefficient viral hosts and that even additional infection duration (possible in this line due to greatly reduced cell death/population depletion) could not overcome this deficit.

In this study, we have investigated the causes of the severely compromised production of functional virus in cells lacking p53 throughout the course of infection. We have found that the absence of p53 contributed to modest reductions in quantifiable parameters at several stages of the viral lifecycle. These events include slower infection kinetics and slightly less efficient nuclear capsid production. However, most significantly, inefficient trafficking of nuclear capsids due to the relative absence of virus-induced infoldings of the inner nuclear membrane (IINM) and a, possibly correlatable, reduction in re-envelopement of virus particles in the cytoplasm were largely responsible for the approximate 25-fold reduction in functional virus production.

2.3 Materials and Methods

2.3.1 Cells and virus growth

Human foreskin fibroblasts (HFFs) were isolated from tissue and propagated in Earle's minimal essential media (MEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (200 U/ml), streptomycin (200 g/ml), and amphotericin B (1.5 g/ml). p53KO telomerase-immortalized human fibroblasts and their parental cell line, Lox (Bunz, Dutriaux et al. 1998; Wei, Hemmer et al. 2001) (both kind gifts from Dr. John Sedivy, Brown University), were maintained in complete medium composed of Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (Ham) 1:1 (DMEM/F-12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (2mM), penicillin (200 U/ml), and streptomycin (200 µg/ml). Cells were grown in incubators maintained at 37^{o}C and 5% CO_2. The Towne strain of HCMV was obtained from the ATCC (#VR 977), propagated under standard procedures (Tamashiro, Hock et al. 1982), and used at a multiplicity of infection (MOI) of 5 for all experiments.

2.3.2 Cell cycle synchronization and infection

G0 synchronization was carried out via serum starvation as previously described (Casavant, Luo et al. 2006). The cells were then trypsinized and reseeded onto plates or flasks in complete DMEM/F-12 medium. After allowing 1-2 h for attachment, cells were infected at an MOI of 5 with HCMV Towne. After 4 h,

medium was replaced with fresh complete medium to remove the excess virus that had not entered cells.

2.3.3 Assays for infectious progeny virion shedding cells

The shedding of infectious virus was assessed using two protocols. First, we used conventional focus forming assays as previously described (Luo and Fortunato 2007). Briefly, infected cell samples (3×10^5), were harvested at 120 hpi. Infected cells were washed with phosphate-buffered saline (PBS) three times before trypsinization and centrifugation to pellet. The cell pellet was washed and repelleted three additional times with complete medium to fully remove any viral particles potentially adhering to the outside of the infected cells. The medium from the final wash was reserved for later use as a negative control and to ensure removal of all extra-cellular virus. The cell pellet was then resuspended in 1 ml of complete culture medium and cells were counted and diluted as necessary. Immediately following dilution, 50 cells were inoculated (in triplicate) onto a monolayer of HFFs. Cells were overlaid with agar and resulting foci were counted after 7 d. The average of the replicate samples were expressed as percent focus-forming cells (focus-forming units/50 cells). The assays were performed five times and the average plus one standard error is presented.

In the second assay the infected cells were assayed for infectious centers via immunofluorescent staining for IE1 and UL44 foci. First, monolayers of HFFs were seeded onto coverslips placed into the wells of 12-well dishes. Then, approximately

40 infected cells were seeded per well. For each cell type and timepoint, three duplicate wells were seeded to provide technical replicates. Medium was replaced daily to contain as much as possible the spread of infection to only directly adjacent cells. Coverslips were harvested each day for three days. HCMV shedding capability from each of the infected cells was assessd by immunofluorescent staining. Seeded cells were identified as the cells with large UL44 foci amidst surrounding infected ($IE1^+$) cells.

2.3.4 Antibodies

Primary antibodies used were several mouse monoclonal antibodies (mAb) anti-IE1 (1:25; IgG2A; a kind gift from Dr. Bill Britt, University of Alabama, Birmingham), anti-UL44 (1:1000; 1202S [IgG1]; Goodwin Institute), anti-Lamin A/C (1:1000; 636 [IgG2B]; Santa Cruz Biotechnology), anti-Lamin A (1:1000; 133A2 [IgG3]; abcam), and a rabbit polyclonal Ab anti-UL50 (1:2000; a kind gift from Dr. James Alwine, University of Pennsylvania, Philadelphia) (Buchkovich, Maguire et al. 2010). Secondary antibodies used were goat anti-mouse IgG 2A TRITC-coupled Ab (Southern Biotech), goat anti-mouse IgG1 Alex Fluor 488-coupled Ab (Molecular Probes), goat anti-mouse IgG2B Alex Fluor 488-coupled Ab (Molecular Probes), goat anti-mouse IgG3 Alex Fluor 488-coupled Ab (Molecular Probes), and donkey anti-rabbit TRITC-coupled Ab (Jackson Immunoresearch).

2.3.5 Immunofluorescence staining assay (IF)

Except where noted below, all coverslips were fixed in 3% formaldehyde followed by permeabilization in 1% Triton as previously described (Rosenke, Samuel et al. 2006). Nuclei were counterstained with Hoechst dye. Visualization of UL50 immunofluorescent localization was optimized by using an "extraction first" methodology. Cells were treated with detergent before fixation in formaldehyde (Cardoso, Leonhardt et al. 1993; Marciniak, Lombard et al. 1998; Lombard and Guarente 2000; Luo, Rosenke et al. 2007). Additionally, this methodology included a blocking solution using 10% human IgG (20 mg/ml), rather than 30% FBS.

Epifluorescent analysis was performed on a Nikon Eclipse E800 flourescence microscope equipped with a Nikon DS-Ri1 high resolution color camera and Nikon NIS Elements Basic Research (Br) imaging software.

2.3.6 Transmission electron microscopy (TEM)

Cells were serum starved as described above, then reseeded, infected at a MOI of five, and harvested at 120 hpi. Then cells were fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M PBS, rinsed extensively in 0.1M Cacodylate buffer with 3.5% sucrose, post fixed in 2% OsO_4 in 0.1M Cacodylate buffer for 2h at RT, and then rinsed extensively in de-ionized distilled (dd) H_2O. Optimal membrane contrast was obtained by staining cells with 1% tannic acid for 1h at RT and then rinsing twice with deionized, distilled (dd) H_2O. Samples were dehydrated through an increasing ethanol series (30-100%), followed by two 100%

acetone dehydrations. To prepare for embedding, cells were infiltrated in acetone and SPURRs (1:1 ratio) overnight. The resin was then replaced three times with fresh 100% SPURRs for 1 h. Samples were stored in SPURRs overnight at RT before they were embedded at 70^{o}C for 48 h. After embedding, samples were sectioned at ~ 100 nm using a diamond knife. Sections were contrast enhanced by staining with 4% uranyl acetate for 10 min, followed by Reynold's lead citrate staining for 5 min in a CO_2 free environment. Samples were examined with an FEI Technai G2 20 Twin transmission electron microscope (FEI TEM) equipped with a 200 kV LaB6 electron source and a 4K Eagle camera. Images were captured using TEM Imaginig Analysis (TIA) software. Images for analysis were captured at a magnification of 2500x and 5000x. Where necessary and for quantitative analysis, images were stitched together using image editing software (PhotoShop) from these individual captures.

2.3.7 Viral particle extraction and negative staining

One T75 flask each of Lox or p53KO synchronized cells was seeded and infected at an MOI of five. At 120 hpi, supernatant (10 ml) containing the viral particles shed from the infected cells was harvested and centrifuged at low speed to remove cellular debris, eight mls of supernatant were then ultracentrifuged at 23,000 rpm for 75 min at 10^{o}C. Viral particle pellets were gently resuspended in 40 ml of PBS. Viral particles were prepared for negative stain and TEM analysis by settling 3 μl of particles onto a 200 mesh Formvar/Carbon coated nickel grid for 3

min at RT. Grids were then stained with 3 μl of 1% phosphotungstic acid (PTA) for 1 min. Samples were examined with FEI TEM as described above.

2.3.8 Statistical analysis

In statistics, conducting a parametric ANOVA assumes a normal distribution of errors. Since our data did not fit this assumption, a non-parametric test was used instead. The Friedman test (Friedman 1937) is designed for analysis of data from a randomized complete block design, but does not assume normal errors. The four biological replicates were performed on different dates. The different dates were the blocks within which each comparison was tested and then combined with the information from the other replicates. SAS (Cary, NC) was used for this analysis.

2.4 Results

2.4.1 The majority of p53KO cells released functional virions.

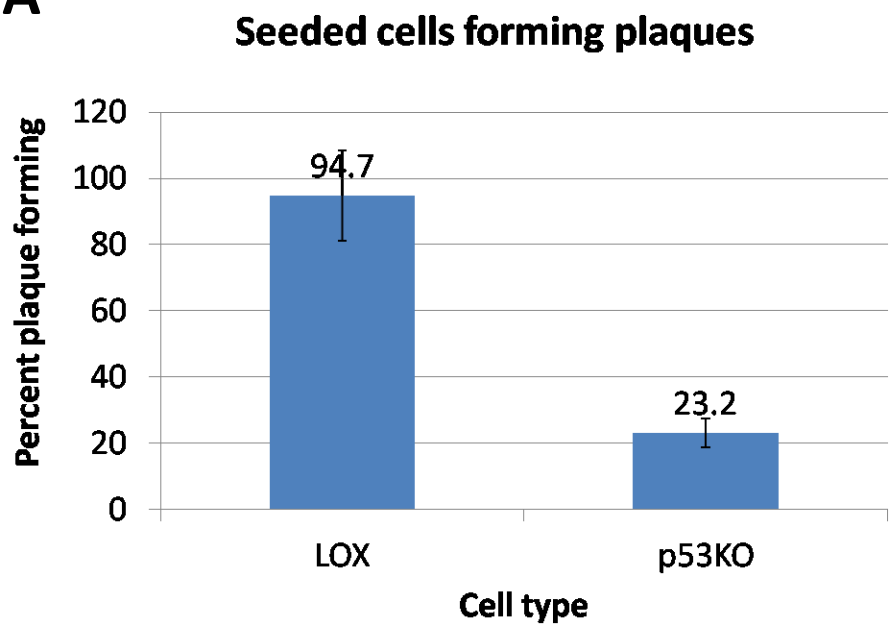
Titration of the virus produced by the p53KOs had found a 25-fold reduction at 120 h pi (Casavant, Luo et al. 2006). Using the initial number of cells infected and the results from the cumulative titer experiments (pfu/ml supernatant), an estimate of approximately 50 pfu/cell were shed by each wt cell through 120 hpi. p53KO cells shed 2 pfu/cell through the same timepoint. This measure of pfu/cell was based on averages from a population of cells. Conventional focus forming assays were performed to determine how many cells within the p53KO population were releasing

enough functional virions at 120 hpi to form identifiable plaques when seeded onto a monolayer of permissive cells. Figure 2.1A displays the percentage of cells producing scoreable/recognizable plaques at 7 days post seeding. Essentially 100% of seeded Lox cells, the p53KO parental wt, produced visible plaques, while only 20-30% of the p53KO cells generated identifiable plaques even at 9 days post seeding. This result paralleled our earlier results, which found ~30% of cells trafficking pp65 into the cytoplasm and containing large VAC formation at these late times pi. Were only these 30% of cells shedding virus, producing identifiable plaques, and accounting for the lower titers? Or were the large majority of cells shedding such small amounts of virus (2 pfu/ml) that identifiable plaques were not generated?

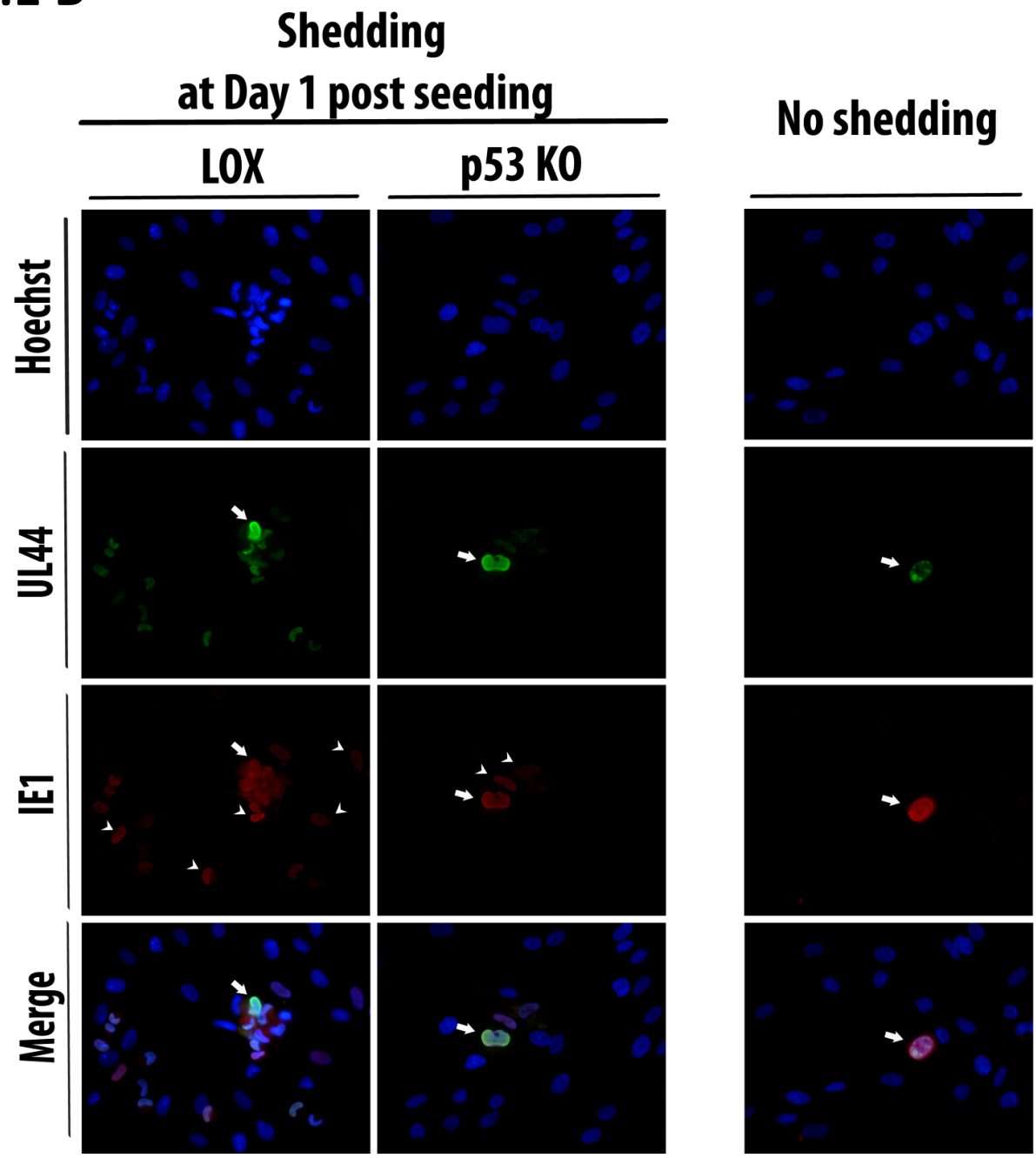
This question was addressed using a more sensitive infectious center assay. Infected cells were again harvested at 120 hpi and known quantities of infected cells were seeded onto monolayers of HFFs grown on coverslips. To allow further processing of these cells no agarose overlays were used in these cultures. Coverslips were harvested for three successive days. The coverslips were stained for UL44 and IE1 to examine the shedding capability of the seeded cells. Since the majority of wt and KO cells harvested at 120 hpi contained advanced stage replication centers (RCs) (Casavant, Luo et al. 2006) , the seeded cells could be identified by staining for the viral processivity factor UL44 (Penfold and Mocarski 1997). Coverslips were co-stained with IE1 in order to recognize newly infected cells neighboring the seeded cells. Figure 2.1B shows examples of the $UL44^{+}$ seeded cells (arrows in panels), surrounded by newly infected cells stained for IE1

(arrowheads in panels). Greater than 95% of the wt cells shed functional virus to an adjacent cell on day 1. Greater than 60% of the KO cells shed functional virus on day 1. The percentage of KO cells increased to almost 80% by day 3 (Figure 2.1C). Of note, the wt cells functionally infected considerably more neighboring cells than KO cells. Quantitation of the number of infected neighboring cells next to the seeded cells found that the majority of the seeded p53KO cells infected smaller numbers of their neighbors than their wt seeded counterparts (Figure 2.1D). Presumably each wt cell was shedding more functional virus than each KO cell. These assays demonstrated that a large majority of the KO cells were shedding functional virus, although it was perhaps only a single functional virion to an adjacent cell.

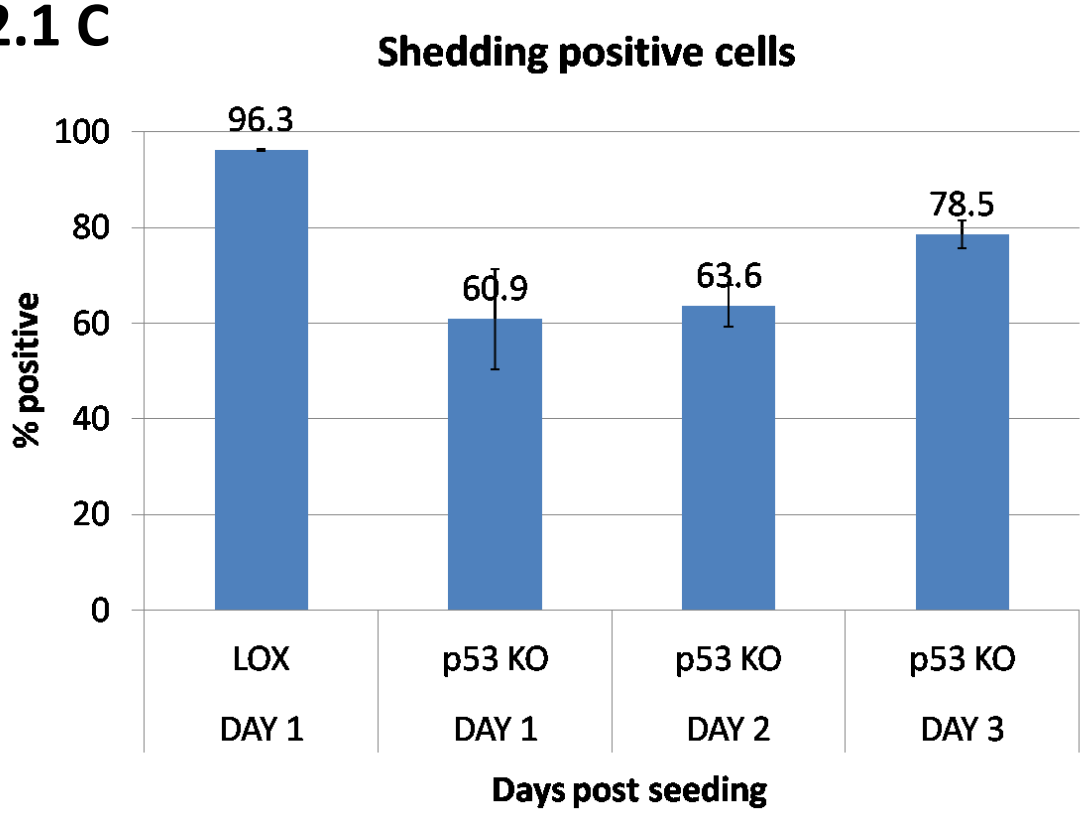
2.1 A



2.1 B


Shedding
at Day 1 post seeding
No shedding
LOX
p53 KO
Hoechst
UL44
IE1
Merge

2.1 C


Shedding positive cells
% positive
100
80
60
40
20
0
96.3
60.9
63.6
78.5
LOX
DAY 1
p53 KO
DAY 1
p53 KO
DAY 2
p53 KO
DAY 3
Days post seeding

2.1 D

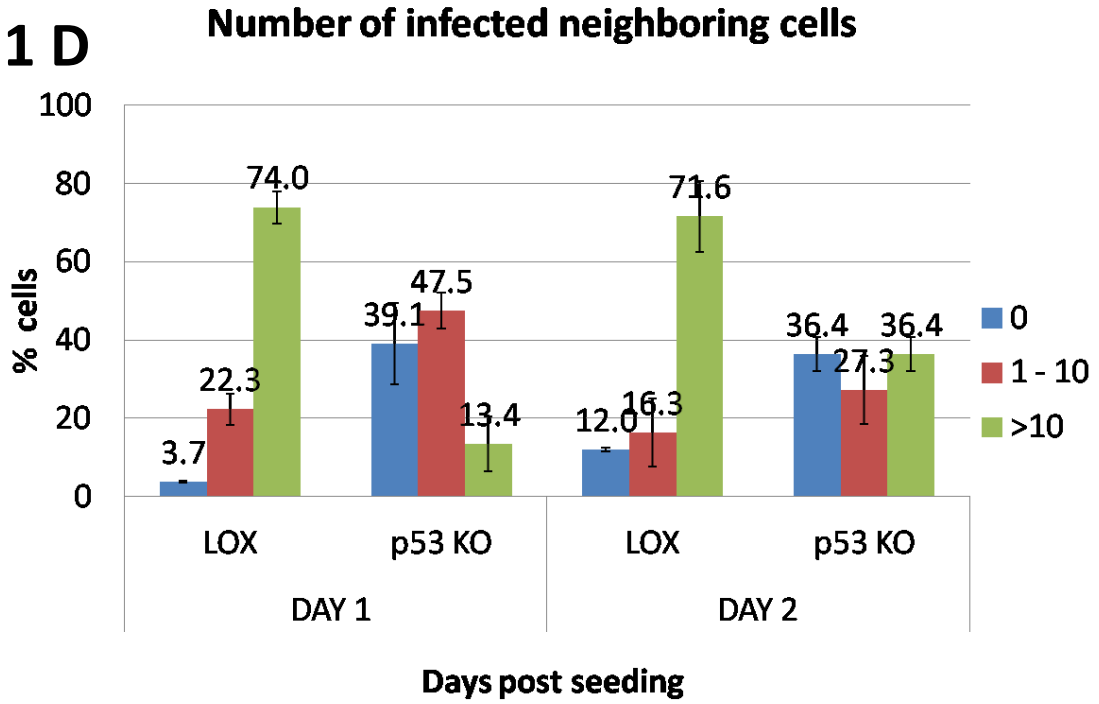

Number of infected neighboring cells
% cells
100
80
60
40
20
0
3.7
22.3
74.0
39.1
47.5
13.4
12.0
16.3
71.6
36.4
27.3
36.4
0
1 - 10
>10
LOX
p53 KO
LOX
p53 KO
DAY 1
DAY 2
Days post seeding

Figure 2.1 Quantitation of shedding positive cells in wt LOX cells compared to p53KO cells. Infected Lox and p53KO cells were harvested for seeding onto monolayers at 120 hpi. (A) The percentage of cells generating visible plaques in conventional plaque forming assays. Error bars represent one standard error calculated from five experiments. (B) Infectious center assays to more clearly quantitate virion shedding. Coverslips were stained for UL44 to detect seeded cells and IE1 to detect newly infected neighboring cells (arrows indicate seeded cells; arrow heads indicate newly infected neighboring cells). (C) Percentage of shedding positive cells detected by immunofluorescent staining of Infectious center assays. Error bars represent one standard error calculated from three technical replicates. (D) Quantitation of numbers of newly infected neighboring cells at day 1 and 2 post seeding. Numbers of infected neighboring cells were binned at 0, 1-10, and more than 10. Error bars represent one standard error calculated from three technical replicates.

2.4.2 Capsid production in the nucleus was modestly reduced in p53KO cells.

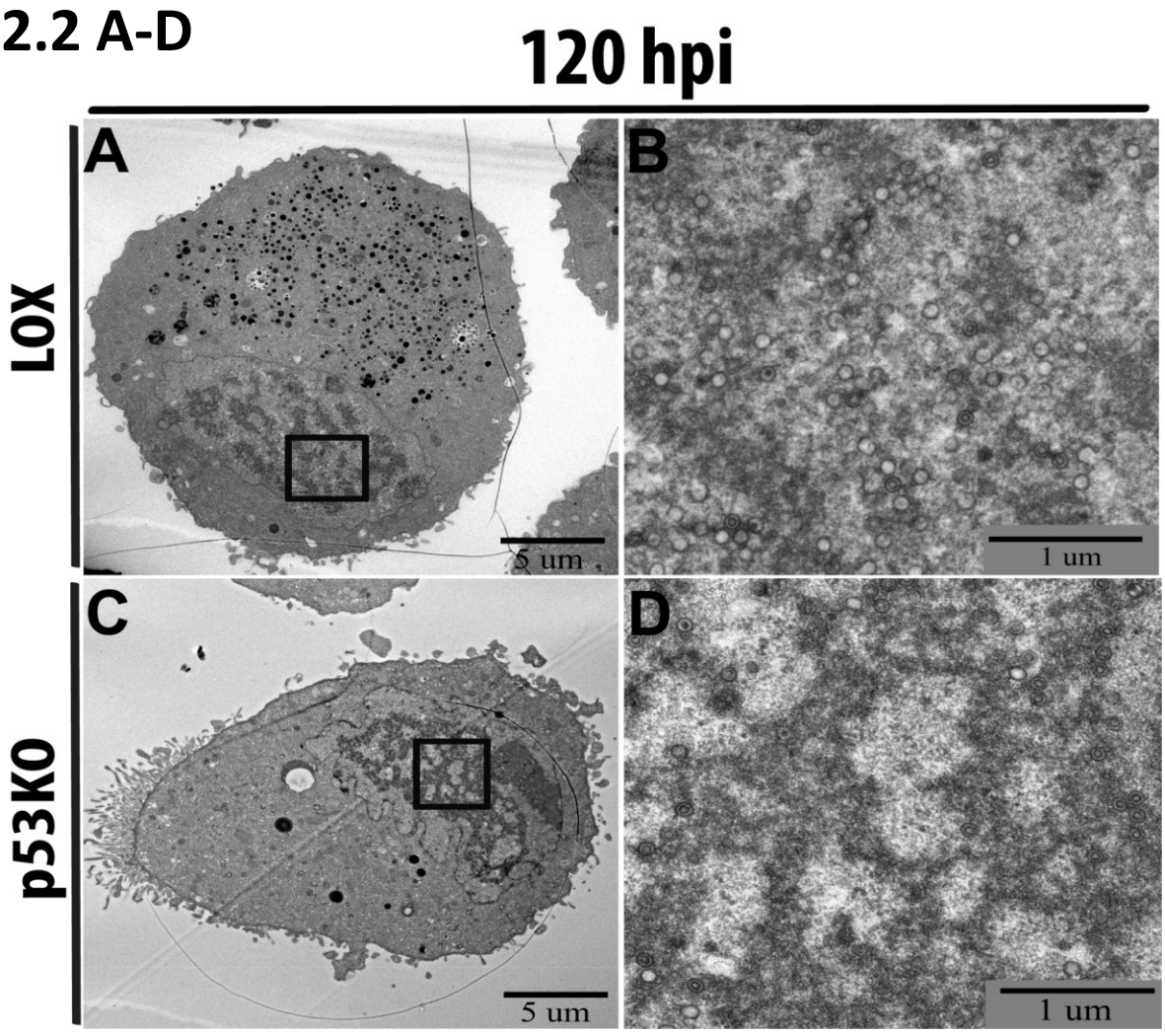
The infectious center assays indicated that the large majority of p53KO cells were capable of shedding small amounts of virus. Then where was the breakdown in capsid production inside of these infected cells? Electron microscopy was used to examine the ultrastructural characteristics of infected wt fibroblasts and p53KOs at 120h pi. By this timepoint the wt cells were beginning to die and had reached their maximum virus production. The KO cells had also reached their maximum production of virus (as evidenced by no further increases in titers at 144 or 168h pi) (Casavant, Luo et al. 2006 and data not shown), however most of the KO cells survived. Cells were processed for TEM and images captured for analysis. Initial experiments included an additinional wt fibroblast human foreskin fibroblasts (HFFs), which had been included in our initial studies (Casavant, Luo et al. 2006), in order to allow correlation to previous experiments and verify that fibroblasts of differing lineages behaved similarly. Cells were selected for imaging based on a number of criteria. First, only cells that could be clearly distinguished at both lower and higher magnifications were imaged. We believe due to the vagaries of processing some cells in a section were much less clear and in some cases damaged or torn by the sectioning process. Second, only cells in which the cross-section of the nucleus constituted ~30-50% of a cell's area were selected. This criteria was used to ensure as nearly as possible that the cross-section was as close as possible to centered through the volume of the nucleus/cell. Our imaging criteria intentionally included not screening cells for particular morphologies. This stemmed in part from the impracticality of attempting to add additional considerations to the already difficult

TEM process. And that we hoped to capture truly representative samples from the populations.

In the TEM images RCs (as visualized with UL44 staining in Figure 2.1B) were seen in a broad range of sizes and densities of both cell types (see Figure 2.2A-B for Lox and 2C-D for p53KO; HFF not shown). The range of sizes and densities of individual structures had considerable overlap between the cell types; with individual wt cells displaying replication centers on the lower end of the size range and individual p53KO cells containing large, dense replication centers as well. HFFs showed an equally wide distribution (data not shown).

In all cell types the vast majority of all the capsids that could be identified were associated with the RCs (images in Figures 2B and D represent higher magnification images of the boxed regions in 2A and C, respectively). In the absence of a large, well-defined replication center, which was less common in wt than p53KO cells, but occurred in both cell types, very few capsids were present inside the nucleus. However, in only a single cell of any of the cell types, a KO cell in this case, were capsids not identified in the cytoplasm. This indicated that these cells all had replication centers, but that the RC was not present in the plane of the particular microtome section. Our intention was to compare the number of capsids present in the nuclei of these cells, and since capsids were highly associated with RCs it would not have been representative to include cells lacking large RCs. Therefore our image library was culled to exclude cells from our analysis that lacked large RCs. Generally, the median number of capsids that could be identified in both wt cells exceeded the number in the p53KO cells by approximately 2-fold (Figure

2.2E). In both wt cells the median number of capsids was ~400 at 120h pi and the median number of capsids in the p53KO cells was ~200. Although statistically different, a difference of two-fold in the number of capsids present between cell types could certainly not account for the 25 fold differential in titers for these cell types. We therefore continued our analysis to look for other differentials.



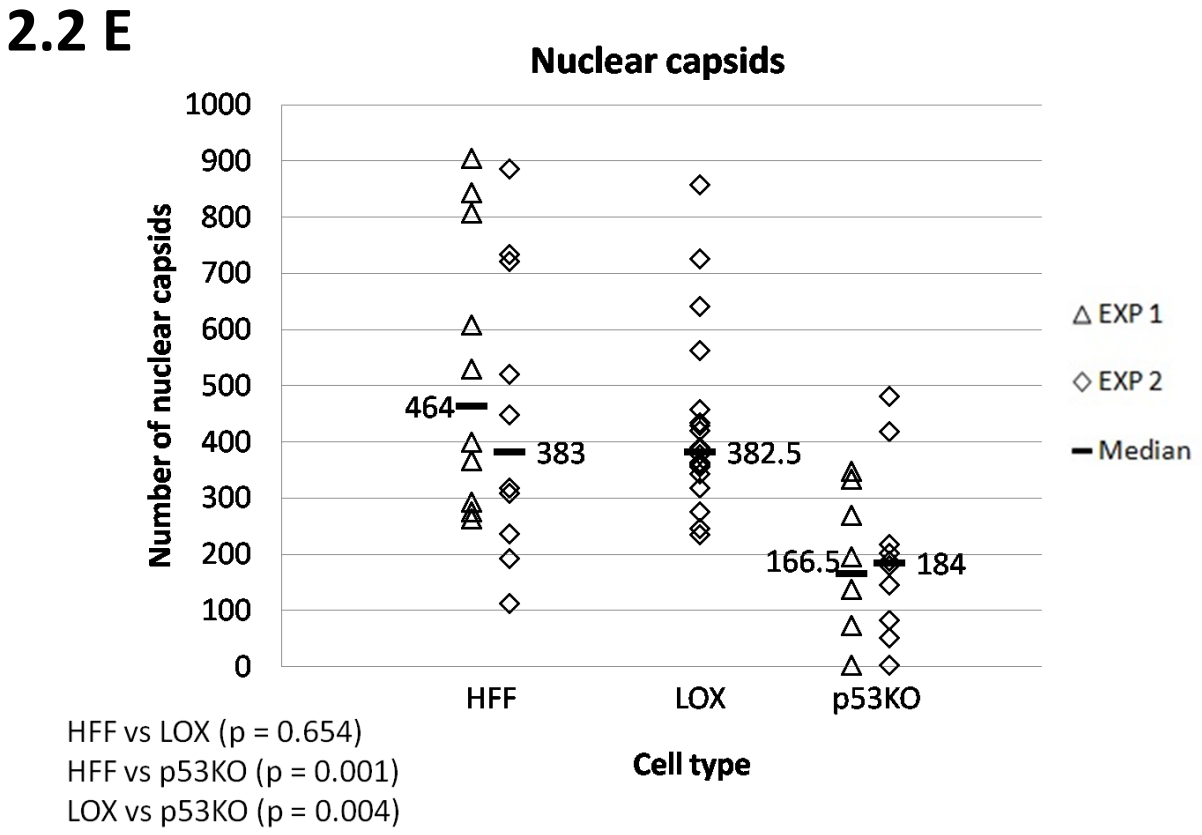
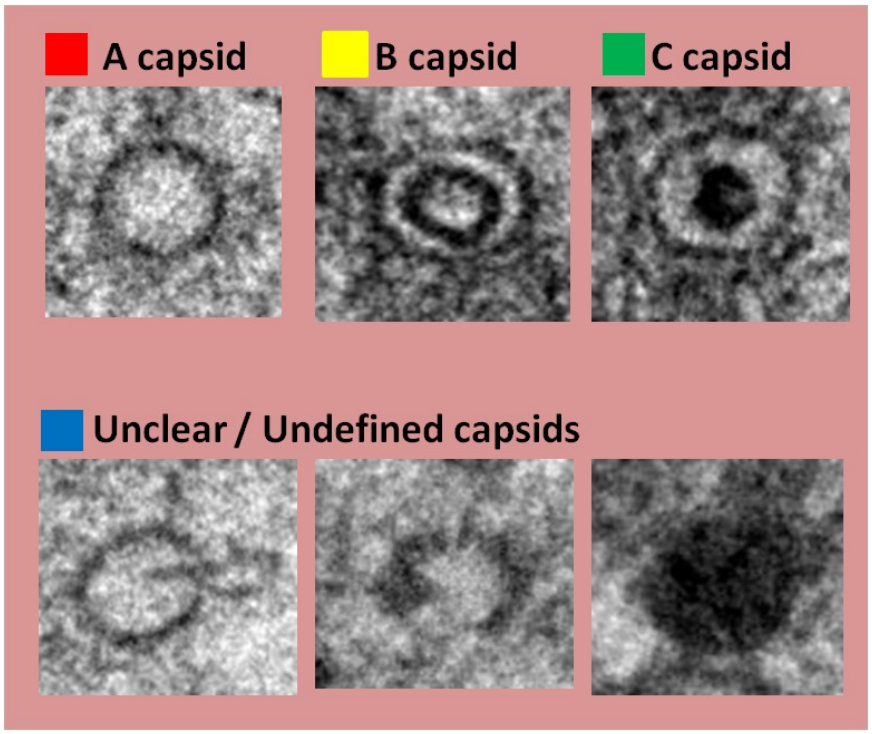


Figure 2.2 Quantitation of capsid production in the nucleus in wt cells compared to p53KO cells. Electron micrographs of infected cells show replication centers with numerous HCMV capsids. Cross sections of entire cells were captured at a magnification of 1,700X (A and C). The insets display the nuclear capsids in Lox (panel B) and p53KO (panel D) at 120 hpi at a magnification of 5,000X. (E) Quantitation of capsids in the nucleus in wt cells (HFF and LOX) compared to p53KO cells in two separate experiments. Each symbol represents an individual analyzed cell in this and all the following figures. Experiment 1 (EXP 1) used HFF as the wt comparison to p53KO cells. Experiment 2 (EXP 2) used both HFF and LOX wt cells compared to p53KO cells. Median capsid counts for each cell type in a given experiment are marked with a horizontal bar in this and all the following figures.

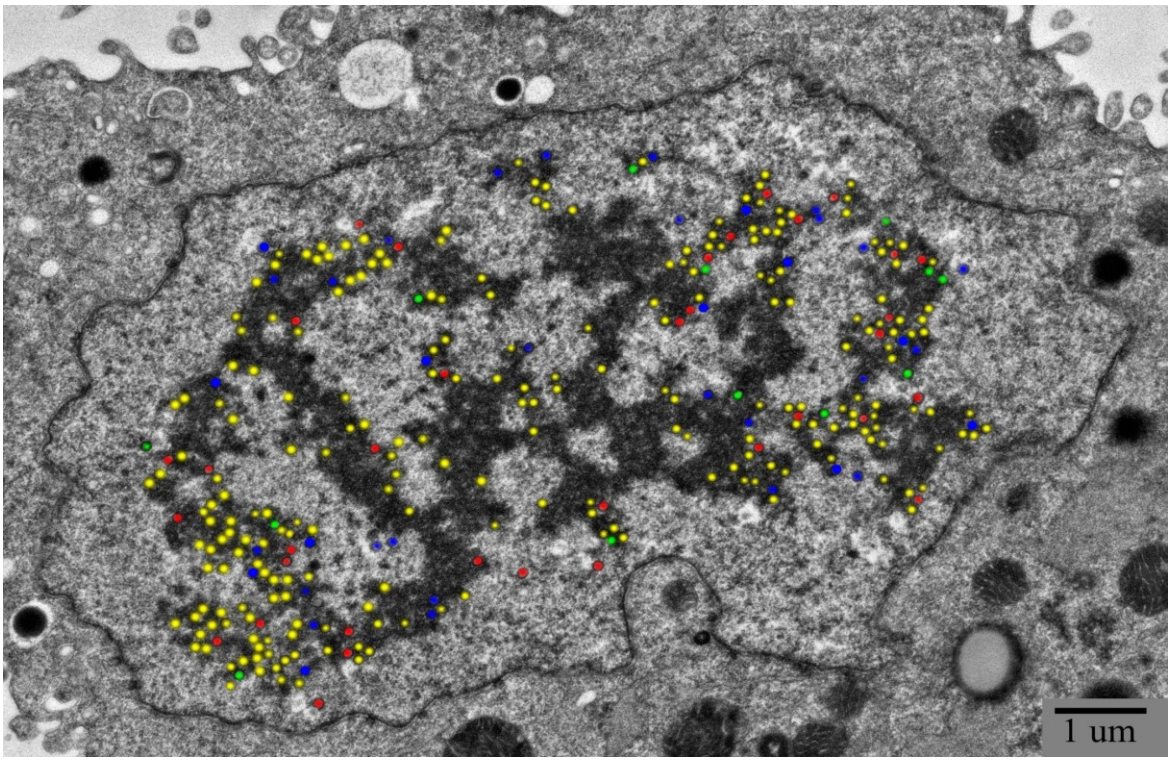
2.4.3 Conversion of capsid B to C was equivalent between wt and p53KO cells.

Electron micrographs were also used to distinguish the types of capsids present in the nuclei of wt and KO cells at 120h pi infection. We used broadly accepted criterion to identify A, B and C capsids (reviewed in reference Mocarski 2001), examples of which can be seen in Figure 2.3A (top panels). We included a fourth category, undetermined (hereafter U capsids), which encompassed a large range of morphologies with the defining characteristic of not fitting clearly into any one of the accepted categories or those capsids not clearly enough imaged to confidently identify. We suspect the U capsids were largely those in transitional states between B and C capsids, since many appeared to be in the process of packaging DNA, as seen by other groups (Gibson 2008) (Fig 3A – bottom panels). An example of our categorizing of capsid types is shown in Figure 2.3B. Figure 2.3C displays the distribution of capsids A, B, C, and U in all three cell types (HFF, Lox and p53KO). U capsids were present in similar percentages in all cell types, and therefore, did not distort comparisons between the other capsid types. According to most models (Chan, Brignole et al. 2002; Loveland, Chan et al. 2005), the scaffolding proteins must be removed from B capsids before, or as, DNA is packaged into the capsid shell. Therefore, we used the ratio of B capsids to C capsids as a measure of the DNA packaging efficiency. At 120h pi the ratio of B to C capsids in KO cells was equivalent to the ratio in both wt cells and they had no significant difference (see Figure 2.3C for p-values). In the absence of p53 the KO cells were capable of packaging DNA as efficiently as both wt controls.

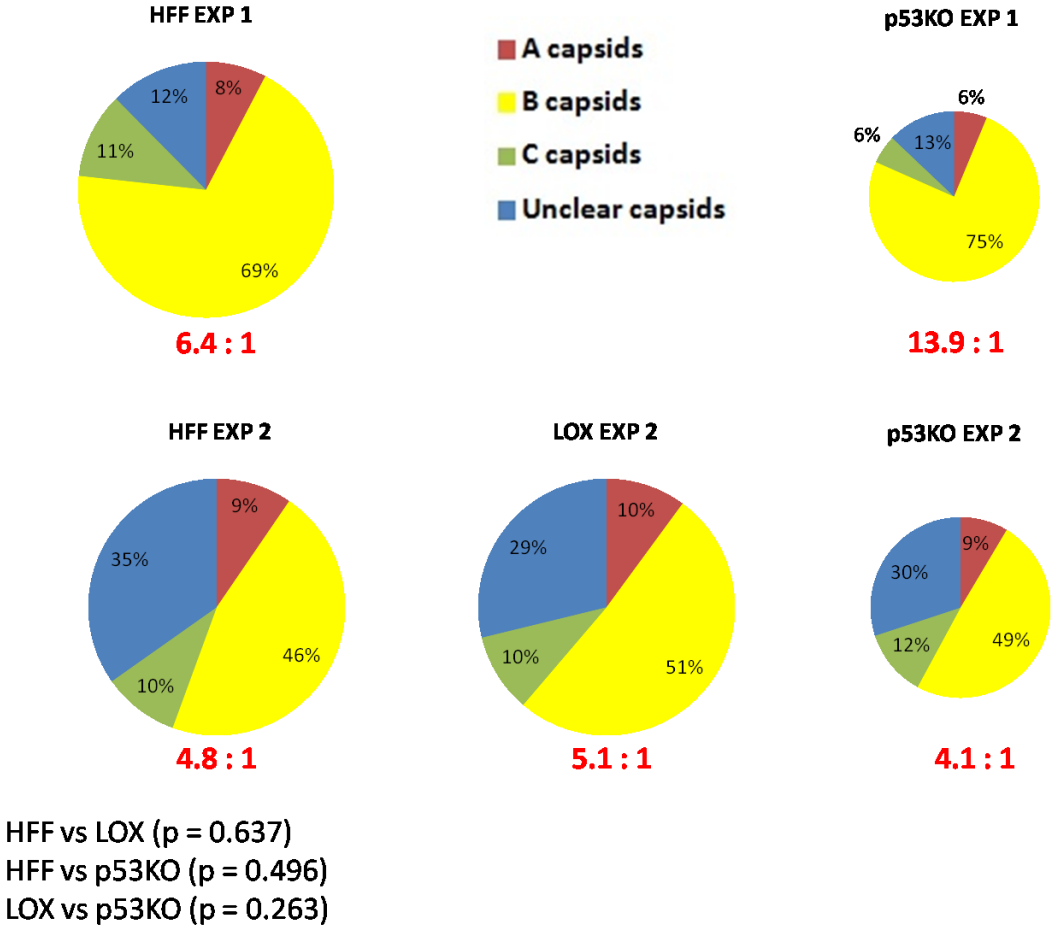
2.3 A



2.3 B



2.3 C



HFF vs LOX (p = 0.637)
HFF vs p53KO (p = 0.496)
LOX vs p53KO (p = 0.263)

Figure 2.3 Identification and quantitation of capsid types A, B, C and U in wt cells compared to p53KO cells. (A) Examples of A, B, C and U (Unclear) capsid types as identified. Capsids were labeled red, yellow, green, and blue, respectively. (B) Example of capsid type analysis. 5,000X magnification was used. (C) Quantitation of A, B, C and U capsids in wt cells compared to p53KO cells. The ratios below each graph represent the B: C ratio that is determined by dividing the average number of B capsids by the average number of C capsids for a given cell type in each experiment. The size of each pie chart represents the medians of the total number of capsids found in the nucleus (see Figure 2.2E). EXP 1 used HFF and p53KO cells. EXP 2 used HFF, LOX and p53KO cells.

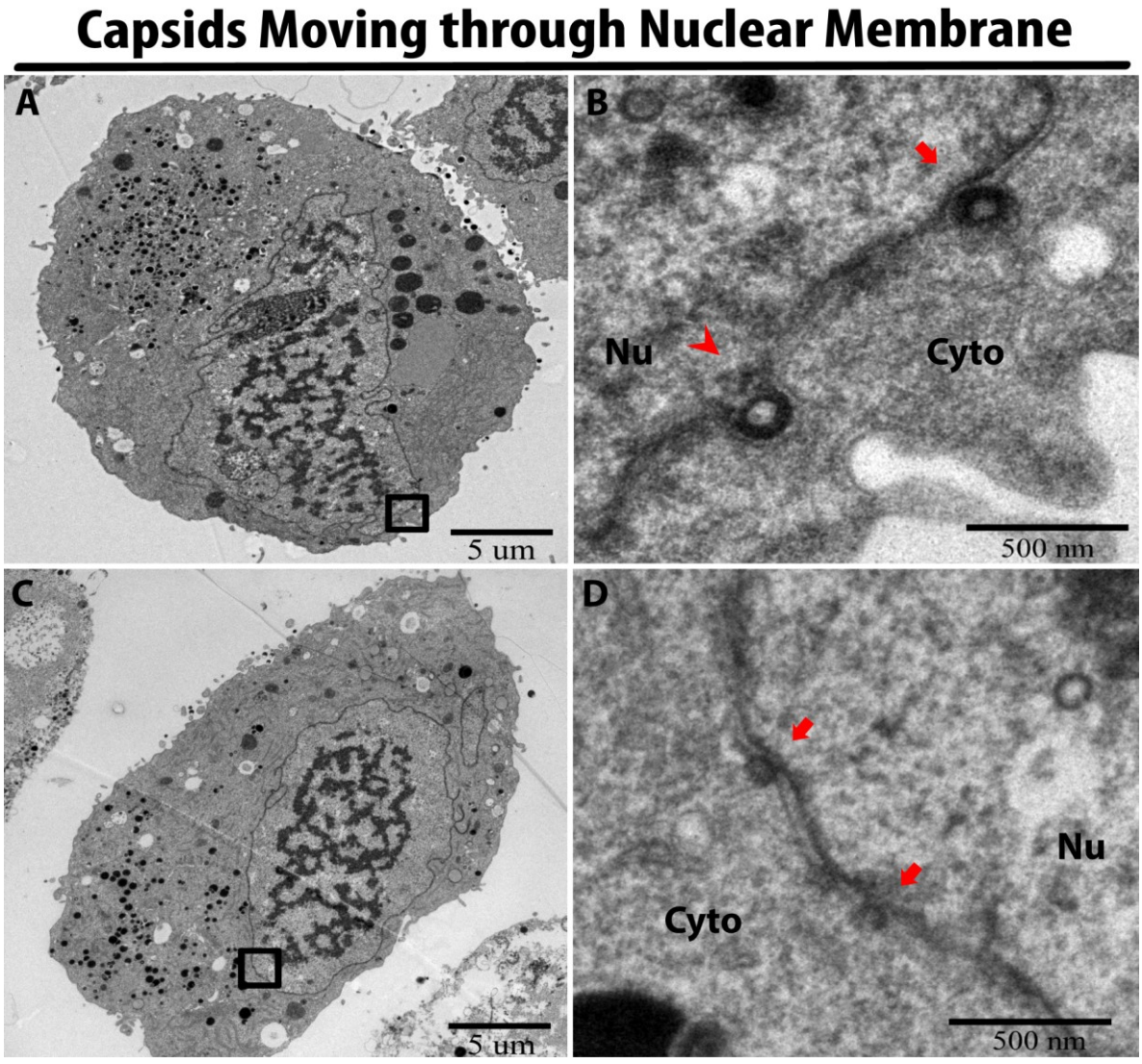
2.4.4 Trafficking of capsids through the nuclear membrane was less efficient in p53KOs.

Our previous studies found that in the majority of p53KO cells the tegument protein pp65 failed to relocate from the nucleus to the cytoplasm by 96h pi (Casavant, Luo et al. 2006). pp65 is the most abundant viral protein in virions. At the early stage of infection, pp65 is delivered to the nucleus independent of the capsid via its nuclear localization signal (NLS) (Revello, Percivalle et al. 1992; Schmolke, Drescher et al. 1995; Arcangeletti, Rodighiero et al. 2011). Starting from 48 hpi, pp65 translocates into the cytoplasm (Sanchez, Greis et al. 2000; Sanchez, Mahr et al. 2007). If the location of the majority of this protein was not in the cytoplasm, it seemed plausible that nuclear capsids might not be transiting out of the nucleus either. Nuclear egress involves envelopment/de-envelopment as capsids cross the nuclear membrane. We returned to our electron micrographs and counted the number of capsids trafficking out of the nucleus (shown in Figure 2.4A-D; B and D are higher magnifications of the boxed areas in A and C). We defined trafficking capsids as those capsids that were 1) directly adjacent to the inner nuclear membrane (Figure 2.4B, arrowhead), 2) found within the perinuclear space (Figure 2.4B, arrow) and 3) capsids still at least in contact with the outer nuclear membrane and perhaps not actually yet deposited into the cytoplasm (Figure 2.4D, arrow heads).

The number of capsids found in these locations varied considerably, ranging in these two separate experiments and three different cell types from as few as zero

to a high of more than 17 in each of the different environments (Figure 2.4E). Within each experiment the median number of the perinuclear capsids were very similar.

2.4 A-D



2.4 E

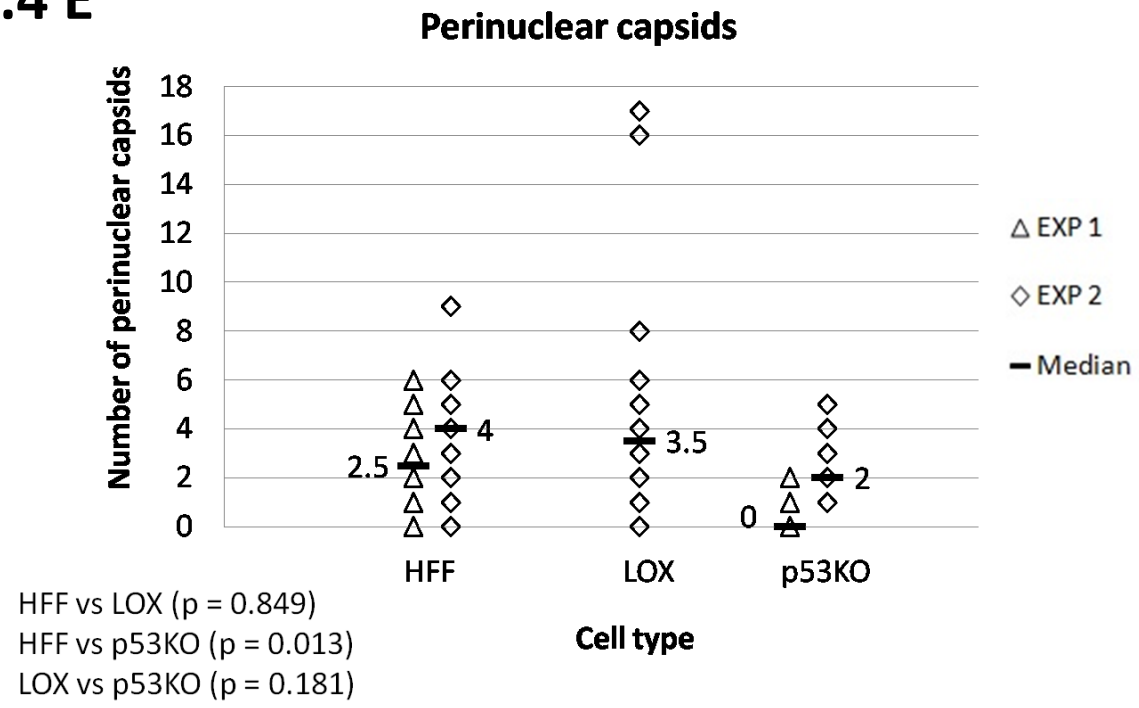
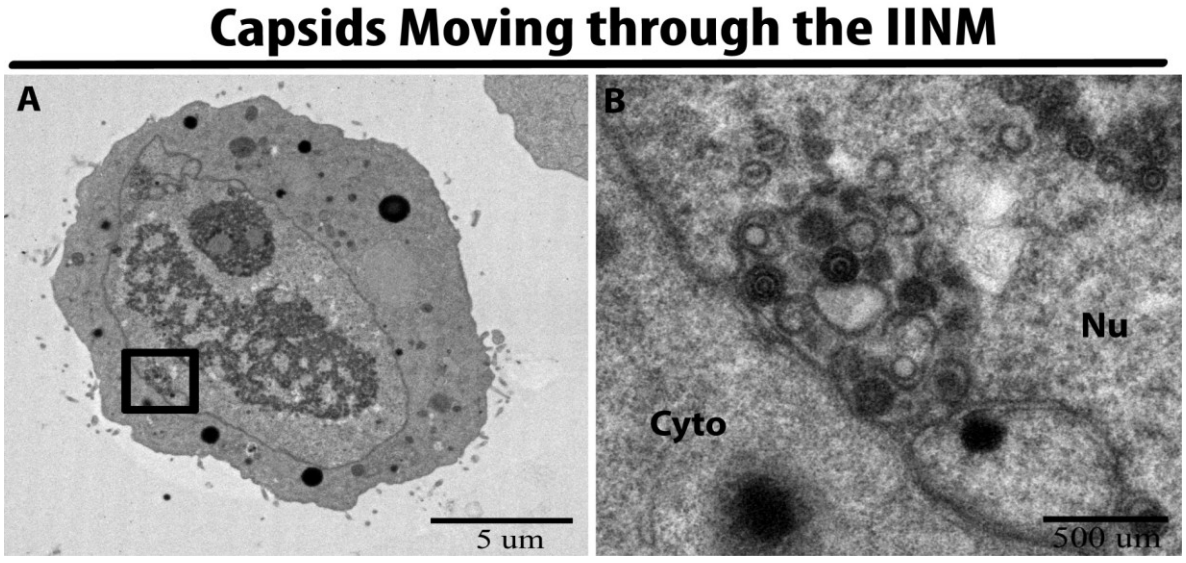


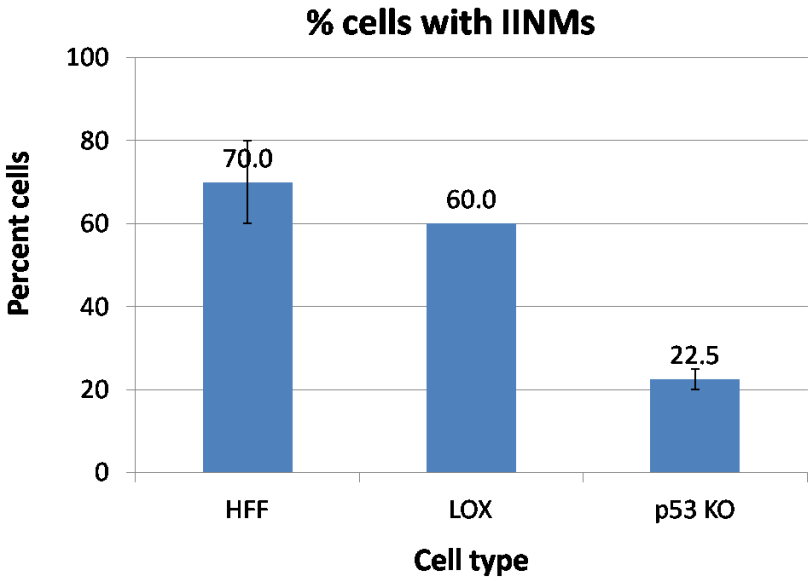
Figure 2.4 Capsids moving through the nuclear membrane. Whole cells were captured at a magnification of 1,700X (A and C). The insets display capsids trafficking into and out of the perinuclear space at a magnification of 5,000 X (B and D). Perinuclear capsids budded through the inner nuclear membrane (B; arrow head). Perinuclear capsids were also located within the perinuclear space (panel B; arrow) or immediately adjacent to the outer nuclear membrane (panel D; arrows). Nu = nucleus; Cyto = cytoplasm. LOX cells are shown. (E) Quantitation of capsids in the perinuclear space in wt cells compared to p53KO cells. EXP 1 used HFF and p53KO cells. EXP2 used HFF, LOX and p53KO cells.

While conducting this analysis and searching the literature it came to our attention that an additional portion of the nuclear capsid population should be included in the actively trafficking category. This second category included all capsids within infoldings of the inner nuclear membrane (IINMs), structures that have been observed by others to contain enveloped capsids (Ruebner, Miyai et al. 1964; Papadimitriou, Shellam et al. 1984; Severi, Landini et al. 1988; Gilloteaux and Nassiri 2000; Dal Monte, Pignatelli et al. 2002; Buser, Walther et al. 2007). Buser *et al*. specifically suggested that these tubular infolding structures were sites of the majority of trafficking capsids (Buser, Walther et al. 2007). These structures (pictured in Figure 2.5A and B) were present in many more of wt cells (an average of 70% of HFF and 60% of Lox contained IINMs) than in the p53KO cells (~20%) (Figure 2.5C).

2.5 A-B



2.5 C



<X-square test>
HFF vs LOX (p = 0.507)
HFF vs p53KO (p = 0.003)
LOX vs p53KO (p = 0.019)

Figure 2.5 Capsids exiting the nucleus via infoldings of the inner nuclear membrane (IINMs). Whole cell was captured at a magnification of 1,700X (A) and IINMs were observed at a magnification of 5,000X (B). Nu = nucleus; Cyto = cytoplasm. LOX cells are shown. (C) Percentage of cells containing IINMs. EXP 1 used HFF and p53KO cells. EXP 2 used HFF, LOX and p53KO cells. Error bars represent one standard error calculated from two experiments.

2.4.5 A detailed analysis of the IINM including cells with reconstituted p53

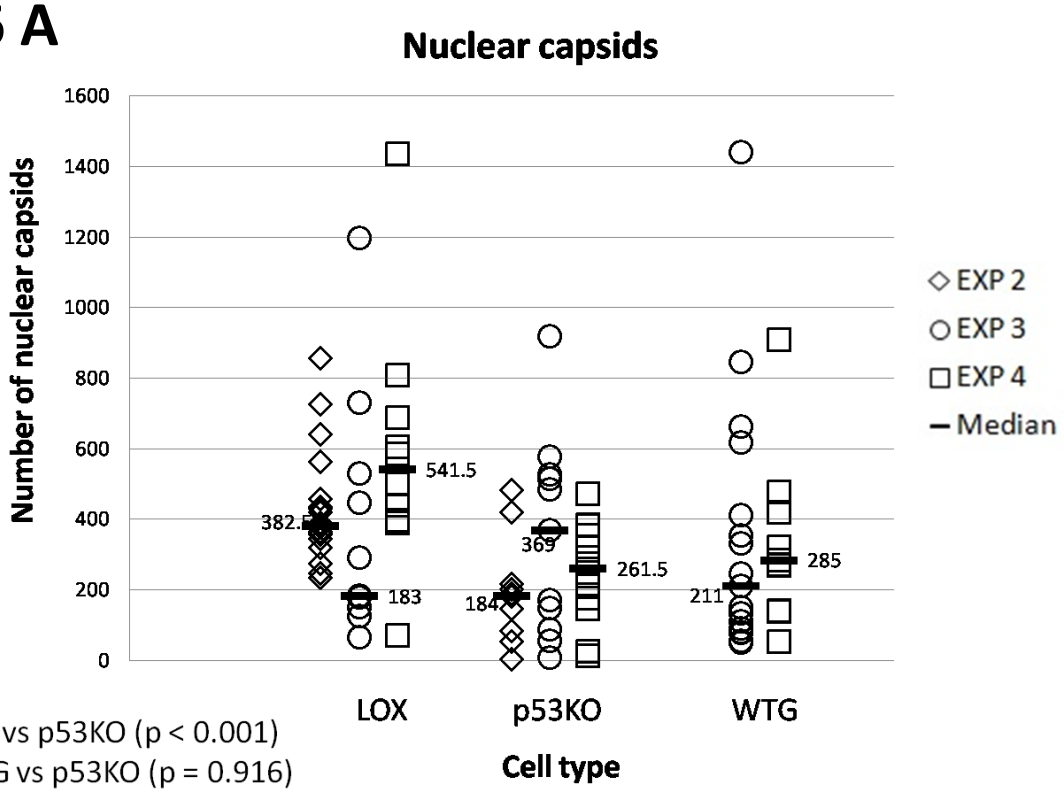
The presence and capacity of IINM tubules was the only parameter found within the continuum of capsid development and trafficking inside the nucleus up to this stage to even approach the degree of disparity in the titer data between the wt and p53KO cells. The suggestion of Buser et al. (Buser, Walther et al. 2007) that these IINM constituted the major route of capsid exit from the nucleus indicated a closer examination of these structures was in order. Given the relatively infrequent occurrence of these structures in any individual microtome section we felt it was necessary to increase our experimental sample size. Two additional experiments were performed. Since both wt cell types had behaved nearly identically to this point, further analysis was limited to study of the p53KO cells and their parental Lox cells. In addition, in order to assess the role that p53 might be playing, a p53 reconstituted KO cell line was included.

As described in our previous work, p53 was transfected into the KO cells to establish several p53-expressing stable clones. The p53-expressing lines were designated WT A-H. All the lines produced similar, and quite low, levels of p53. Any expression of p53 above very low levels appeared to be lethal to these cells (Casavant, Luo et al. 2006). All the stable lines behaved similarly with respect to all parameters tested. Titers from infection of these cell lines were reduced approximately two to three fold from wt, but increased approximately 10 fold over their p53KO parental strain (Casavant, Luo et al. 2006). In this study, we captured ultrastructural EM images of infected WTG cells, as well as wt and p53KO cells in parallel infections. These images were analyzed in the same manner as described

above. Since the absence of p53 had not altered the proportional representation of A,B,C and U capsids, we did not identify individual capsid types in these parallel experiments.

Two additional experiments (experiments 3 and 4) and EM analyses were performed, each using the three different cell types (wt, p53KO and WTG). In order to ensure that our initial evaluations were valid, the numbers of capsids within the nucleus and perinuclear space were enumerated. As can be seen in Figures 6A (nuclear capsids) and 6B (perinuclear capsids), there were only minor differences between the three cell types as observed previously (Figure 2.2E and 2.4E).

2.6 A



LOX vs p53KO ($p < 0.001$)
WTG vs p53KO ($p = 0.916$)
LOX vs WTG ($p = 0.091$)

2.6 B

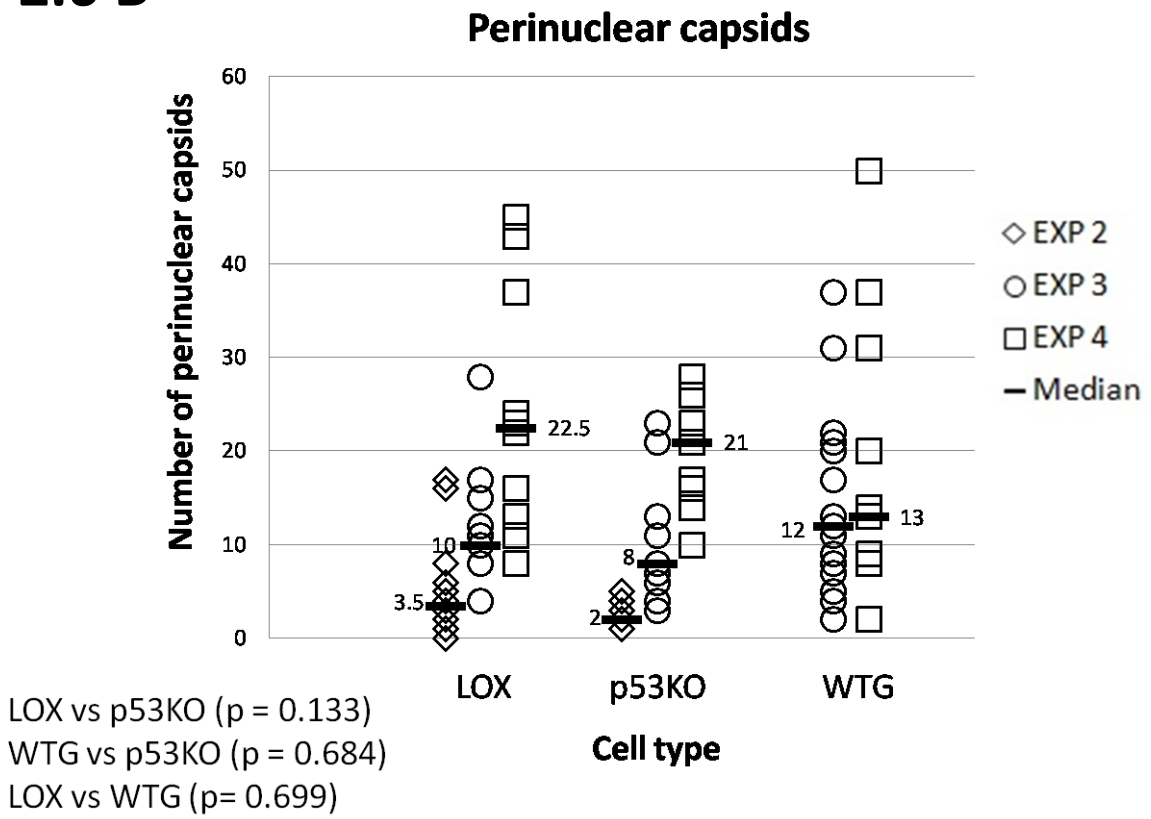
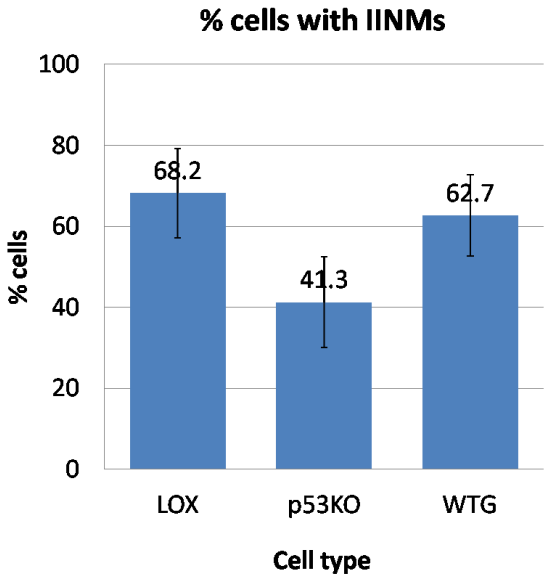


Figure 2.6 Number of nuclear and perinuclear capsids in LOX compared to p53KO and WTG cells. Numbers of nuclear capsids (A) and perinuclear capsids (B) are compared in three cell types in three separate experiments (EXP 2, 3, and 4). Hereafter, LOX and p53KO cells were used in EXP 2, 3, and 4. WTG was used in EXP 3 and 4. Note that the data from EXP 2 was already shown in Figures 2 and 4 above.

Next the presence and content of the IINMs in the three cell types was evaluated. Figure 2.7A shows that the number of cells with IINMs increased from an average of ~40% in the KO population to an average of ~60% in the WTG population (matching the wt percentage). The IINMs were examined more closely to identify any further differences between the cell types. Figure 2.7B displays the wide range in the numbers of IINMs/cell. Although there appeared to be a minor increase in the median number of IINMs/cell in the WTG cells compared to the KO cells, analysis of the differences between the medians was not statistically different (see Figure 2.7B for p-values).

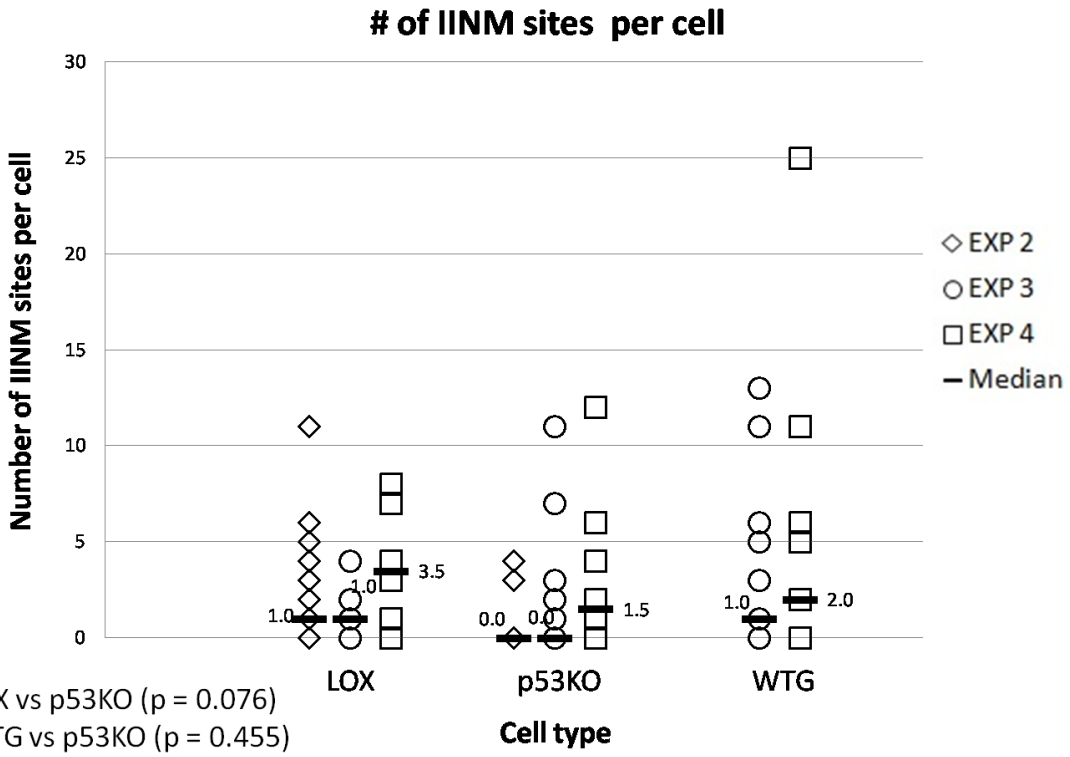
Interestingly, in all cell types the IINMs were all almost completely full of capsids, with very little unoccupied space within the tubules. This was true of the largest and smallest tubes. In some instances the capsids could be identified. All three types of capsids were seen within the tubules, with no discernible pattern of distribution. The three types of capsids appeared equally likely to enter the tubules. The absence of any “empty” space inside the tubes suggested that they were formed and extended by the entrance of capsids into these extensions of the perinuclear space. We therefore looked to see whether there were differences in the average number of capsids found within these IINM on a per cell basis for each cell type. There was a substantial difference between the Lox and p53KO cells in this parameter. Although slightly increased, the difference between WTG and p53KO was not substantial (Figure 2.7C). The reintroduction of p53 appeared to have increased the number of cells that containing IINMs, but the infoldings remained small when compared to wt.

2.7 A



<X-square test>
LOX vs p53KO (p = 0.044)
WTG vs p53KO (p = 0.163)
LOX vs WTG (p= 0.613)

2.7 B



LOX vs p53KO (p = 0.076)
WTG vs p53KO (p = 0.455)
LOX vs WTG (p= 0.985)

2.7 C

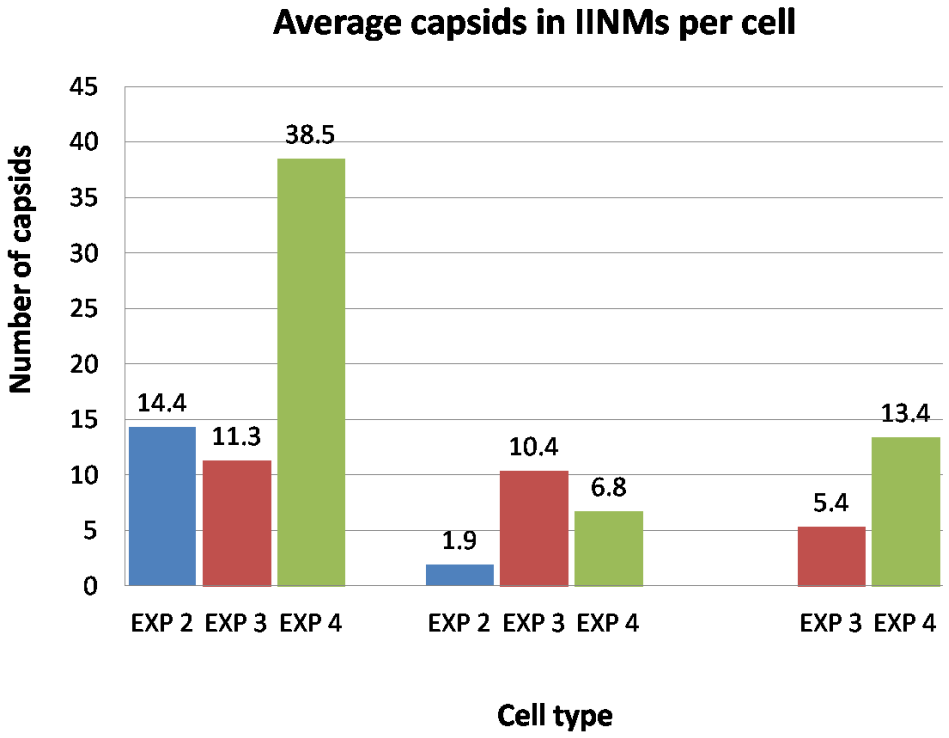
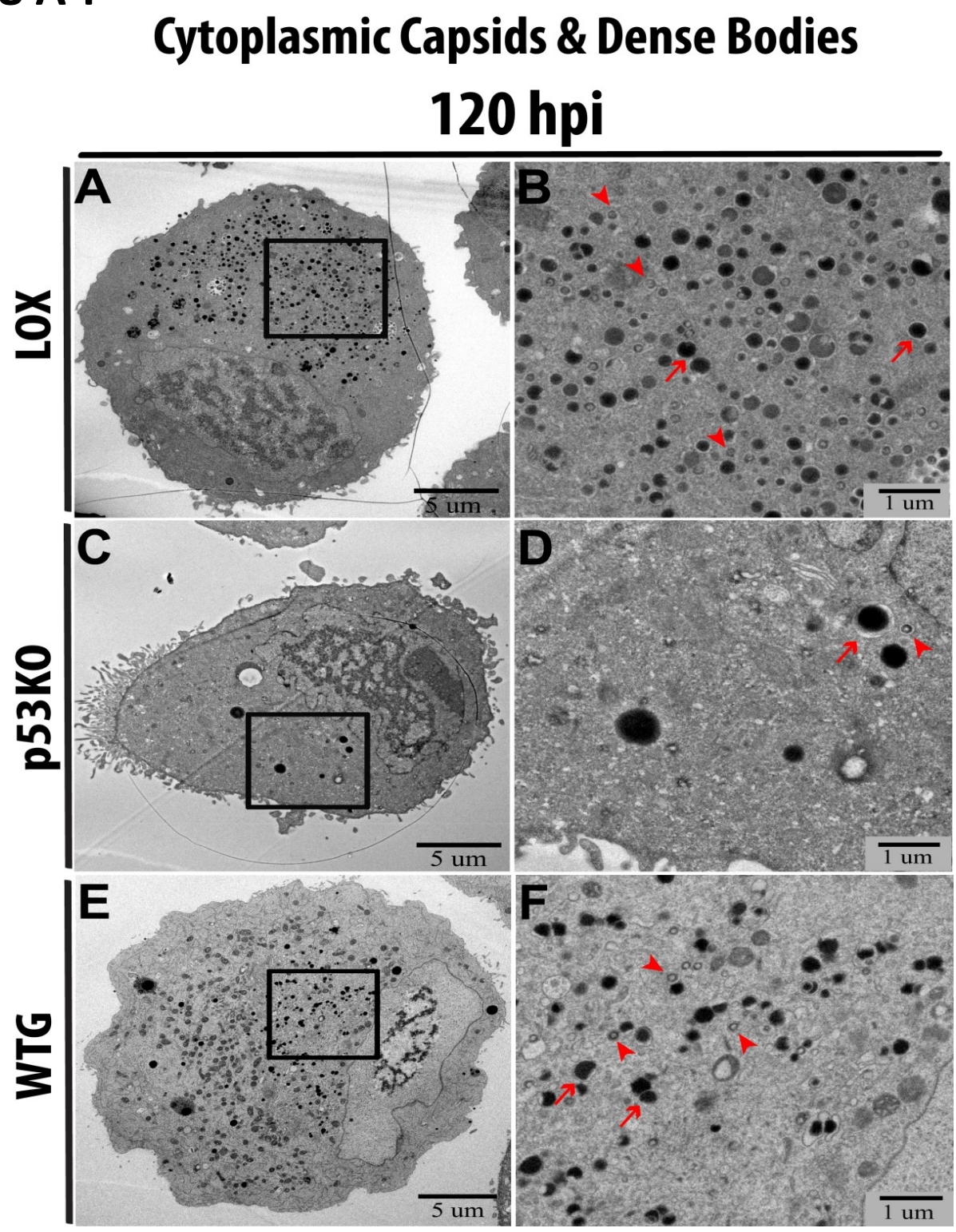


Figure 2.7 Investigation of cells with IINMs and capsids in these structures. (A) Percentage of cells containing IINMs in LOX, p53KO, and WTG. Error bars represent one standard error calculated from two (WTG) or three (Lox and KO) experiments. (B) Number of IINMs per cell. All cells in each experiment are represented whether they contain IINMs or not. (C) Average number of capsids contained in IINMs per cell. Numbers were calculated by dividing total number of capsids in IINMs by number of cells for a given cell type in a given experiment.

2.4.6 Capsids were largely absent in the cytoplasm of p53KOs.

After analyzing the number of capsids transiting the nuclear membrane, we continued our enumeration out into the cytoplasm for each cell type. Large numbers of virions were found in the cytoplasm in the wt Lox cells (Figure 2.8A and B). Cytoplasmic virions are indicated by the red arrowheads in Figures 2.8B. Virions were largely absent from the cytoplasm of p53KOs (Figures 2.8C and D; notice the lack of arrowheads). At 120 hpi, noticeably more capsids were present in the WTG cells than in the KOs (Figure 2.8E and F; compare D and F). By 120h pi, the median number of capsids found in the cytoplasm of infected wt cells ranged from approximately 45-90 (Figure 2.8G). In contrast, medians of approximately 12-13 capsids were found in the cytoplasm of infected p53KOs, a difference that was statistically significant ($p < 0.0001$). This 5.3 fold differential considerably exceeded the ~2 fold differential of capsids found inside the nucleus. Clearly the nuclear "egress gate" was not as widely opened in the KO cells. The median number of capsids in the cytoplasm of the WTG cells, median of ~21-33, was significantly increased over that found in the KO cells ($p = 0.0027$), indicating that reintroduction of p53 enhanced the ability of the capsids to exit the nucleus.

2.8 A-F


Cytoplasmic Capsids & Dense Bodies
120 hpi
LOX
p53KO
WTG
A
B
C
D
E
F
5 um
1 um
5 um
1 um
5 um
1 um

2.8 G

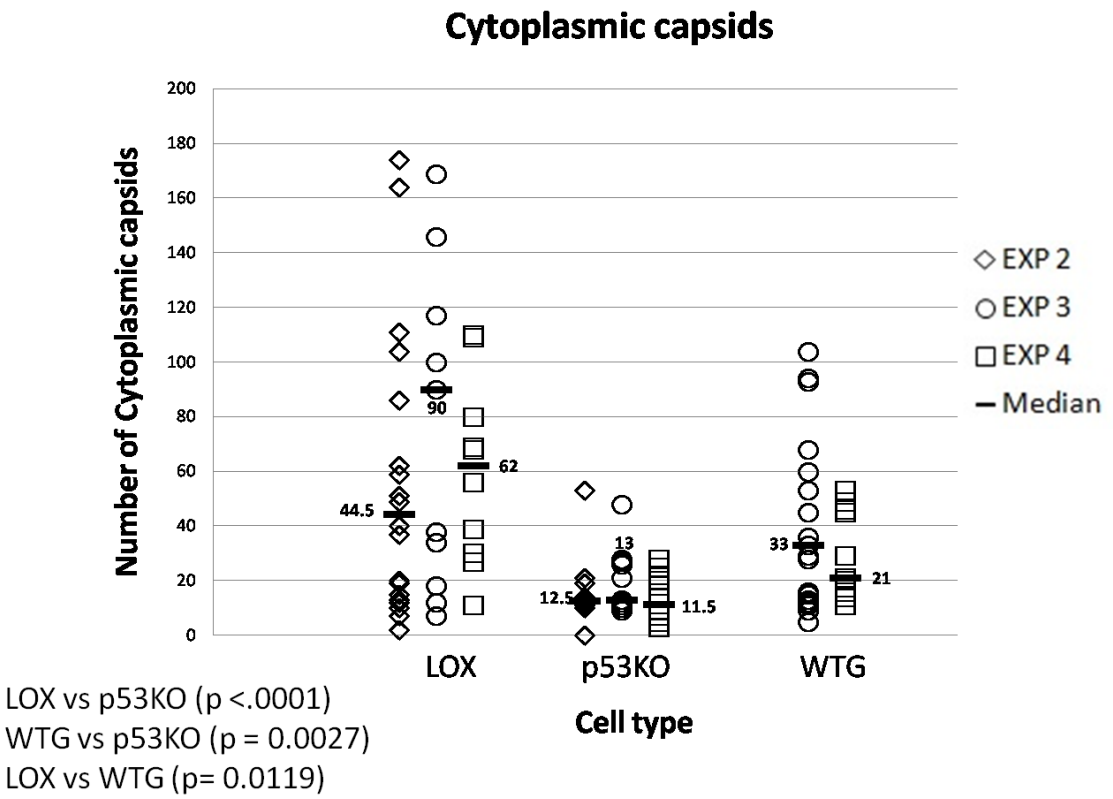
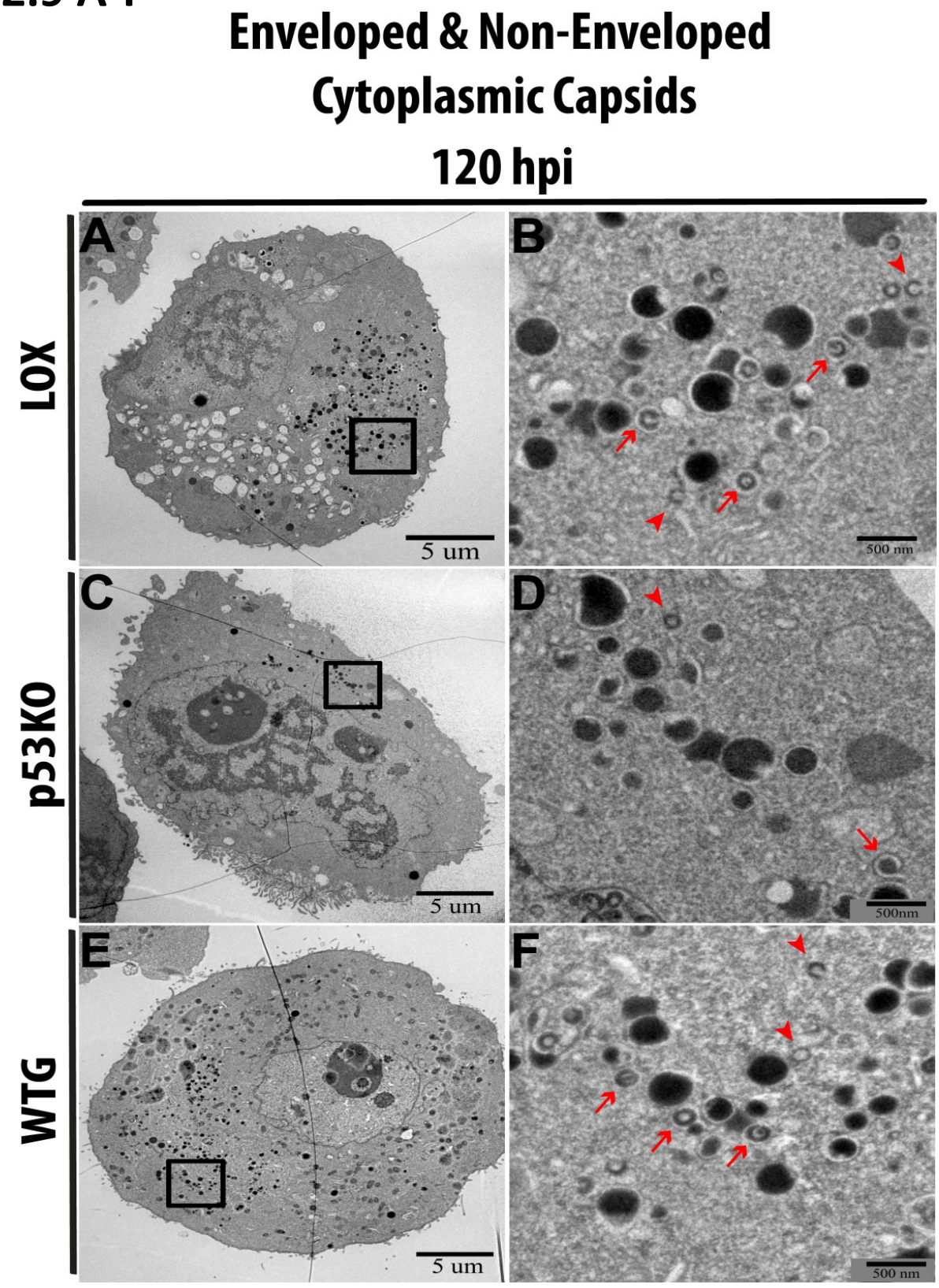


Figure 2.8 Cytoplasmic capsids and dense bodies. Whole cells were captured at a magnification of 1,700X (A, C, and E). Insets display cytoplasmic regions in LOX, p53KO and WTG cells at 120 hpi at a magnification of 2,500X (B, D, and F). Arrows indicate dense bodies; arrow heads indicate capsids. (G) Quantitation of capsids in the cytoplasm in LOX, p53KO, and WTG.

2.4.7 Fewer of the capsids present in the cytoplasm of p53KO cells were enveloped.

The reduced virus-induced production of nuclear membrane “portals” was mirrored in the cytoplasm. Dense bodies (DBs) (arrows in Figure 2.8B, D, and F) were present in approximately similar proportions, capsids/DBs, in the three cells types (data not shown). This indicated that the presence of these non-functional virus particles was not disproportionately reducing the prevalence of functional virions in KO cells compared to wt. However, the proportions and numbers of re-enveloped capsids (Figure 2.9A-F) in the cytoplasm were higher in the wt cells (Figure 2.9G). The ratio of all cytoplasmic capsids to re-enveloped capsids in wt cells was approximately two to one (2:1) and seven to one (7:1) in the p53KO cells. In absolute numbers the difference was even more marked, with an average median of 16 fold more re-enveloped capsids present in the cytoplasm of the wt cells than KO cells. Re-introduction of p53 into the KO cell line also increased the number of membrane-enveloped capsids found in the cytoplasm. The number of enveloped capsids in WTG cells increased an average of seven fold over their p53KO parental cell line. The absence of p53 allowed only reduced virus-triggered membrane production, inhibiting the normal cytoplasmic re-envelopment of capsids, thereby contributing to the drastically reduced production of virions and virus particles.

2.9 A-F


Enveloped & Non-Enveloped
Cytoplasmic Capsids
120 hpi
LOX
p53KO
WTG
A
B
C
D
E
F
5 um
500 nm
5 um
500nm
5 um
500 nm

2.9 G

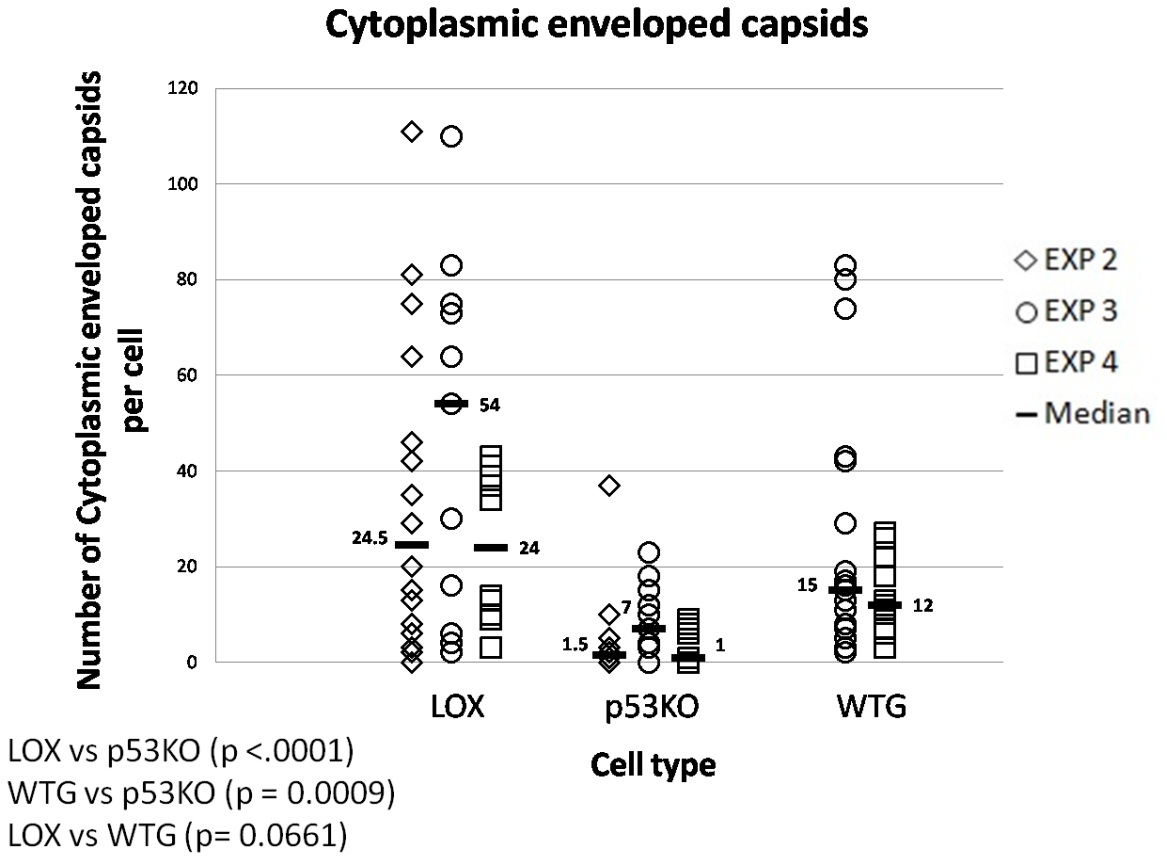


Figure 2.9 Enveloped cytoplasmic capsids. (A-F) Whole cells were captured at a magnification of 1,700X (A, C, and E). Insets display enveloped and non-enveloped capsids in cytoplasmic regions in LOX, p53KO, and WTG at 120 hpi at a magnification of 2,500X (B, D, and F). Arrows indicate enveloped capsids; arrow heads indicate non-enveloped (naked) capsids. (G) Quantitation of enveloped capsids in the cytoplasm in LOX, p53KO, and WTG cells.

2.4.8 Fewer viral particles were released from p53KOs.

To confirm the above results beyond the confines of individual cells medium was collected from infected Lox, p53KO and WTG at 120 h pi. The medium was ultracentrifuged for 1 hour to precipitate all viral particles. An equivalent number of cells produced substantially different sized pellets of viral particles (data not shown). These viral particles were resuspended in PBS and spotted onto EM grids. The grids were then negatively stained. Vastly more viral particles were released from the infected wt Lox cells than from the infected p53KO cells (Figure 2.10). Particles released from WTG displayed an intermediate phenotype, which was considerably increased from the KO cells, but not to the level of wt. While there were many fewer virions released from p53KOs, those virions present appeared indistinguishable in morphology from those released from the Lox cells. Unfortunately, the viral particles adhered to the EM grids in unevenly distributed patterns. The particles expressed by wt cells were found in many more higher density regions than p53KO cells. Random sampling of the highest density regions of each cell type found a range of 15 to 30-fold more wt particles than were released from the KO cells. This bracketed the original titer data and implicated the failure in the p53KO cells to efficiently traffick capsids into the cyotplasm by not forming adequate membraneous IINM structures or allow proper re-envelopment of those virus particles able to reach the cytoplasm as the probable cause for the titer decrease.

2.10

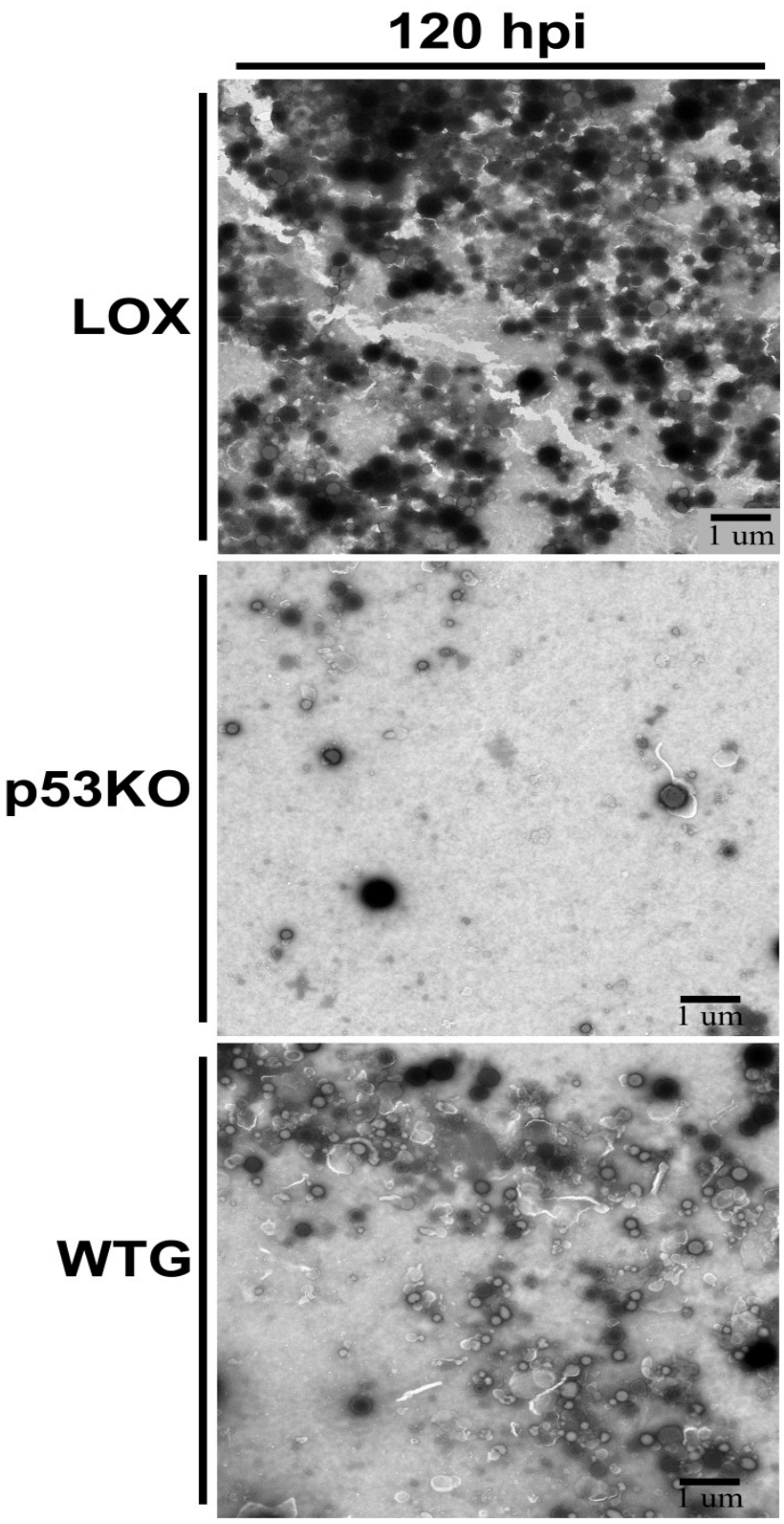


Figure 2.10 Negative staining of shed viral particles. Viral particles were observed at a magnification of 5,000X.

2.5 Discussion

In our previous work, we observed that virus titers of the wt cells were ~25-fold higher at 120h pi than those of p53KO cells. We had previously established that in p53KOs virus entry and IE gene expression were essentially normal (Casavant, Luo et al. 2006). We have extended those studies to systematically isolate various stages of HCMV development in order to discover if the substantial decrease in functional virus production in the absence of p53 could be attributed to any particular defect in the virus life cycle. The data presented here demonstrate that the failure of nuclear capsids to properly exit the nucleus was the most significant blockade to functional virus production, in particular, p53's absence largely prevented typical IINM formation. Additionally, the paucity of these membrane-derived structures in the p53KO cells was paralleled by the inefficient envelopment of capsids able to reach the cytoplasm. Preliminary results have indicated that, although p53KO cells upregulated lipid biosynthesis equivalently to wt cells, the lipid produced was not efficiently incorporated into new membrane, as evidenced by the accumulation of free lipid in droplets in the cytoplasm of the KO cells.

Standard plaque-forming assays found that while wt cells expressed reasonably large quantities of functional virus, only ~30% of the p53KOs expressed appreciable amounts; i.e., enough virions to form readily discernable plaques. The more sensitive fluorescence-based focus forming assays allowed us to determine that the very large majority of KO cells did in fact express virus, albeit at perhaps vanishingly low levels. This indicated that while the absence of p53 led to inefficient expression of functional virions, it did not render the virus incapable of replicating its

genome, producing/packaging capsids, or exiting from most of the KO cells. Where then were the blockades that could explain the 25 fold decrease in functional particle release?

EM images of the replication centers in wt cells were qualitatively examined and found the RCs to be generally larger and more dense than those present in KO cells. However, despite this disparity in morphology, analysis of the quantity of nuclear capsids in the KO cells found them decreased by an average of only ~2 fold compared to wt. Additionally, the ratio of B to C capsids in p53KOs at 120h pi was nearly identical in proportion to the wt populations, indicating equivalently efficient packaging in the absence of p53.

It is difficult to extrapolate even approximate numbers of capsids found within the entire volume of the nuclei of the cells used in these experiments. However, even admittedly very rough approximations could offer some useful insight. Given that the EM sections were ~100 nm thick, a reasonable guestimate of the volume of the entire sectioned nucleus would be 10 fold greater and, further, assuming an RC comprised ~50% of the volume of the nucleus, the number of capsids counted represented roughly 10% of the actual number within a given cell's nucleus. These approximations put the mean numbers of capsids present in a "generic" wt and KO cell at 2500 and 1000, respectively; numbers vastly in excess of those the titer data from either cell type found were shed over an entire 120h time course (~50 functional virions from wt cells and only ~2 from KO cells). Undoubtedly, many of the capsids present were empty (NIEPs) or contained damaged/incomplete virus genomes, however, the virus's DNA replication and capsid packaging machinery

seemed to function at rates far exceeding the later stages of the virus life cycle. More pertinent to this study, a far greater percentage of properly packaged nuclear capsids were ultimately shed from the wt cells. The modestly reduced average number of capsids found within the nuclei of p53KO cells fell well within the range of wt production and the conversion from B to C capsids was equivalent in both cell types. Thus the modest reduction in nuclear capsid production could not account for the greatly reduced capacity of the knockout cells to shed functional virus.

Quantitative analysis of capsids/virions in the cytoplasm found ~5.3 fold fewer of these particles in KO cells compared to wt. Interestingly, there was no correlation in either wt or KO cells between capsid production within the nucleus and the number of cytoplasmic capsids/virions. Analysis of the EM images had not suggested any delay in the production of nuclear capsids in the p53KO cells at late times, which might have led to reduced transit into the cytoplasm. Each EM section served as a snapshot of activity at a given moment. If all systems were functioning properly, the presence of near wt-like numbers of capsids within the nucleus of KO cells by 120h pi would have been expected to elicit wt-like nuclear membrane transit, which has been suggested to be a rapid process (Buser, Walther et al. 2007). If nuclear egress were not inhibited we would have expected analysis of the EM snapshots to produce more similar medians of cytoplasmic capsid quantities in the two cell types by 120h pi. Our data suggested that the absence of p53 was abrogating viral remodeling of the inner nuclear membrane and the formation of IINMs. Our parallel protein localization study showed that the lamin structure in KO

cells was less disrupted than wt suggesting interaction between p53 and the nuclear egress complex (NEC).

Invaginations in the inner nuclear membrane have been reported in the cytomegaloviruses to be the site of the most prolific capsid nuclear exit (Buser, Walther et al. 2007). We observed that only approximately 20-40% of the p53KOs contained IINMs. This group likely represented the majority of the ~30% found expressing virus by conventional plaque assay (i.e., the cells with the highest level of secretion).

Certainly the literature regarding the use of extended tubes or IINM for trafficking of capsids is most abundant for the beta herpesviruses, although there have also been indications of their use in the alpha herpesvirus PrV (Klupp, Granzow et al. 2007). Much of the literature on HSV and PrV (both alphaherpes viruses) indicate that these viruses traffic directly through a remodeled INM without the formation of tubules (Klupp, Granzow et al. 2000; Roller, Zhou et al. 2000; Reynolds, Ryckman et al. 2001; Fuchs, Klupp et al. 2002; Reynolds, Wills et al. 2002). In addition, it has been shown that the UL50/UL53 equivalents in these viruses are capable of distinct deformation of membranes, indicating that these components of the NEC can accomplish the primary envelopment process alone (Bigalke, Heuser et al. 2014). However, despite this apparent difference, it is clear that all the herpesviruses require remodeling of the lamina and that they utilize components of the NEC, and perhaps other cellular components, to accomplish this task. Lacking formation of the tubules, HCMV capsid trafficking through the INM becomes, at best, exceedingly inefficient.

It should be noted that identifying capsids in the cytoplasm was much less precise than within the nucleus. This was due in part to the necessity of using lower magnification to capture the entire cytoplasm of each cell using a manageable number of individual images. Additional layers of tegument, envelope and many more obscuring cellular structures all contributed to difficulties in identification of capsid types, despite these hindrances capsids could be identified generically in the cytoplasm. Use of higher magnification would also have greatly increased the data gathering time and forced use of a much smaller sampling of cells. Due to the phenotypic variability observed within the p53KO cells in our earlier experiments, we decided that a larger sample size would produce the most robust data set.

As mentioned above, approximately 5.3-fold fewer capsids were identified in the cytoplasm of KO cells. Egress from the nucleus was significantly inhibited, but not to the degree of the ~25-fold reduced titers. DBs were found in both cell types in similar proportions to capsids, although the absolute number of DBs was higher in the wt cells (see Fig 9 for an example of prevalence). This indicated these non-replicative virus particles were not diluting the presence of functional virions disproportionately in the KO cells. Inhibition of synthesis of IINM membrane in the KO cells suggested that perhaps less membrane overall (and Golgi-derived membrane in particular) might be available in the KO cells for re-envelopment of virions. Re-enveloped virions were 16-fold more common in wt cells compared to KO. It appeared that p53's absence not only inhibited virus-mediated membrane production of IINMs within the nucleus and their associated capsid egress, but also compromised the virus's capacity to force its host to produce sufficient additional

membrane for wt-like levels of virion re-envelopment. The reduction in membrane production in KO cells may have been caused by the reduced capsid trafficking and a consequentially reduced signal to ramp up membrane production, or a propagated effect which did not allow lipid incorporation into new membranes, as evidenced by a large accumulation of lipid droplets within the p53KO cells. In either case it was noteworthy that the IINMs of both cell types were almost entirely full of capsids. This suggested that these tubule-like structures were formed as the result of the entrance of capsids into these extensions of the perinuclear space. Extrapolation of the formation of tubes in this manner further suggests that the “remodeling” of the nuclear lamina could perhaps more properly be described as a rearrangement or dispersal of the phosphorylated lamins via on going extension and expansion of the IINMs. The phosphorylated lamins were unzipped and capable of being moved, but a separate process seems to have been responsible for the actual large lamin rearrangement observed in wt cells.

The reintroduction of p53 recovered nearly all the stages of the viral life cycle examined, including the presence of tubules in the WTG population. However, an exception to recovery was the reduced size of IINMs formed in the WTG cells. Although individual IINMs were slightly more numerous within the WTG cells than in the KO parental line, they were at most comparable, or even reduced, in size. This suggested that, while reintroducing a very small amount of p53 enabled nuclear capsids to traffic into the cytoplasm in increased numbers, it was not sufficient to fully reconstitute the formation of IINMs to wt numbers, sizes or trafficking

capabilities. This suggested that a larger quantity of p53 was needed to accomplish tubule synthesis, a hypothesis we plan to explore in the future.

Finally, qualitative observations of expressed virus particles via negative staining reinforced our impression of the cytoplasmic environment, finding only a small fraction of, unfortunately impossible to definitively quantify, particles. We believe there were tens of fold less virus-generated particles in the supernatant of p53KOs compared to their wt parental line or the reconstituted WTG cells. This confirmed our initial virus titer data and we believe combined with our other findings suggests a critical role for p53 in the normal IINM mediated egress of HCMV capsids from their nuclear factory and the subsequent re-envelopment of those particles able to transit to their cytoplasmic distribution hub.

References

Adler, B. and C. Sinzger (2013). Cytomegalovirus Interstrain Variance in Cell Type Tropism. Cytomegaloviruses: From Molecular Pathogenesis to Intervention. M. J. Reddehase and N. A. W. Lemmermann. Norfolk, UK, Caister Academic Press. **1:** 297-321.

Arcangeletti, M. C., I. Rodighiero, et al. (2011). "Cell-cycle-dependent localization of human cytomegalovirus UL83 phosphoprotein in the nucleolus and modulation of viral gene expression in human embryo fibroblasts in vitro." Journal of cellular biochemistry **112**(1): 307-317.

AuCoin, D. P., G. B. Smith, et al. (2006). "Betaherpesvirus-conserved cytomegalovirus tegument protein ppUL32 (pp150) controls cytoplasmic events during virion maturation." Journal of virology **80**(16): 8199-8210.

Baxter, M. K. and W. Gibson (2001). "Cytomegalovirus basic phosphoprotein (pUL32) binds to capsids in vitro through its amino one-third." Journal of virology **75**(15): 6865-6873.

Bigalke, J. M., T. Heuser, et al. (2014). "Membrane deformation and scission by the HSV-1 nuclear egress complex." Nature communications **5**: 4131.

Biron, K. K. (2006). "Antiviral drugs for cytomegalovirus diseases." Antiviral research **71**(2-3): 154-163.

Boppana, S. B. and W. J. Britt (1995). "Antiviral antibody responses and intrauterine transmission after primary maternal cytomegalovirus infection." The Journal of infectious diseases **171**(5): 1115-1121.

Boppana, S. B., K. B. Fowler, et al. (1999). "Symptomatic congenital cytomegalovirus infection in infants born to mothers with preexisting immunity to cytomegalovirus." Pediatrics **104**(1 Pt 1): 55-60.

Boyle, K. A. and T. Compton (1998). "Receptor-binding properties of a soluble form of human cytomegalovirus glycoprotein B." Journal of virology **72**(3): 1826-1833.

Bresnahan, W. A., I. Boldogh, et al. (1996). "Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1." Virology **224**(1): 150-160.

Britt, W., and C. Alford (1996). Cytomegalovirus. Fields Virology. B. N. Fields, Lippincott-Raven Publishers, Philadelphia, Pa.**:** 2493-2523.

Buchkovich, N. J., T. G. Maguire, et al. (2010). "Role of the endoplasmic reticulum chaperone BiP, SUN domain proteins, and dynein in altering nuclear morphology during human cytomegalovirus infection." Journal of virology **84**(14): 7005-7017.

Buchkovich, N. J., T. G. Maguire, et al. (2009). "The endoplasmic reticulum chaperone BiP/GRP78 is important in the structure and function of the human cytomegalovirus assembly compartment." Journal of virology **83**(22): 11421-11428.

Bunz, F., A. Dutriaux, et al. (1998). "Requirement for p53 and p21 to sustain G2 arrest after DNA damage." Science **282**(5393): 1497-1501.

Buser, C., P. Walther, et al. (2007). "Cytomegalovirus primary envelopment occurs at large infoldings of the inner nuclear membrane." Journal of virology **81**(6): 3042-3048.

Cardoso, M. C., H. Leonhardt, et al. (1993). "Reversal of terminal differentiation and control of DNA replication: cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication." Cell **74**(6): 979-992.

Casavant, N. C., M. H. Luo, et al. (2006). "Potential role for p53 in the permissive life cycle of human cytomegalovirus." Journal of virology **80**(17): 8390-8401.

Castillo, J. P., A. D. Yurochko, et al. (2000). "Role of human cytomegalovirus immediate-early proteins in cell growth control." Journal of virology **74**(17): 8028-8037.

Chan, C. K., E. J. Brignole, et al. (2002). "Cytomegalovirus assemblin (pUL80a): cleavage at internal site not essential for virus growth; proteinase absent from virions." Journal of virology **76**(17): 8667-8674.

Chen, Z., E. Knutson, et al. (2001). "Degradation of p21cip1 in cells productively infected with human cytomegalovirus." Journal of virology **75**(8): 3613-3625.

Cinque, P., R. Marenzi, and D. Ceresa (1997). "Cytomegalovirus infections of the nervous system." Intervirology **40**: 85-97.

Colberg-Poley, A. M. and C. D. Williamson (2013). Intracellular Sorting and Trafficking of Cytomegalovirus Proteins during Permissive Infection.

Cytomegaloviruses: From Molecular Pathogenesis to Intervention. M. J. Reddehase and N. A. W. Lemmermann. Norfolk, UK, Caister Academic Press. **1:** 196-229.

Compton, T. (2004). "Receptors and immune sensors: the complex entry path of human cytomegalovirus." Trends in cell biology **14**(1): 5-8.

Compton, T., D. M. Nowlin, et al. (1993). "Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate." Virology **193**(2): 834-841.

Dai, X., X. Yu, et al. (2013). "The smallest capsid protein mediates binding of the essential tegument protein pp150 to stabilize DNA-containing capsids in human cytomegalovirus." PLoS pathogens **9**(8): e1003525.

Dal Monte, P., S. Pignatelli, et al. (2002). "Analysis of intracellular and intraviral localization of the human cytomegalovirus UL53 protein." The Journal of general virology **83**(Pt 5): 1005-1012.

Das, S. and P. E. Pellett (2011). "Spatial relationships between markers for secretory and endosomal machinery in human cytomegalovirus-infected cells versus those in uninfected cells." Journal of virology **85**(12): 5864-5879.

Das, S., A. Vasanji, et al. (2007). "Three-dimensional structure of the human cytomegalovirus cytoplasmic virion assembly complex includes a reoriented secretory apparatus." Journal of virology **81**(21): 11861-11869.

Feire, A. L. and T. Compton (2013). Virus Entry and Activation of Innate Defence. Cytomegaloviruses: From Molecular Pathogenesis to Intervention. M. J. Reddehase and N. A. W. Lemmermann. Norfolk, UK, Caister Academic Press. **1:** 125-140.

Fortunato, E. A. and D. H. Spector (1998). "p53 and RPA are sequestered in viral replication centers in the nuclei of cells infected with human cytomegalovirus." Journal of virology **72**(3): 2033-2039.

Fowler, K. B., F. P. McCollister, et al. (1997). "Progressive and fluctuating sensorineural hearing loss in children with asymptomatic congenital cytomegalovirus infection." The Journal of pediatrics **130**(4): 624-630.

Fowler, K. B., S. Stagno, et al. (1992). "The outcome of congenital cytomegalovirus infection in relation to maternal antibody status." The New England journal of medicine **326**(10): 663-667.

Friedman, M. (1937). "The use of ranks to avoid the assumption of normality implicit in the analysis of variance." Journal of the American Statistical Association **32**(200): 675–701.

Fuchs, W., B. G. Klupp, et al. (2002). "The interacting UL31 and UL34 gene products of pseudorabies virus are involved in egress from the host-cell nucleus and represent components of primary enveloped but not mature virions." Journal of virology **76**(1): 364-378.

Gaytant, M. A., E. A. Steegers, et al. (2002). "Congenital cytomegalovirus infection: review of the epidemiology and outcome." Obstetrical & gynecological survey **57**(4): 245-256.

Gibson, W. (2008). "Structure and formation of the cytomegalovirus virion." Current topics in microbiology and immunology **325**: 187-204.

Gibson, W. and A. Irmiere (1984). "Selection of particles and proteins for use as human cytomegalovirus subunit vaccines." Birth defects original article series **20**(1): 305-324.

Gilloteaux, J. and M. R. Nassiri (2000). "Human bone marrow fibroblasts infected by cytomegalovirus: ultrastructural observations." Journal of submicroscopic cytology and pathology **32**(1): 17-45.

Hsu, C. H., M. D. Chang, et al. (2004). "HCMV IE2-mediated inhibition of HAT activity downregulates p53 function." The EMBO journal **23**(11): 2269-2280.

Indran, S. V., M. E. Ballestas, et al. (2010). "Bicaudal D1-dependent trafficking of human cytomegalovirus tegument protein pp150 in virus-infected cells." Journal of virology **84**(7): 3162-3177.

Isaacson, M. K. and T. Compton (2009). "Human cytomegalovirus glycoprotein B is required for virus entry and cell-to-cell spread but not for virion attachment, assembly, or egress." Journal of virology **83**(8): 3891-3903.

Johnson, D. C. and J. D. Baines (2011). "Herpesviruses remodel host membranes for virus egress." Nature reviews. Microbiology **9**(5): 382-394.

Kalejta, R. F. (2008). "Tegument proteins of human cytomegalovirus." Microbiology and molecular biology reviews : MMBR **72**(2): 249-265, table of contents.

Kari, B. and R. Gehrz (1992). "A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope." Journal of virology **66**(3): 1761-1764.

Kari, B. and R. Gehrz (1993). "Structure, composition and heparin binding properties of a human cytomegalovirus glycoprotein complex designated gC-II." The Journal of general virology **74 (Pt 2)**: 255-264.

Klupp, B. G., H. Granzow, et al. (2007). "Vesicle formation from the nuclear membrane is induced by coexpression of two conserved herpesvirus proteins." Proceedings of the National Academy of Sciences of the United States of America **104**(17): 7241-7246.

Klupp, B. G., H. Granzow, et al. (2000). "Primary envelopment of pseudorabies virus at the nuclear membrane requires the UL34 gene product." Journal of virology **74**(21): 10063-10073.

Lombard, D. B. and L. Guarente (2000). "Nijmegen breakage syndrome disease protein and MRE11 at PML nuclear bodies and meiotic telomeres." Cancer research **60**(9): 2331-2334.

Loveland, A. N., C. K. Chan, et al. (2005). "Cleavage of human cytomegalovirus protease pUL80a at internal and cryptic sites is not essential but enhances infectivity." Journal of virology **79**(20): 12961-12968.

Luo, M. H. and E. A. Fortunato (2007). "Long-term infection and shedding of human cytomegalovirus in T98G glioblastoma cells." Journal of virology **81**(19): 10424-10436.

Luo, M. H., K. Rosenke, et al. (2007). "Human cytomegalovirus disrupts both ataxia telangiectasia mutated protein (ATM)- and ATM-Rad3-related kinase-mediated DNA damage responses during lytic infection." Journal of virology **81**(4): 1934-1950.

Marciniak, R. A., D. B. Lombard, et al. (1998). "Nucleolar localization of the Werner syndrome protein in human cells." Proceedings of the National Academy of Sciences of the United States of America **95**(12): 6887-6892.

Mettenleiter, T. C., B. G. Klupp, et al. (2009). "Herpesvirus assembly: an update." Virus research **143**(2): 222-234.

Mocarski, E. S., and C.T. Courcelle (2001). Cytomegaloviruses and Their Replication. Fields Virology. D. M. Knippe, and Howley, P.M., Lippincott Williams & Wilkins. **2:** 2629-2673.

Muganda, P., R. Carrasco, et al. (1998). "The human cytomegalovirus IE2 86 kDa protein elevates p53 levels and transactivates the p53 promoter in human fibroblasts." Cellular and molecular biology **44**(2): 321-331.

Muganda, P., O. Mendoza, et al. (1994). "Human cytomegalovirus elevates levels of the cellular protein p53 in infected fibroblasts." Journal of virology **68**(12): 8028-8034.

Murphy, E. A., D. N. Streblow, et al. (2000). "The human cytomegalovirus IE86 protein can block cell cycle progression after inducing transition into the S phase of permissive cells." Journal of virology **74**(15): 7108-7118.

O'Dowd, J. M., A. G. Zavala, et al. (2012). "HCMV-infected cells maintain efficient nucleotide excision repair of the viral genome while abrogating repair of the host genome." PLoS pathogens **8**(11): e1003038.

Papadimitriou, J. M., G. R. Shellam, et al. (1984). "An ultrastructural investigation of cytomegalovirus replication in murine hepatocytes." The Journal of general virology **65 (Pt 11)**: 1979-1990.

Penfold, M. E. and E. S. Mocarski (1997). "Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis." Virology **239**(1): 46-61.

Poland, S. D., P. Costello, et al. (1990). "Cytomegalovirus in the brain: in vitro infection of human brain-derived cells." The Journal of infectious diseases **162**(6): 1252-1262.

Revello, M. G., E. Percivalle, et al. (1992). "Nuclear expression of the lower matrix protein of human cytomegalovirus in peripheral blood leukocytes of immunocompromised viraemic patients." The Journal of general virology **73 (Pt 2)**: 437-442.

Reynolds, A. E., B. J. Ryckman, et al. (2001). "U(L)31 and U(L)34 proteins of herpes simplex virus type 1 form a complex that accumulates at the nuclear rim and is required for envelopment of nucleocapsids." Journal of virology **75**(18): 8803-8817.

Reynolds, A. E., E. G. Wills, et al. (2002). "Ultrastructural localization of the herpes simplex virus type 1 UL31, UL34, and US3 proteins suggests specific roles in primary envelopment and egress of nucleocapsids." Journal of virology **76**(17): 8939-8952.

Roller, R. J., Y. Zhou, et al. (2000). "Herpes simplex virus type 1 U(L)34 gene product is required for viral envelopment." Journal of virology **74**(1): 117-129.

Rosenke, K., M. A. Samuel, et al. (2006). "An intact sequence-specific DNA-binding domain is required for human cytomegalovirus-mediated sequestration of p53 and may promote in vivo binding to the viral genome during infection." Virology **348**(1): 19-34.

Ruebner, B. H., K. Miyai, et al. (1964). "Mouse Cytomegalovirus Infection. An Electron Microscopic Study of Hepatic Parenchymal Cells." The American journal of pathology **44**: 799-821.

Ryckman, B. J., M. C. Chase, et al. (2008). "HCMV gH/gL/UL128-131 interferes with virus entry into epithelial cells: evidence for cell type-specific receptors." Proceedings of the National Academy of Sciences of the United States of America **105**(37): 14118-14123.

Ryckman, B. J., M. A. Jarvis, et al. (2006). "Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion." Journal of virology **80**(2): 710-722.

Ryckman, B. J., B. L. Rainish, et al. (2008). "Characterization of the human cytomegalovirus gH/gL/UL128-131 complex that mediates entry into epithelial and endothelial cells." Journal of virology **82**(1): 60-70.

Sanchez, V., K. D. Greis, et al. (2000). "Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly." Journal of virology **74**(2): 975-986.

Sanchez, V., J. A. Mahr, et al. (2007). "Nuclear export of the human cytomegalovirus tegument protein pp65 requires cyclin-dependent kinase activity and the Crm1 exporter." J Virol **81**(21): 11730-11736.

Sanchez, V., E. Sztul, et al. (2000). "Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment." Journal of virology **74**(8): 3842-3851.

Sarov, I. and I. Abady (1975). "The morphogenesis of human cytomegalovirus. Isolation and polypeptide characterization of cytomegalovirions and dense bodies." Virology **66**(2): 464-473.

Schmolke, S., P. Drescher, et al. (1995). "Nuclear targeting of the tegument protein pp65 (UL83) of human cytomegalovirus: an unusual bipartite nuclear localization signal functions with other portions of the protein to mediate its efficient nuclear transport." Journal of virology **69**(2): 1071-1078.

Severi, B., M. P. Landini, et al. (1988). "Human cytomegalovirus morphogenesis: an ultrastructural study of the late cytoplasmic phases." Archives of virology **98**(1-2): 51-64.

Shan, R., X. Wang, et al. (2009). "Growth and development of infants with asymptomatic congenital cytomegalovirus infection." Yonsei medical journal **50**(5): 667-671.

Soderberg-Naucler, C. (2006). "Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer?" Journal of internal medicine **259**(3): 219-246.

Speir, E., R. Modali, et al. (1994). "Potential role of human cytomegalovirus and p53 interaction in coronary restenosis." Science **265**(5170): 391-394.

Tamashiro, J. C., L. J. Hock, et al. (1982). "Construction of a cloned library of the EcoRI fragments from the human cytomegalovirus genome (strain AD169)." Journal of virology **42**(2): 547-557.

Tandon, R. and E. S. Mocarski (2011). "Cytomegalovirus pUL96 is critical for the stability of pp150-associated nucleocapsids." Journal of virology **85**(14): 7129-7141.

Tsai, H. L., G. H. Kou, et al. (1996). "Human cytomegalovirus immediate-early protein IE2 tethers a transcriptional repression domain to p53." The Journal of biological chemistry **271**(7): 3534-3540.

Vanarsdall, A. L., B. J. Ryckman, et al. (2008). "Human cytomegalovirus glycoproteins gB and gH/gL mediate epithelial cell-cell fusion when expressed either in cis or in trans." J Virol **82**(23): 11837-11850.

Wang, D. and T. Shenk (2005). "Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism." Proceedings of the

National Academy of Sciences of the United States of America **102**(50): 18153-18158.

Wei, W., R. M. Hemmer, et al. (2001). "Role of p14(ARF) in replicative and induced senescence of human fibroblasts." Molecular and cellular biology **21**(20): 6748-6757.

Zhu, H., Y. Shen, et al. (1995). "Human cytomegalovirus IE1 and IE2 proteins block apoptosis." Journal of virology **69**(12): 7960-7970.

Chapter 3

The absence of p53 during human cytomegalovirus infection interrupts UL50/UL53 localization to the inner nuclear membrane and subsequent capsid egress

3.1 Abstract

Human Cytomegalovirus (HCMV) virion production decreases in the absence of p53. Here the effect of p53's absence on the virus's nuclear egress machinery has been investigated. In our parallel electron microscopy study most p53 knockout cells (KOs) released functional virions. KOs produced capsids in nearly wild type (wt) quantities, but formation of infoldings of the inner nuclear membrane (IINMs) was substantially reduced. Capsid nuclear egress is known to require phosphorylation and remodeling of the nuclear lamina. We have found that KOs expressed the viral kinase UL97, thought to initiate lamina phosphorylation, at close to wt levels. UL97 phosphorylated specific substrates in the KOs and the lamina in essentially all KO cells, however the lamina was not remodeled. This suggested the nuclear egress complex (NEC) was not functioning properly. UL53 was only expressed in 50% of KOs. UL53's NEC binding partner, UL50, failed to re-localize to the nucleus of the large majority of infected KOs. In the proportion of KOs with UL50 in the nucleus, UL50 displayed rim staining and was always co-localized with UL53. These cells were likely able to traffic capsids out of their nuclei. p53 was reintroduced into KO cells (WTGs). Expression of UL53 and accumulation of UL50 at the INM in infected

WTGs recovered to near wt levels. Confocal IF found that localizations of UL50/53 presented as “threads” extending from the INM into the nucleus. These structures were smaller or absent in infected KOs. We believe these structures were tubular IINMs. The absence of p53 inhibited the wt behavior of UL50-53 and the NEC, preventing formation of tubular IINMs, thereby drastically reducing the production of functional virions.

3.2 Introduction

Human Cytomegalovirus (HCMV) is a linear double stranded DNA virus. This virus is ubiquitous in most human populations. HCMV is an opportunistic pathogen that causes medical problems in immunocompromised individuals such as AIDS patients and transplant recipients (Zhang, Hanff et al. 1995; Fornara, Lilleri et al. 2011; Shi, Lu et al. 2011). Some studies also report that HCMV may contribute to certain types of cancers including malignant, prostate carcinomas, and colorectal cancers (Cobbs, Harkins et al. 2002; Harkins, Volk et al. 2002; Samanta, Harkins et al. 2003; Soderberg-Naucler 2006). Perhaps most problematic is that HCMV is a major cause of congenital birth defects. Approximately 8,000 infants are significantly negatively affected each year in the United States. Half of these cases have severe neurological defects at birth that include blindness, deafness, mental retardation, microencephaly, and cerebral calcification. The remaining cases manifest sequelae within 1-2 years after birth (Britt 1996; Cinque 1997; Fowler, McCollister et al. 1997; Boppana, Fowler et al. 1999).

HCMV infection can lead not only to significant negative consequences for its host at large, but also highly disrupts individual cellular functions including normal cell cycling. Virus-induced relocalization, and association of, certain cellular proteins into the viral replication centers (RCs) may, at least in part, be responsible for these disruptions occurrences. The RCs are non-membrane bound structures located within the nucleus of infected cells. The RCs are the site of viral DNA replication (Rixon, Atkinson et al. 1983; Quinlan, Chen et al. 1984; de Bruyn Kops and Knipe 1988; Liptak, Uprichard et al. 1996; Lukonis and Weller 1996; Lukonis, Burkham et al. 1997; Uprichard and Knipe 1997). One of the tightly RC-associated proteins is the cell cycle regulatory protein p53 (Fortunato and Spector 1998). Amongst a myriad of functions, p53 is a specific transcription factor that mediates the cellular damage response. Activation of p53 can lead to either p53-mediated cell cycle arrest (presumably for repair of damaged DNA) or apoptosis (reviewed in reference Sax and El-Deiry 2003). These p53-mediated responses do not occur during HCMV infection.

We have previously shown that the presence of p53 was involved in or required for several viral activities or functions. Rosenke et al reported 21 viral genes have p53 binding sites. p53 was differentially bound at some of these sequence-specific DNA binding domains over the course of infection (Rosenke, Samuel et al. 2006). p53 was found to influence viral gene expression, through either direct or indirect interaction, at early times of infection (Hannemann, Rosenke et al. 2009). Most significantly the presence of p53 in the infected cell was found to be necessary for wild type (wt) levels of functional virion production

(Casavant, Luo et al. 2006). The production of functional virions was reduced by approximately 25-fold at late times pi in knockout cells (KOs) completely absent wt p53.

During the normal course of infection, localization of expressed pp65, the most abundant tegument protein, is first seen in the nucleus at 12h pi (arcangeletti, 2003 human). Later in infection (~48h pi), a shift in pp65 localization begins and the vast majority of the pp65 is present in the cytoplasm (Sanchez, Greis et al. 2000; Sanchez, Mahr et al. 2007), although there is undoubtedly still protein present to a lesser degree in the nucleus, where it is associated with nuclear capsids. In the KOs, however, pp65 was found to remain largely confined to the nucleus throughout infection (Casavant, Luo et al. 2006). pp65 has been used as a proxy for virion maturation from the nucleus (Britt and Vugler 1987; Sanchez, Clark et al. 2002; Casavant, Luo et al. 2006). If this protein was absent from the cytoplasm it suggested to us that other viral proteins and nuclear capsids might not be exiting the nucleus as well. Our parallel electron microscopy study (see Chapter 2) found that the absence of p53 disrupted the efficient egress of HCMV capsids from the nucleus.

Understanding of the precise molecular mechanism of HCMV virion nuclear egress is limited. The nuclear pore complex capable of transporting ~40 nm molecules (Pante and Kann 2002) does not have the capacity to transport a ~130 nm diameter capsid. Recently it has been proposed that a Nuclear Egress Complex (NEC), at least in part, facilitates virion egress from the nucleus by destabilization of the nuclear lamins (Muranyi, Haas et al. 2002; Marschall, Marzi et al. 2005; Bjerke

and Roller 2006; Park and Baines 2006; Camozzi, Pignatelli et al. 2008; Cano-Monreal, Wylie et al. 2009; Hamirally, Kamil et al. 2009; Leach and Roller 2010; Milbradt, Webel et al. 2010; Sharma, Kamil et al. 2014). The nuclear lamina is a dense meshwork that mechanically supports the shape of the nucleus. This meshwork is composed of lamins and their associated membrane proteins (reviewed in references Goldman, Gruenbaum et al. 2002; Dechat, Pfleghaar et al. 2008). The lamina must be dissociated in order for the virus to exit the nucleus (reviewed in references Mettenleiter, Klupp et al. 2009; Johnson and Baines 2011).

The NEC of HCMV is comprised of both viral and cellular proteins. Viral proteins include UL50, UL53, UL97 and perhaps some other, yet undefined, proteins. Cellular proteins of the NEC include the Lamin B receptor (LBR), protein kinase C (PKC), p32, peptidyl-prolyl *cis-trans* isomerase (Pin1), and emerin (Krosky, Baek et al. 2003; Marschall, Marzi et al. 2005; Camozzi, Pignatelli et al. 2008; Hamirally, Kamil et al. 2009; Milbradt, Auerochs et al. 2009; Sam, Evans et al. 2009; Milbradt, Webel et al. 2010; Miller, Furlong et al. 2010; Milbradt, Kraut et al. 2014; Sharma, Kamil et al. 2014). UL50 is a key component required to recruit UL53 and PKC to the nuclear lamina, and acting as a “hook” to secure the NEC to the inner nuclear membrane (INM) (Muranyi, Haas et al. 2002; Camozzi, Pignatelli et al. 2008; Milbradt, Auerochs et al. 2009; Sam, Evans et al. 2009; Miller, Furlong et al. 2010). p32 is recruited to the lamina through interaction with the LBR (Milbradt, Auerochs et al. 2009) and in turn recruits UL97 (Marschall, Marzi et al. 2005). UL97 has been found to contribute to phosphorylating lamins A/C (Hamirally, Kamil et al. 2009; Milbradt, Webel et al. 2010)). Phosphorylation of the lamins generates a binding site

for Pin1, which in turn may promote conformational changes of the lamins, and lead to their localized depletion (Milbradt, Webel et al. 2010). Infoldings of the inner nuclear membrane (IINMs), structures that have been observed by several groups to contain enveloped capsids (Ruebner, Miyai et al. 1964; Papadimitriou, Shellam et al. 1984; Severi, Landini et al. 1988; Gilloteaux and Nassiri 2000; Dal Monte, Pignatelli et al. 2002; Buser, Walther et al. 2007), have been proposed to be the principal site of transit through the nuclear membrane (Buser, Walther et al. 2007). These tubule-like structures apparently facilitate capsid transport into the perinuclear space and through the outer nuclear membrane.

This study has focused on isolating which viral and cellular mechanisms failed to allow normal nuclear egress of capsids in the absence of p53. UL97 was expressed and functional in these cells. Lamin A/C was phosphorylated in the KOs, presumably by UL97, however the nuclear lamina were found to be substantially less remodeled in the KO cells compared to wt, with fewer, if any, substantial gaps in the lamin structure. The accumulation of several components of the NEC at the nuclear rim, including UL50 and UL53, were greatly decreased and frequently absent in KOs. Confocal imaging of UL50 and UL53 found them co-localized in “strings” extending from the nuclear rim into the interior of the nucleus. Lamin A/C was present only at the base of these structures, but not along the length or at the tips. These structures were much less prevalent in KOs and smaller when present. We believe these formations were tubules derived from the INM. The absence of lamins along these tubules suggested they formed from regions of the nuclear membrane where UL97-phosphorylation had allowed disruption of the lamina

structure. Reintroduction of p53 into the KOs increased the number of cells expressing UL50/UL53 staining at the nuclear rim and a slightly increased number of tubules within these nuclei. We propose that the absence of p53 inhibited proper expression of UL53 and localization of UL50 to the INM, thereby preventing formation of the optimal method of tubule transport for HCMV capsid nuclear egress.

3.3 Materials and Methods

3.3.1 Cells and virus growth.

p53KO telomerase-immortalized human fibroblasts, their parental cell line, Lox (Bunz, Dutriaux et al. 1998; Wei, Hemmer et al. 2001) (both kind gifts from Dr. John Sedivy, Brown University), and the p53 re-introducted WTG cells (Casavant, Luo et al. 2006) were maintained in complete medium composed of Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (Ham) 1:1 (DMEM/F-12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (2mM), penicillin (200 U/ml), and streptomycin (200 µg/ml). Cells were grown in incubators maintained at 37^{o}C and 5% CO_2. The Towne strain of HCMV was obtained from the ATCC (#VR 977), propagated under standard procedures (Tamashiro, Hock et al. 1982), and used at a multiplicity of infection (MOI) of 5 for all experiments.

3.3.2 Cell cycle synchronization and infection.

Confluent cells were synchronized in G0 by serum starvation and infected (Salvant, Fortunato et al. 1998; Fortunato, Sanchez et al. 2002). Cells were then trypsinized and reseeded into 1) plates containing coverslips for fluorescent analysis at a density of 5×10^5 cells per plate and 2) reseeded at 1×10^6 cells per plate in complete DMEM/F-12 medium for Western analysis. After 1-2 hours to allow for attachment, cells were infected with Towne supernatant. After 4 hours of infection, supernatant was removed and cells were rinsed with PBS to remove any remaining unadsorbed virus, and finally re-fed with complete medium.

3.3.3 Antibodies

Primary antibodies (Abs) used were several mouse monoclonal Abs including anti-Lamin A/C (636 [IgG2B]; Santa Cruz Biotechnology), anti-Lamin A (133A2 [IgG3]; Abcam), anti-UL97 (IgG2A; a kind gift from Dr. Mark Prichard, University of Alabama School of Medicine, Birmingham), anti-UL44 (1202S [IgG1]; Goodwin Institute), anti-UL53 (IgG1; a kind gift from Dr. Stipan Jonjic, University of Rijeka, Croatia), anti-Pan Actin (Ab-5; NeoMarkers), and two rabbit Abs including anti-UL50 (a kind gift from Dr. James C. Alwine, University of Pennsylvania, Philadelphia) (Buchkovich, Maguire et al. 2010), and anti-Phospho-Lamin A/C (Ser22; Cell Signaling). Secondary Abs used were goat anti-mouse IgG1 Alex Fluor 488-coupled Ab (Molecular Probes), goat anti-mouse IgG 2A TRITC-coupled Ab (Southern Biotech), goat anti-mouse IgG2B Alex Fluor 488-coupled Ab (Molecular

Probes), goat anti-mouse IgG3 Alex Fluor 488-coupled Ab (Molecular Probes), and donkey anti-rabbit TRITC-coupled Ab (Jackson Immunoresearch).

3.3.4 Immunofluorescence staining assay (IF)

Coverslips were harvested and washed in PBS. In this study a “fix first” method was used for investigating all proteins, except UL50 and its specific location in the nucleus as described. Cells were fixed in 3% formaldehyde for 10 min at RT, washed in PBS, then permeabilized with 1% Triton X-100 for 5 min, followed by three washes in PBS. Cells on coverslips were blocked with a 30% FBS blocking solution (PBS with 1% bovine serum albumin and 0.01% Tween 20) for 30 min at RT. Coverslips were then incubated with primary antibodies diluted in blocking solution for 15-30 minutes (mins) at RT. After extensive washes in PBS, cells were incubated with isotype-specific secondary antibodies diluted in blocking solution for 10 min at RT. Coverslips were then washed again in PBS and mounted in glycerol containing paraphenylene diamine to inhibit photobleaching. Nuclei were counterstained with Hoechst dye.

An “extraction first” methodology was used to detect UL50 protein within the nucleus. Cells were treated with detergent before fixation in formaldehyde (Cardoso, Leonhardt et al. 1993; Marciniak, Lombard et al. 1998; Lombard and Guarente 2000). Harvested coverslips were washed twice in PBS supplemented with 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (hereafter PBS/CM), then washed once in CytoSkeleton (CSK) stabilization buffer (10 mM PIPES KOH pH 7, 100 mM NaCl, 300 mM

sucrose, 3 mM $MgCl_2$, stored at 4^{o}C). Extraction included permeabilization of cells with 0.5% Triton X-100 and protease inhibitors (PI; 100 mM dithiothreitol, 2 ug/ml each of aprotinin and leupeptin) in CSK buffer for 5 min at RT. Sucrose was added to CSK buffer to stabilize and preserve cell integrity during the extraction process. Cells were washed twice with CSK buffer prior to extraction. After extraction cells were fixed with 3% formaldehyde in PBS/CM for 10 min at RT, followed by three washes with PBS. After extraction and fixation, cells were blocked and incubated with primary and secondary antibodies. Otherwise all conditions and procedures were as described above with the exception of blocking with 10% human IgG (20 mg/ml).

Epifluorescent analysis was perfomed on a Nikon Eclipse E800 flourescence microscope equipped with a Nikon DS-Ri1 high resolution color camera and Nikon NIS Elements Basic Research (Br) imaging software. Confocal analysis was performed using an Olympus Fluoview 1000 confocal microscope equipped with a 60X Plan Apo oil objective lens (1.42 NA) and FluoView ASW software. Samples were excited using lasers at 488 nm for both lamin A/C and UL53, 559 nm for UL50, and 405 nm for Hoechst. Images were analyzed using Imaris software version 7.2.1.

3.3.5 Western analysis

Cells were seeded and infected as described above, and harvested over a timecourse. Cells were trypsinized, washed, counted, and lysed in Laemmli reducing sample buffer, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 100 mM

dithiothreitol [dTT], 50 mM Tris-Cl pH 6.8, bromophenol blue dye, PI, 20 mM sodium fluoride, 80 mM betaglycerophosphate, 1 mM sodium orthovanadate, and 1% beta-mercaptoethanol. Volume was adjusted with RIPA buffer [50 mM Tris pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP40], to a concentration of 10^4 cells/ul. Lysates were then sonicated and boiled for 5 mins. Cellular debris was pelleted by centrifugation at 13,000 rpm for 3 mins at RT. Lysates were placed on ice for 5 m. Equivalent amounts of cell lysates were run on SDS-polyacrylamide gels, and then transferred to a Protran membrane (GE Healthcare Life Sciences). Proteins were blocked in 2.5% milk in TBS with 0.1% Tween 20, and probed with primary antibodies (3 h for UL50 and overnight for all other proteins). Membranes were washed extensively and then probed with goat anti-rat secondary antibody conjugated with horseradish peroxidase (Amersham), or donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase (GE Healthcare). Proteins were visualized with enhanced-chemiluminescence reagents (Advansta).

3.4 Results

3.4.1 Infection initiated remodeling of the nuclear lamina was much less substantial in KOs.

It has been shown that the nuclear lamina must be substantially remodeled for HCMV capsid egress to occur (Marschall, Marzi et al. 2005; Camozzi, Pignatelli et al. 2008; Hamirally, Kamil et al. 2009; Milbradt, Webel et al. 2010; Sharma, Kamil

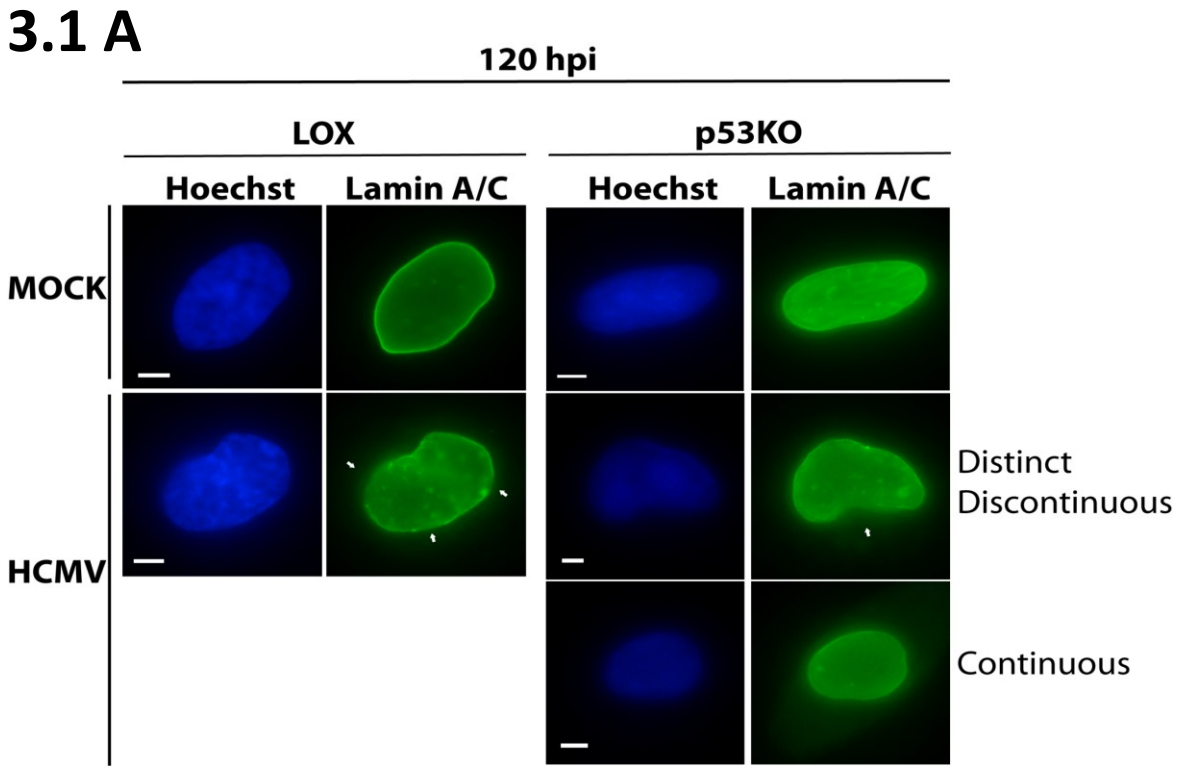
et al. 2014). The viral protein UL97 has been implicated in this process (Krosky, Baek et al. 2003; Hamirally, Kamil et al. 2009; Milbradt, Webel et al. 2010; Reim, Kamil et al. 2013; Sharma, Bender et al. 2014; Sharma, Kamil et al. 2014). Lox and KOs were infected and harvested over a timecourse. Cells were fixed and stained for Lamin A/C. Epifluorescent analysis of nuclear lamina staining found regions of discontinuity in both cell types (Figure 3.1A; arrows).

Confocal imaging of lamin A/C stained cells distinguished much greater detail of the lamin remodeling. When viewed as a single “slice”, the lamin ring was not fully continuous in either mock or virus-infected cell types (see Figure 3.1B with insets). The lamin staining could be more accurately described as a "string of beads" with recurrent small regions of at least reduced expression around the nuclear rim. We suspect these gaps could be nuclear pores, which have been shown to lack lamination (reviewed in reference Goldman, Goldman et al. 2005). Most notably, in the wt cells substantial breaches (Figure 3.1B; arrowhead), amounting to large fractions of the nuclear circumference, were easily identifiable. Such distinct large breaches were largely absent in the KO cells.

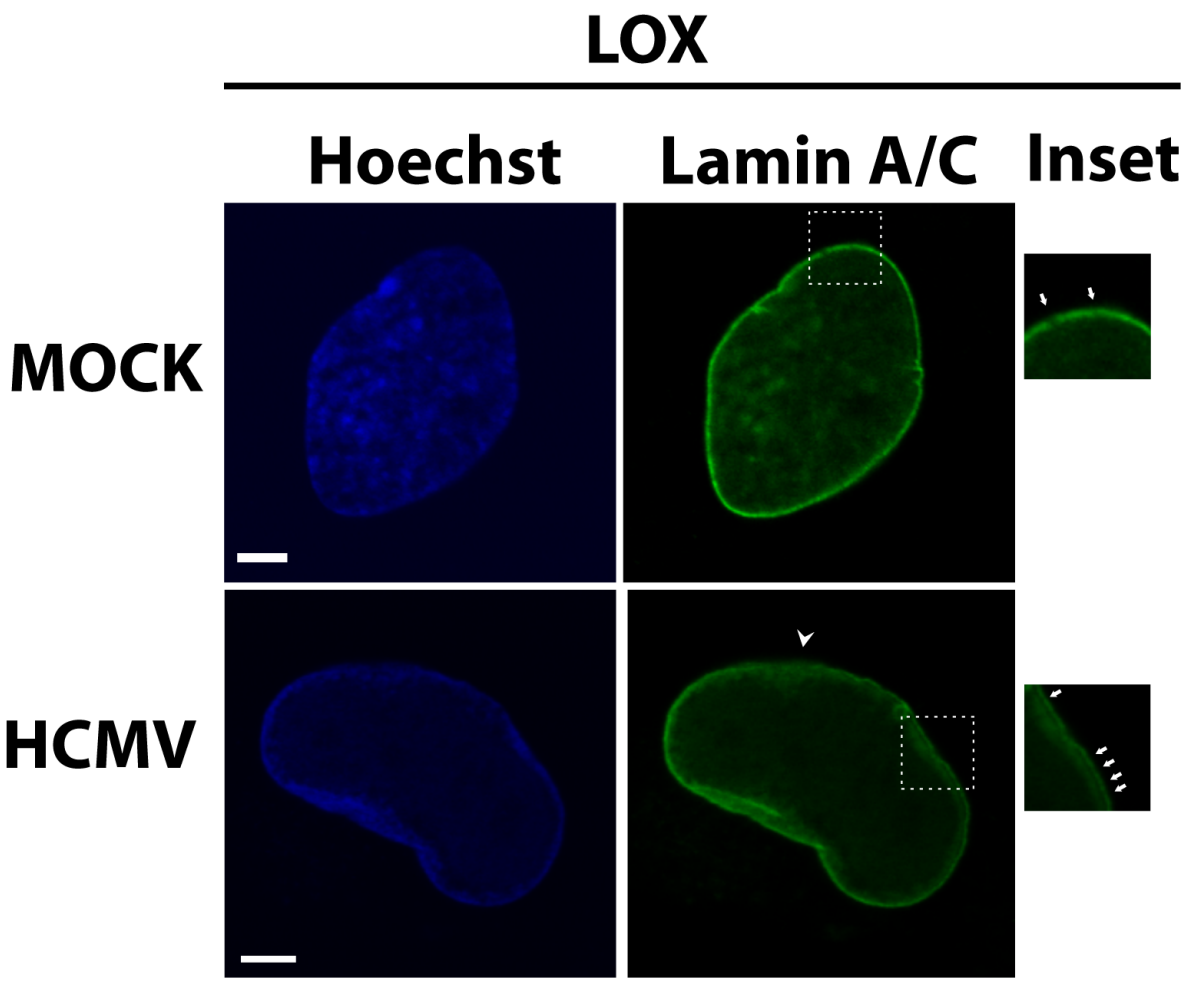
Since minor discontinuities were visible in both mock and virus-infected cells, we focused our attention on the large breaches that were easily visible by both confocal and epifluorescent imaging. Cells were scored for the presence of discontinuous lamin A/C staining (Figure 3.1C). In wt cells the percentage of discontinuity steadily increased from a low of 17% at 24h pi to a high of 67% at 144h pi. The KO cells displayed a similar low of 14% at 24h pi, with very little increase until 96-144h pi when the percentage of cells with a discontinuous staining

pattern rose to 33-36% of the population. This was approximately the same level wt cells reached at 72h pi and only half the maximal level wt cells achieved at late times pi. Similar results were obtained following infection of both cell types transiently expressing lamin A-GFP. The Lamin A-GFP signal remained continuous in the large majority of infected KO cells at late times pi (data not shown).

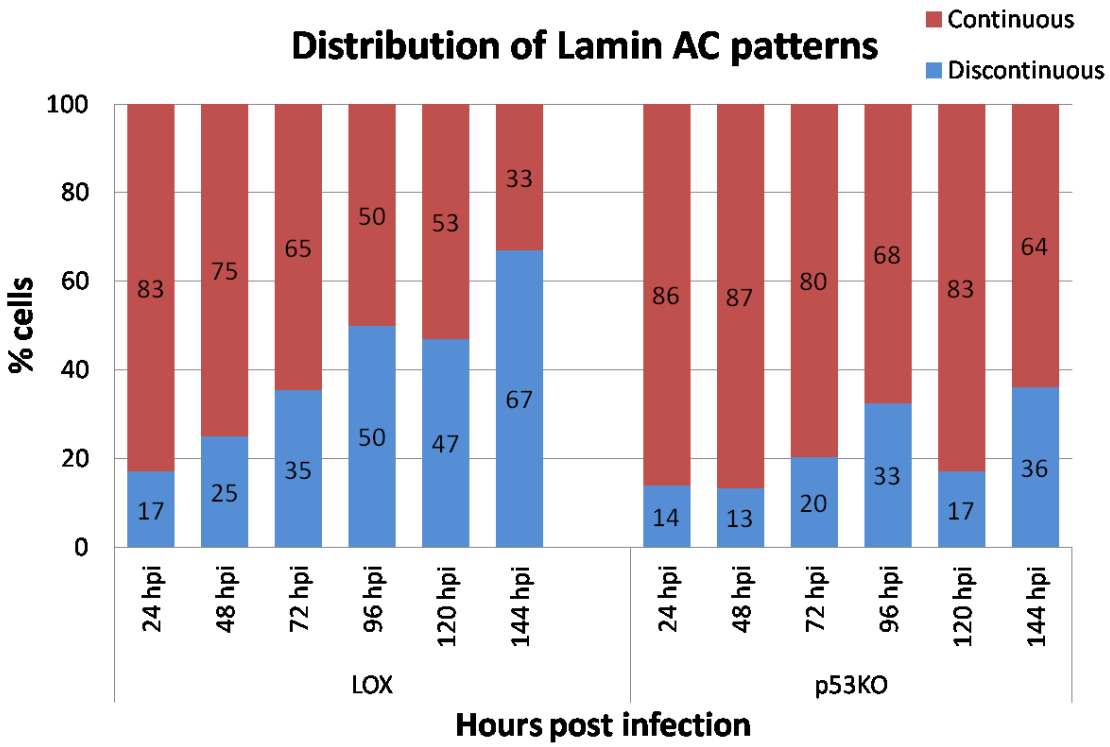
Western blot analysis of endogenous lamin A/C was performed to determine the steady state levels of protein in the two cell types. The total lamin A/C present in the infected cells was approximately equivalent in wt and KOs (Figure 3.1D). The differential in discontinuous lamin staining between the cell types was not due to the presence of more or less lamin in one cell type versus the other.



3.1 B **120 hpi**



3.1 C



3.1 D

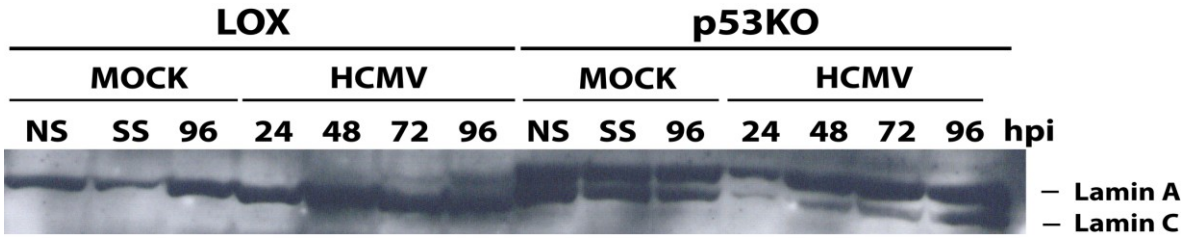
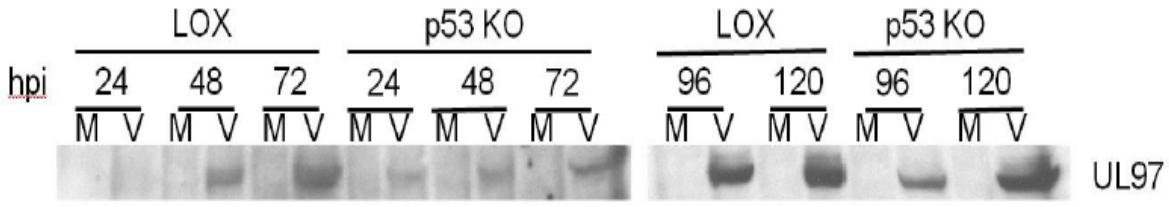


Figure 3.1 Lamin rearrangement induced by HCMV infection. (A) Immunofluorescent staining displays discontinuity of lamin A/C ring in both Lox and p53KO cells (arrows). A continuous lamin ring was seen in mock- and the majority of infected p53KO cells. (B) Confocal imaging of lamin A/C staining. “String of beads” appearance of lamin A/C (arrows) in both mock- and infected wt and p53KOs cells, not observable by epifluorescence, differs from large discontinuity in infected wt cells (arrow head). (C) The percentage of cells displaying large discontinuities in Lox cells compared to p53KO cells over a time course of infection. Bars represent an average of 3 experiments. (D) Western blot analysis of lamin A/C in both Lox and p53KO cells over a time course. NS = non-serum starved, SS = serum starved. All size bars in immunofluorescence images represent 5 um.

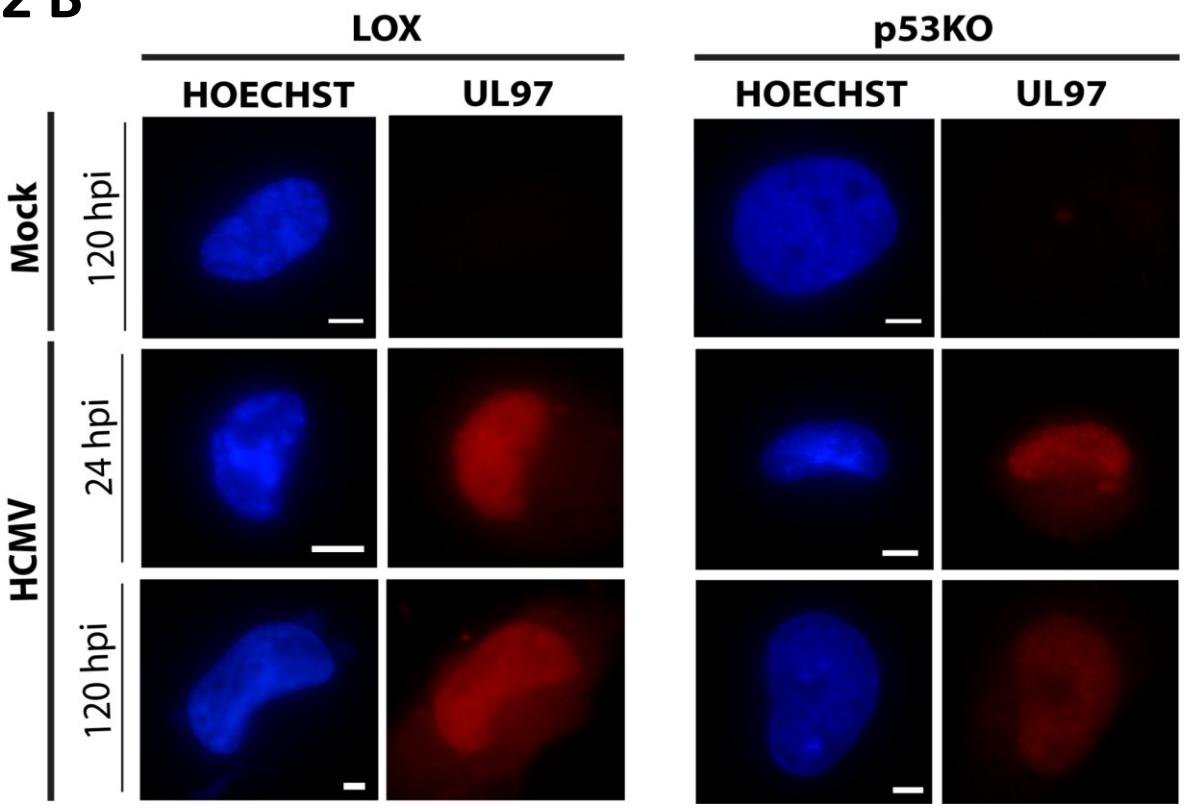
3.4.2 UL97 was present and functional in the majority of p53KO cells.

The viral protein UL97 must be present and functional for normal capsid egress from the nucleus during a functional infection (Krosky, Baek et al. 2003; Hamirally, Kamil et al. 2009; Milbradt, Webel et al. 2010; Reim, Kamil et al. 2013). Western blot analysis of the steady state level of UL97 found it present in both cells types starting at 24h pi, but expressed at a slightly lower level in the KO cells until very late pi (Figure 3.2A). This analysis found slightly less total protein in the KO cell population, but could not distinguish the presence or absence of the protein from individual cells. Therefore, approximately 200 cells of each type were examined by IF for the presence of UL97 over a 120h timecourse. The IF signal was somewhat less intense in the KOs compared to the wt (Figure 3.2B), which likely paralleled the reduced total protein measured by Western blot. The percentage of the populations expressing UL97 primarily in the nucleus was maintained at approximately 70% throughout the time course in both wt and KO cells (Figure 3.2C). Importantly, another 10-20% of both cell types displayed staining of UL97 over the entire cell, presumably located in both the nucleus and cytoplasm throughout infection.

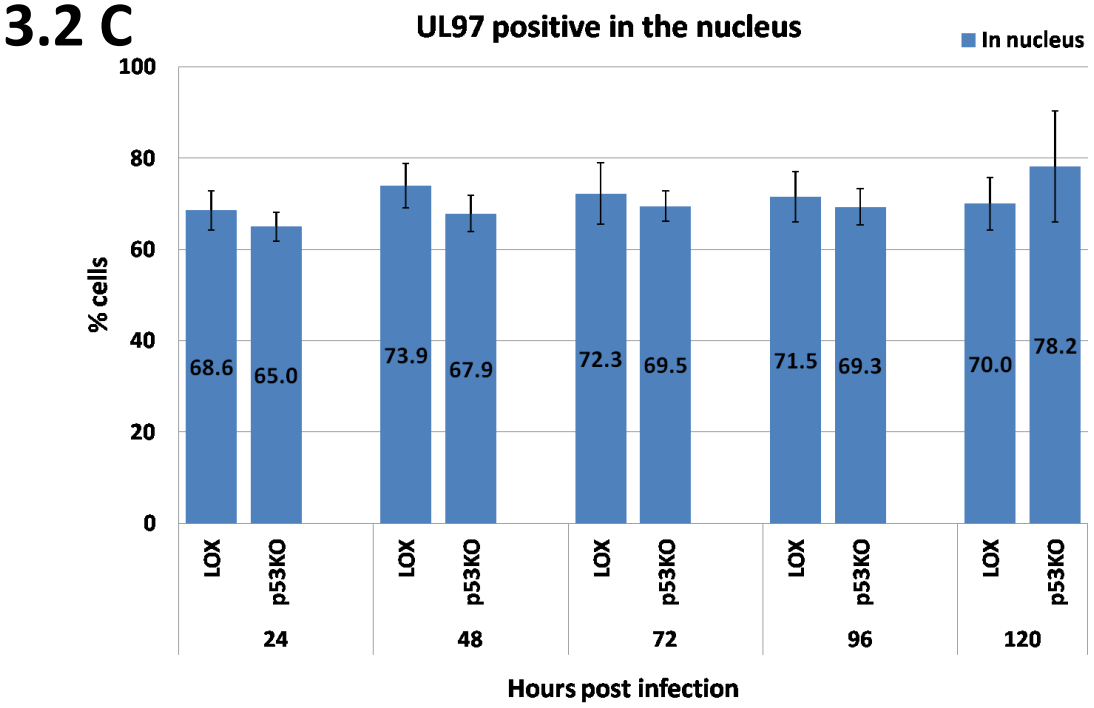
3.2 A



3.2 B


LOX
p53KO
HOECHST
UL97
HOECHST
UL97
Mock
120 hpi
HCMV
24 hpi
120 hpi

3.2 C


UL97 positive in the nucleus
In nucleus
100
80
60
40
20
0
% cells
68.6
65.0
73.9
67.9
72.3
69.5
71.5
69.3
70.0
78.2
LOX
p53KO
LOX
p53KO
LOX
p53KO
LOX
p53KO
LOX
p53KO
24
48
72
96
120
Hours post infection

3.2 D

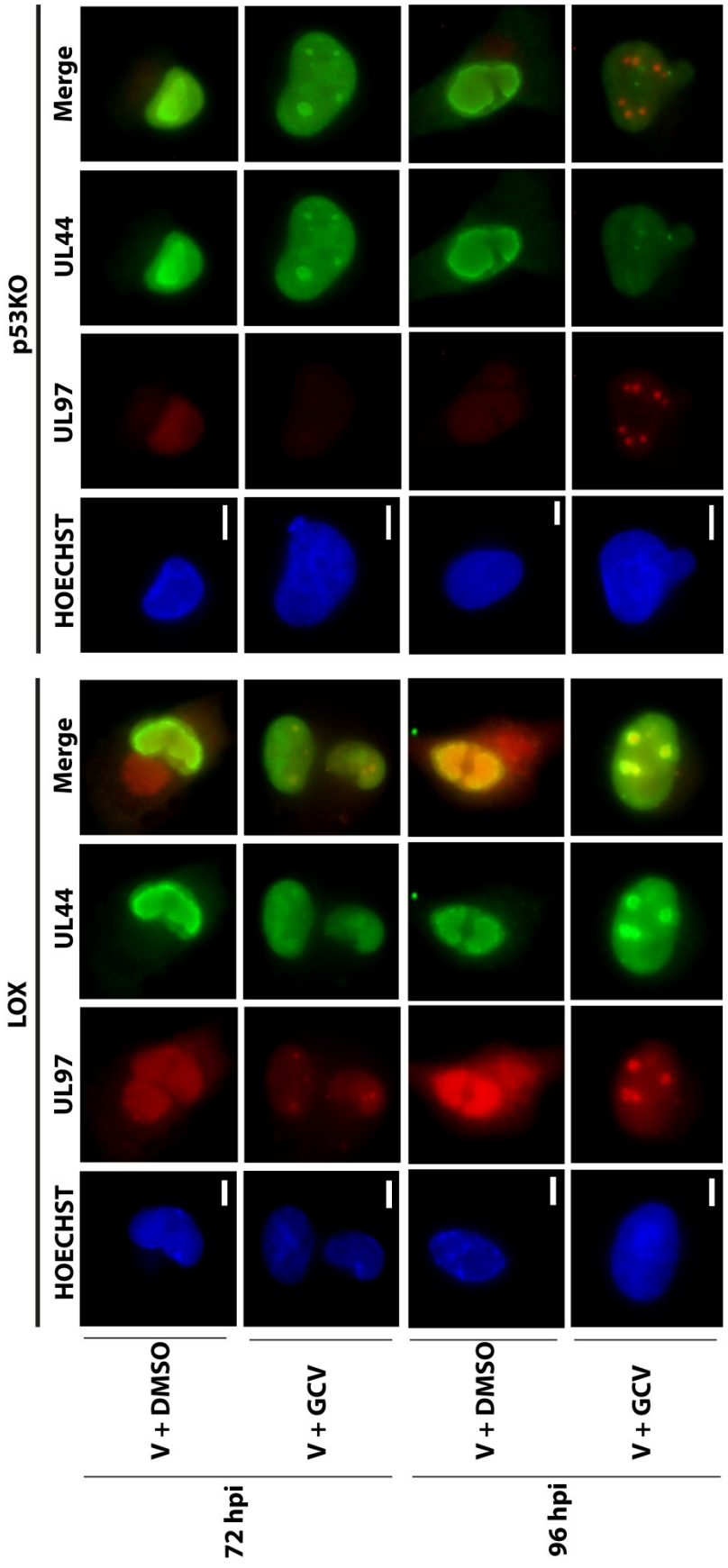
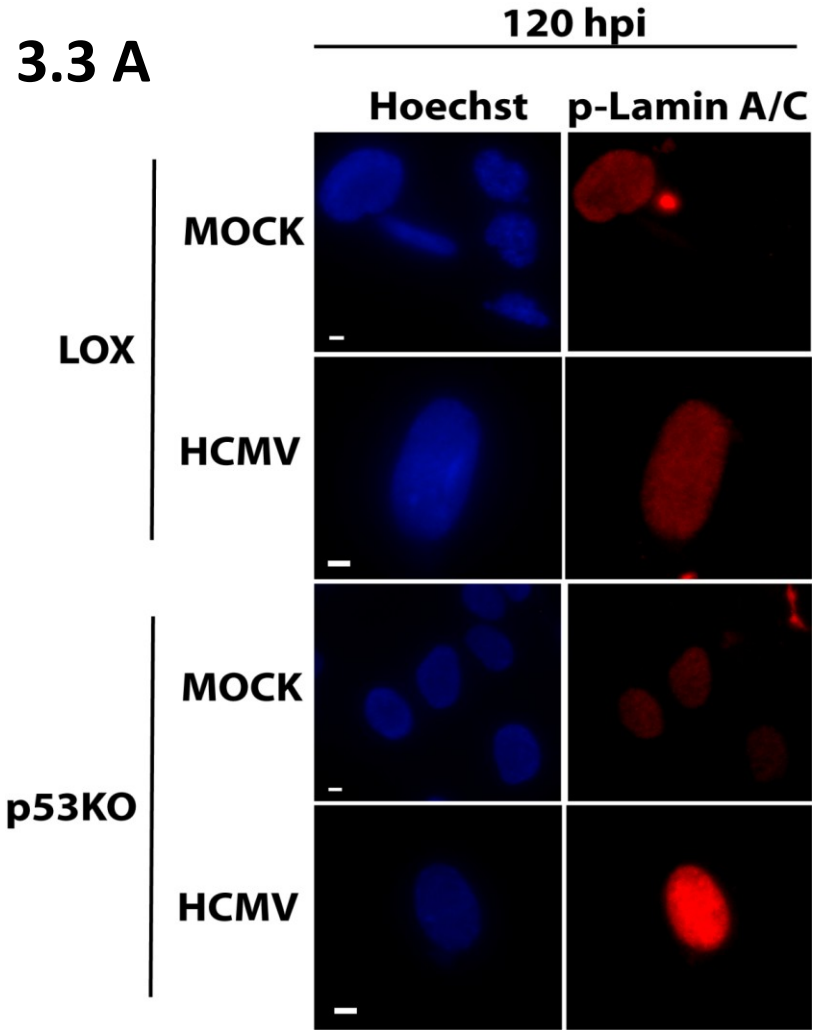


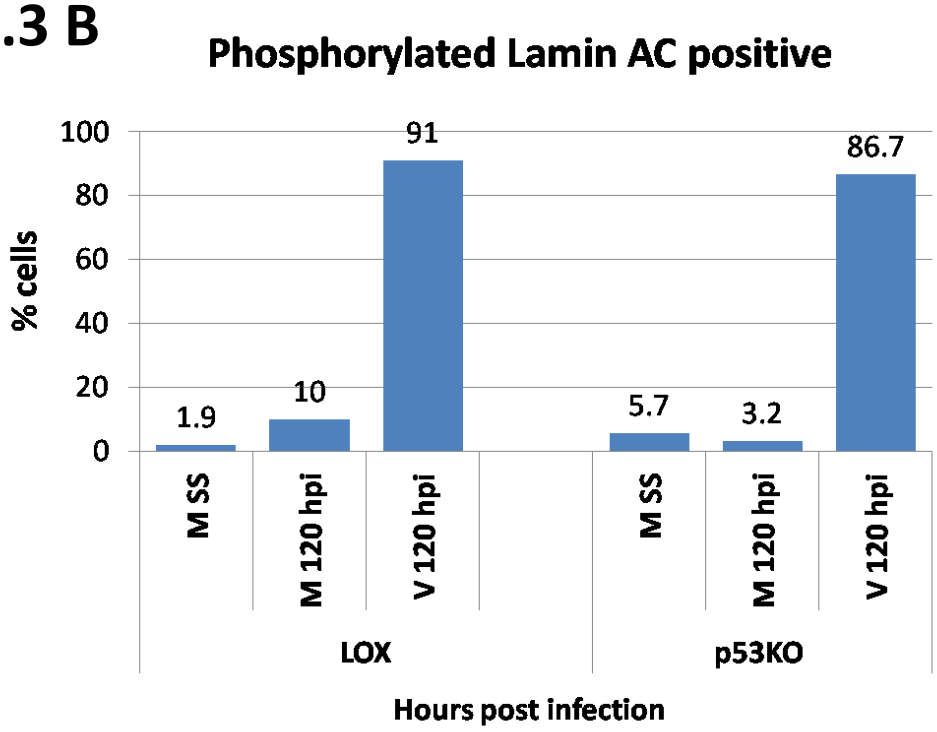
Figure 3.2 UL97 was present and functional in p53KO cells. (A) UL97 expression by Western analysis and (B) immunofluorescent staining at 24 and 120 hpi. (C) Comparison of UL97 expression in Lox and p53KO cells by immunofluorescent staining. Error bars represent one standard error calculated from 6 experiments. (D) Investigation of the functionality of UL97 after GCV treatment. GCV-treated and untreated cells were co-stained for UL97 and UL44. UL97 functionality was indirectly determined by development of viral replication centers.

UL97 was present in p53KO cells, but was it functional? Ganciclovir (GCV) is used as an antiviral against HCMV infection (reviewed in reference Biron 2006). In order for GCV to work effectively, it must be phosphorylated by UL97 (Littler, Stuart et al. 1992; Sullivan, Talarico et al. 1992). Phosphorylated GCV is added to the growing viral DNA chain, arresting its extension and terminating viral DNA replication (reviewed in references McGavin and Goa 2001; Biron 2006). The termination of viral DNA replication can be visualized by examining the viral replication centers (RCs). UL44 is a viral protein, which serves as a processivity factor of the viral DNA polymerase. UL44 is highly associated with the RCs. During HCMV infection RCs progress from multiple small foci at early times pi to single large foci (frequently occupying the majority of the nucleus) at the late times pi (Penfold and Mocarski 1997). IF analysis of late times pi (72 and 96 hpi) found that GCV treatment inhibited formation of advanced stage foci in both cell types (Figure 3.2D). Western blot analysis of UL44 after GCV treatment found similar downregulation of UL44 levels in both cell types at 96 hpi (data not shown). Therefore UL97 was functional for this exogenously introduced molecule.

Previous studies have shown that HCMV-induced nuclear lamina dissociation only occurs following their phosphorylation, particularly phosphorylation of lamin A on the serine at residue 22 (Ser 22). Phospho-lamin A/C (p-lamin A/C) antibody was used for IF analysis of infected cells. In both infected cell types almost all cells (~90%) were positive for p-lamin A/C staining at 120 hpi (Figure 3.3A and B), somewhat surprisingly at considerably higher levels of expression in the KO cells. Western blot analysis of p-lamin A/C also found higher levels of p-lamin A/C in the KO cells compared to their wt counterparts (Figure 3.3C – late timepoints shown). Taken together, our data suggested that UL97 was present in the large majority (~90%) of both cell types and was capable of phosphorylating cellular proteins, including at least one of its targets implicated in HCMV-induced nuclear lamina remodeling.


3.3 A
120 hpi
Hoechst
p-Lamin A/C
LOX
MOCK
HCMV
p53KO
MOCK
HCMV

3.3 B



3.3 C

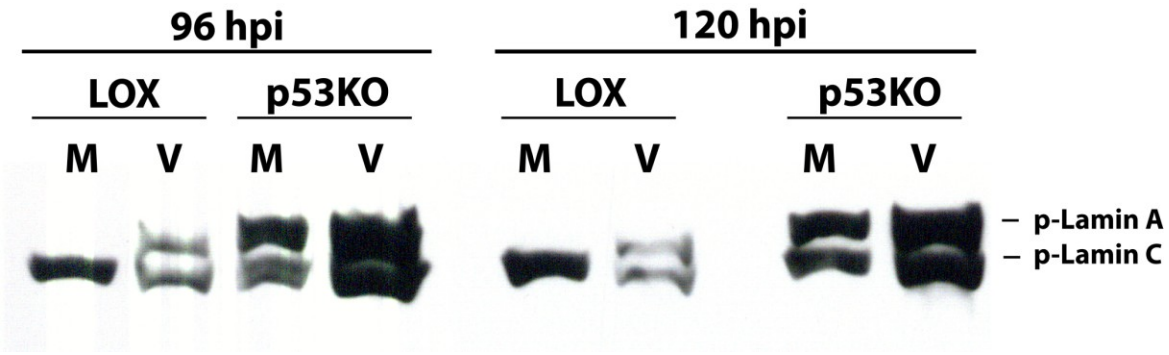


Figure 3.3 Investigation of lamin A/C phosphorylation. (A) Immunofluorescent staining of phosphorylated lamin A/C at 120 hpi and (B) percentage of serum starved mock or 120 hpi mock- and infected cells displaying lamin A/C phosphorylation. Percentages are from a single experiment but are representative of multiple rounds of staining. (C) Western blot analysis of the steady state level of phosphorylated lamin A/C.

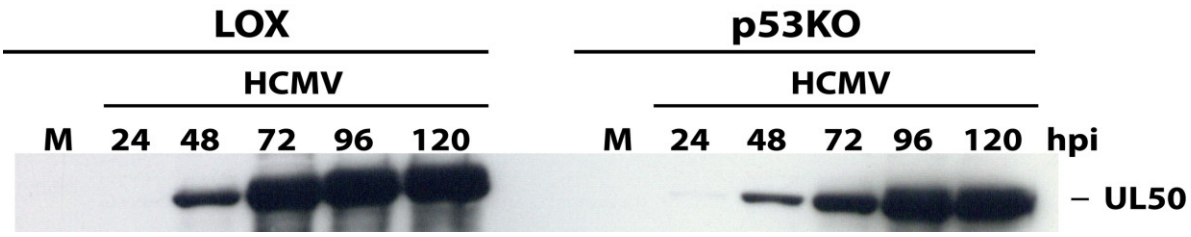
3.4.3 UL50, a required component of the NEC, was present, but did not re-localize properly.

Lamin remodeling is required for HCMV capsid egress from the nucleus (Marschall, Marzi et al. 2005; Camozzi, Pignatelli et al. 2008; Hamirally, Kamil et al. 2009; Milbradt, Webel et al. 2010; Sharma, Kamil et al. 2014). UL97 was present and functional in the infected KO cells, including phosphorylating the nuclear lamina in preparation for remodeling, however substantial modifications to the lamina structure in the KOs did not occur. Recent studies report that both UL50 and UL53 are crucial for efficient capsid nuclear egress during HCMV infection (Dal Monte, Pignatelli et al. 2002; Muranyi, Haas et al. 2002; Milbradt, Auerochs et al. 2007; Camozzi, Pignatelli et al. 2008; Sharma, Kamil et al. 2014). UL50 is a key component of the NEC, which directly recruits UL53, and indirectly recruits other NEC components to the nuclear membrane (Marschall, Marzi et al. 2005; Camozzi, Pignatelli et al. 2008; Milbradt, Auerochs et al. 2009; Sam, Evans et al. 2009; Milbradt, Webel et al. 2010; Miller, Furlong et al. 2010; Sharma, Kamil et al. 2014) Western blot analysis of UL50 confirmed that this protein was only slightly less robustly expressed in the KO cells at earlier timepoints, but was comparable to wt by 120 hpi (Figure 3.4A). IF analysis found UL50 present in the large majorities (~90% each) of both infected wt and KO cell populations at late times, with localization primarily in the cytoplasm, but also at the nuclear rim, where the NEC forms (Figure 3.4B and 4C).

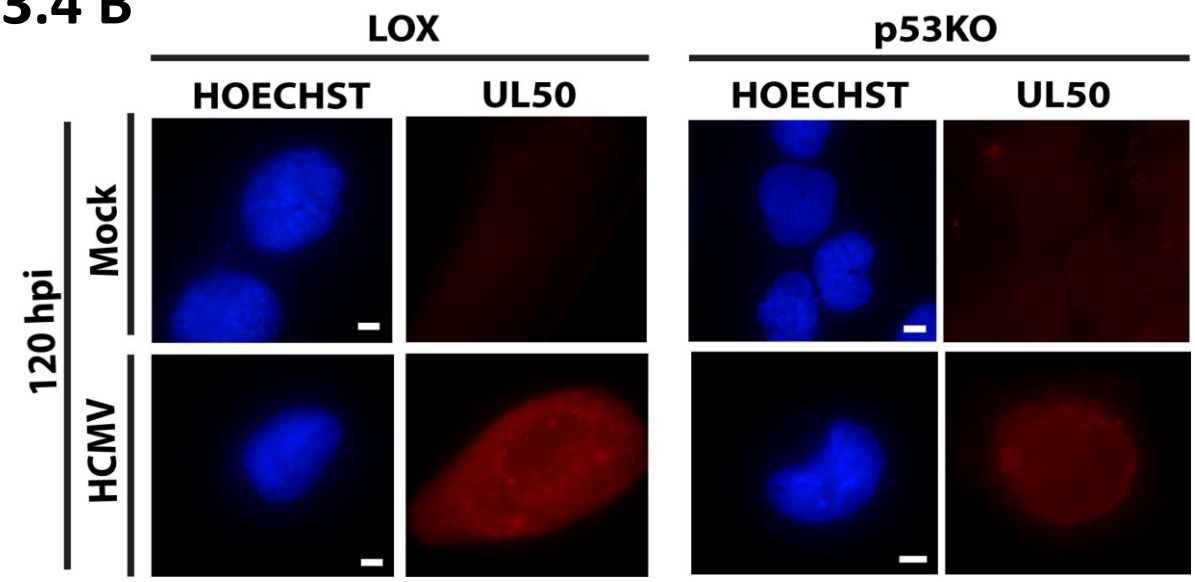
In order to more clearly visualize the nuclear localization of UL50 we used an "extraction first" methodology. This technique utilizes detergent extraction of

cytoplasmic and loosely bound nuclear proteins, prior to fixation in formaldehyde (Cardoso, Leonhardt et al. 1993; Marciniak, Lombard et al. 1998; Lombard and Guarente 2000). When UL50 was localized within the nucleus under these extraction conditions, the signal in all cells showed staining at the nuclear rim (Figure 3.4D). The nuclear UL50 signal, when present, was always weaker in the KO cells. In the figures the signal has also been shown enhanced to allow visualization of the similar staining pattern. UL50 is the “hook” that tethers the NEC into the INM (Milbradt, Auerochs et al. 2007), hence its localization at the nuclear rim. If UL50 was not in close proximity to the INM it could not perform its normal function.

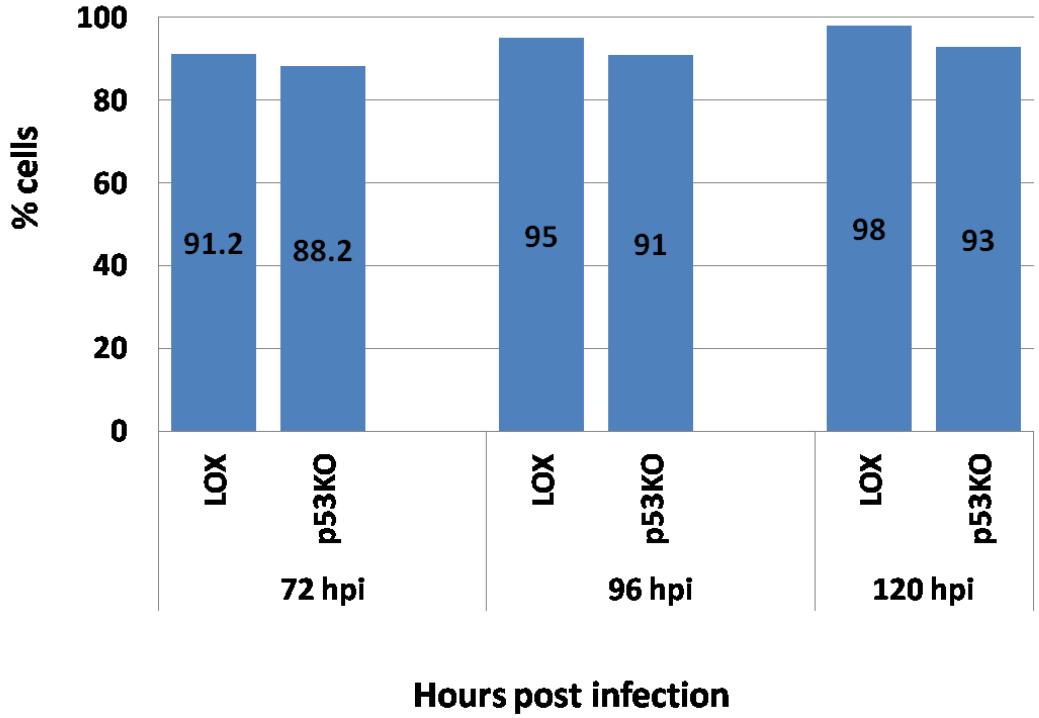
3.4 A



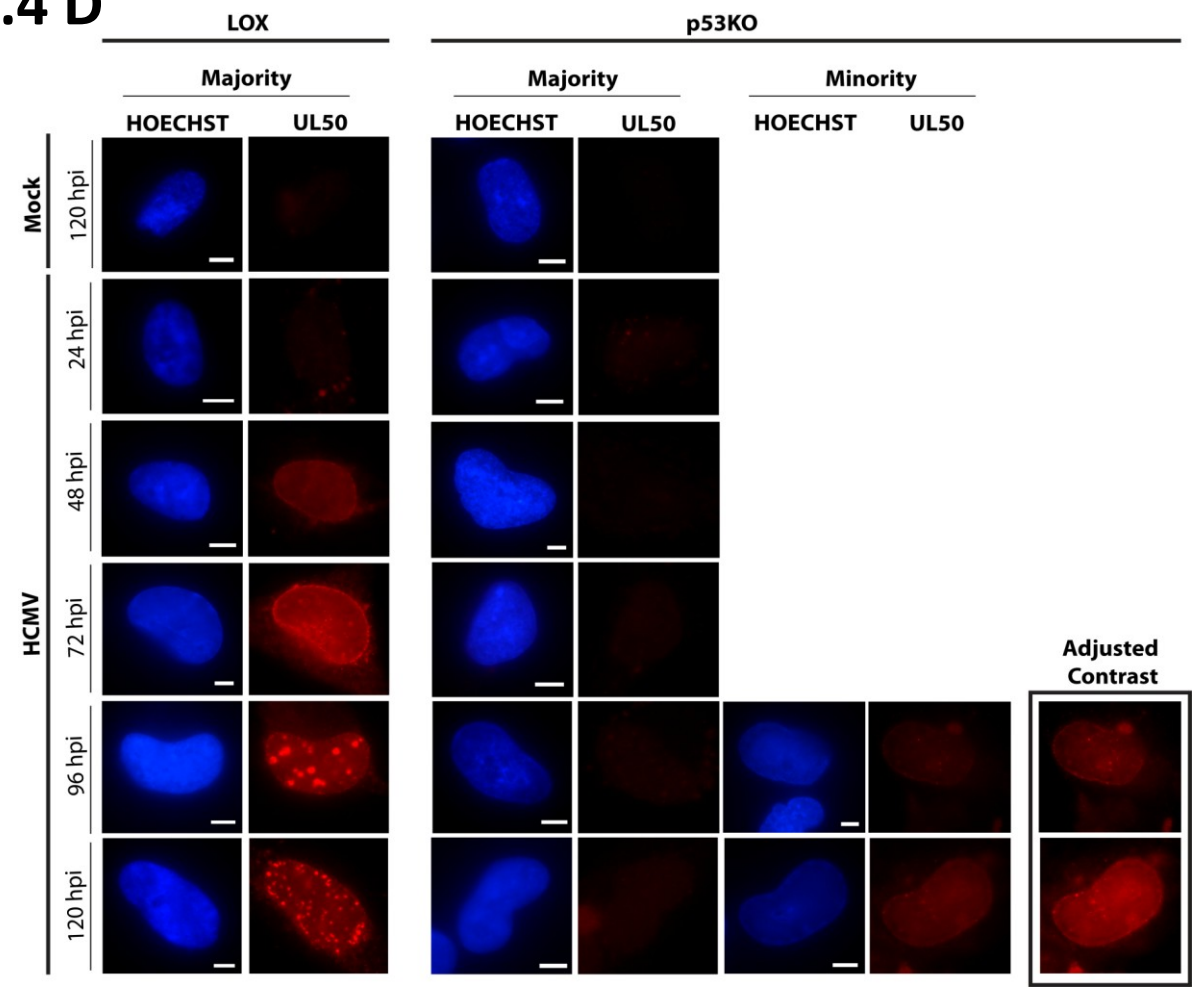
3.4 B


LOX
p53KO
HOECHST
UL50
HOECHST
UL50
120 hpi
Mock
HCMV

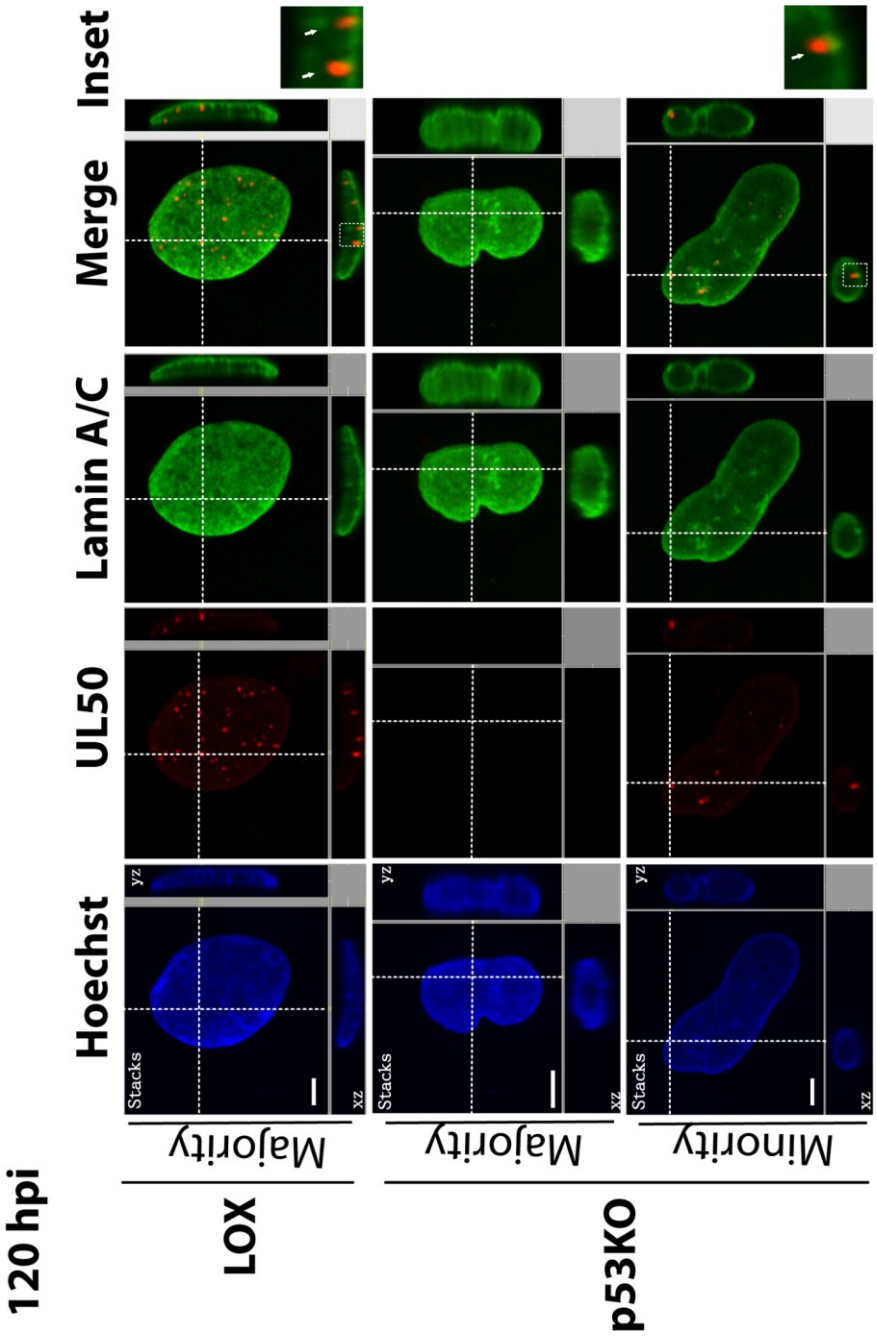
3.4 C


UL50 expression
% cells
100
80
60
40
20
0
91.2
88.2
95
91
98
93
LOX
p53KO
LOX
p53KO
LOX
p53KO
72 hpi
96 hpi
120 hpi
Hours post infection

3.4 D


LOX
p53KO
Majority
Majority
Minority
HOECHST
UL50
HOECHST
UL50
HOECHST
UL50
Mock
120 hpi
HCMV
24 hpi
48 hpi
72 hpi
96 hpi
120 hpi
Adjusted Contrast

3.4 E


120 hpi
LOX
p53KO
Majority
Majority
Minority
Hoechst
UL50
Lamin A/C
Merge
Inset
Stacks
yz
xz

3.4 F

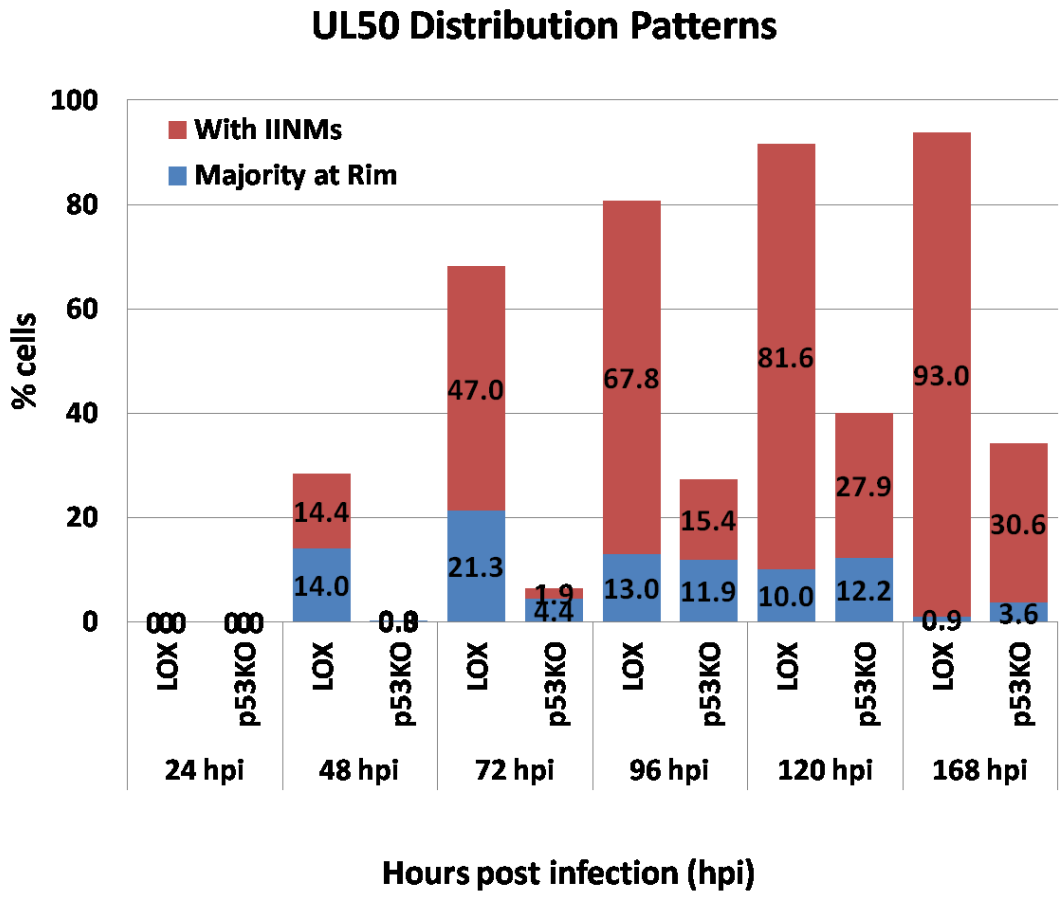


Figure 3.4 UL50 protein presence and localization in p53KO cells. (A) Western blot analysis of UL50 in both cell types. (B) Total (nuclear and cytoplasmic) UL50 protein immunofluorescent staining in both cell types at 120 hpi and (C) the percentage of cells displaying overall UL50 protein expression. Percentages are from a single experiment but are representative of multiple rounds of staining. (D) UL50 localization using “Extraction first” method in Lox cells compared to p53KO cells throughout a timecourse of infection. The minority of the p53KO population expressing UL50 required image contrast enhancement in order to clearly visualize its expression and localization. (E) Confocal analysis of infoldings of the inner nuclear membrane (IINMs). The large planar views (upper left regions of each image) are stacks comprised of the combined individual confocal xy planes of a cell. Both xz and yz represent a single section captured at the dashed crossing lines perpendicular to the large planar view (here and in all subsequent confocal images). (F) The percentage of cells with IINMs in Lox compared to p53KO cells. Percentages represent the average of three separate experiments.

IF analysis of UL50 revealed another relevant pattern. Over the timecourse of infection in wt cells, the UL50 staining was seen to first re-localize to the nucleus, forming a uniform “ring” adjacent to the INM starting at 48-72h pi. The UL50 signal then became punctate in a steadily increasing proportion of the $UL50^{+}$ nuclei population as time progressed. The KO UL50 nuclear rim positive sub-population was delayed by ~48h in conversion to punctate staining

Other research groups have reported similar punctate patterns when investigating UL50 binding partners, including UL53 and RASCAL, by infection and transfection (Dal Monte, Pignatelli et al. 2002; Camozzi, Pignatelli et al. 2008; Miller, Furlong et al. 2010). These puncta were examined more thoroughly by Confocal IF. Imaging of the puncta determined them to be thread-like projections extending from the nuclear rim into the interior of the nucleus (Figure 3.4E). UL50 was seen to stain along the length of these formations (Figure 3.4E - Inset). The portions of these formations contiguous with the nuclear membrane were commonly found within regions of disorganized lamin A/C staining and Lamin A/C was not present in along the length of the structures as they extended away from the nuclear rim. We believe these thread-like formations were infoldings of the INM (IINMs), which have been proposed as the principal sites of HCMV capsid nuclear egress (Buser, Walther et al. 2007). In our parallel EM studies, we observed these IINMs as capsid filled tubules within the interior of the nucleus.

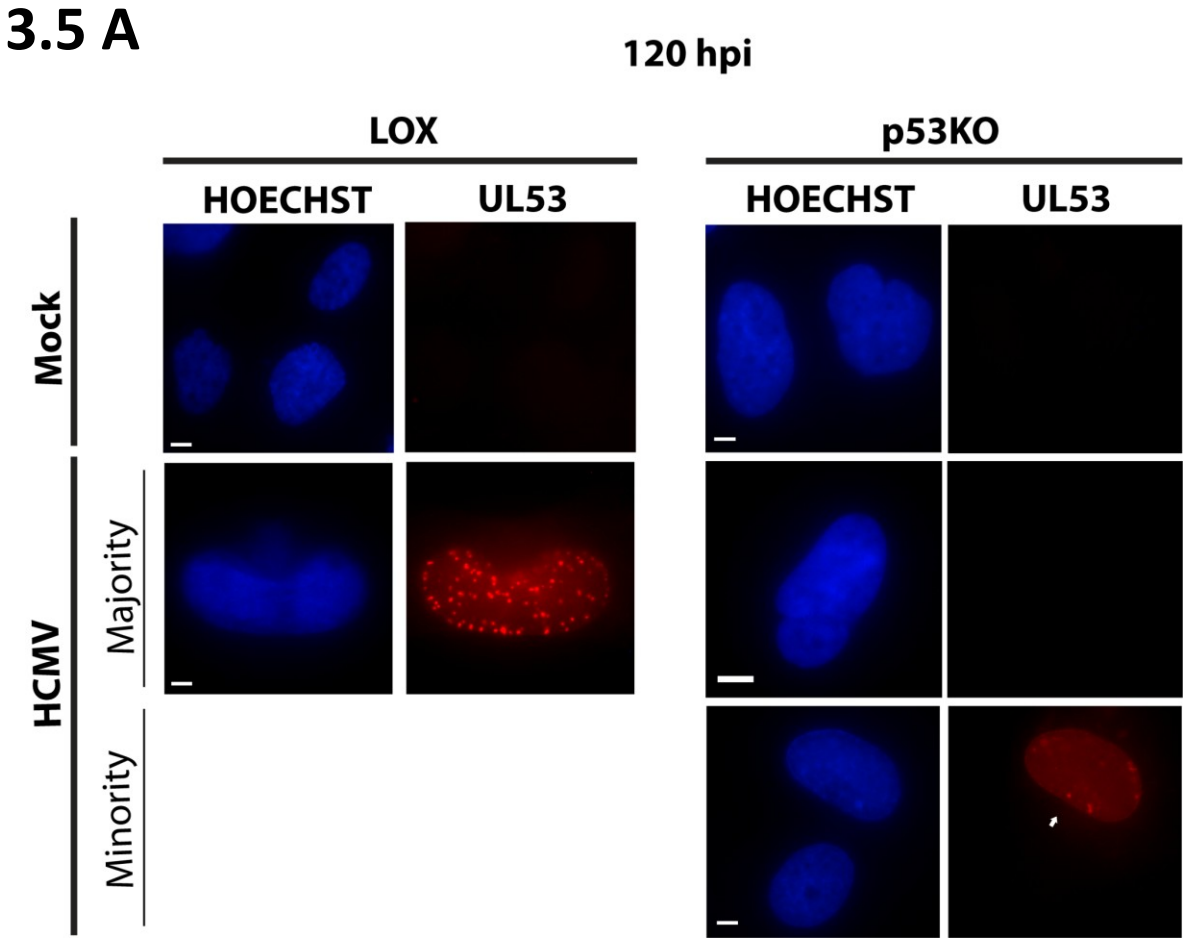
The extracted cells were analyzed and the numbers of cell with UL50 staining within the nucleus, and the signal's location, were enumerated (Figure 3.4F). This analysis found UL50 localized within the nucleus in greater than 90% of

wt cells at 120h pi, however localization of this critical NEC protein was delayed by at least 48h pi and seen in at most 40% (averaging ~30% between 96 and 168h pi) of infected KO cells' nuclei. The conversion of the UL50 staining pattern from ring-like to punctate over time was analyzed. In the large majority of the UL50 positive sub-population staining did convert to the punctate pattern, albeit with significantly fewer puncta per cell in the KOs compared to wt. (Figure 3.4F). Our parallel EM study had detected fewer of these IINMs in the KO population as well and identified capsid nuclear egress as a highly significant factor contributing to the reduced virus titers produced by the KO cells. The failure of UL50 to re-localize properly suggested the NEC was not functioning normally.

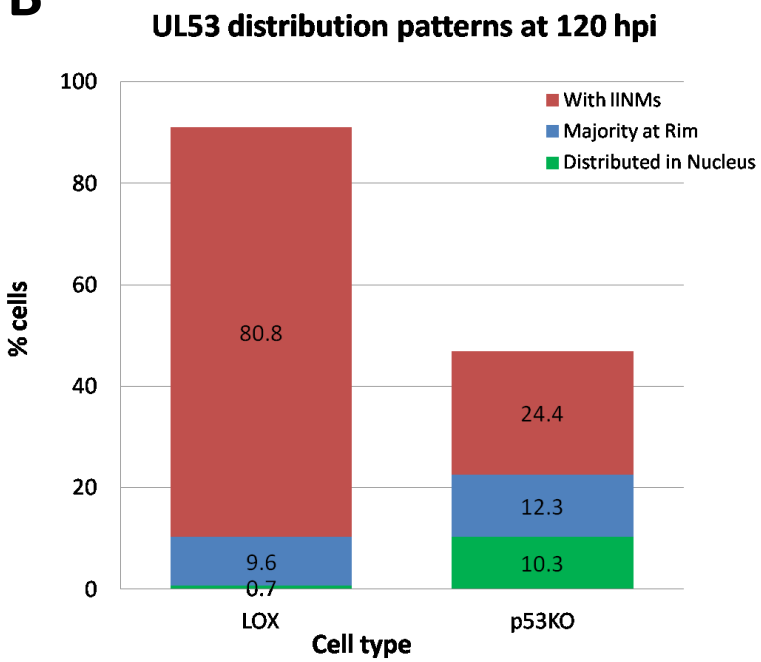
3.4.4 UL53 was only expressed in half the KO cells and its presence was required for proper UL50 localization.

The failure of UL50 to re-localize properly suggested that other components of the NEC might also not be present at the nuclear membrane. One of UL50's binding partners is UL53. The presence of UL50-UL53 complex is crucial for facilitating capsid nuclear egress during HCMV infection (Dal Monte, Pignatelli et al. 2002; Muranyi, Haas et al. 2002; Milbradt, Auerochs et al. 2007; Camozzi, Pignatelli et al. 2008; Sharma, Kamil et al. 2014). IF staining of UL53 produced surprising results. At 120 hpi, a large proportion of KO cells appeared to not express UL53 (Figure 3.5A – majority KO). A subset displayed a similar pattern to the wt cells, localizing to the nuclear rim and/or at IINMs (Figure 3.5A – minority KO; IINM

indicated by arrow). Quantitation of this staining revealed that the large majority of wt cells (>90%) displayed UL53 at the nuclear rim (or in puncta), while only approximately 30-40% of KO cells were seen with similar distributions (and an additional 10% distributed throughout the nucleus) (Figure 3.5B). Confocal analysis of dual-stained UL50 and UL53 cells revealed that UL53 co-localized with UL50 at the IINMs (shown in Figure 3.5C) and/or the nuclear rim when UL50 was present in the nucleus. Here again, the NEC was not behaving in a wt manner in the large majority of KO cells, and tellingly, a large proportion of KO cells completely lacked a major component of this complex.



3.5 B



3.5 C

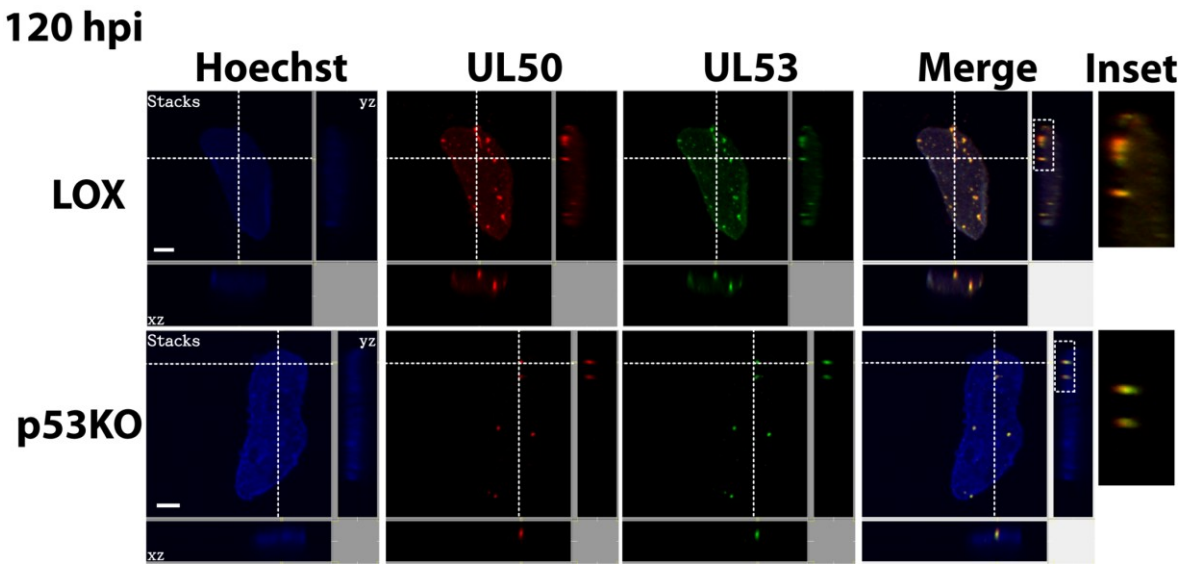
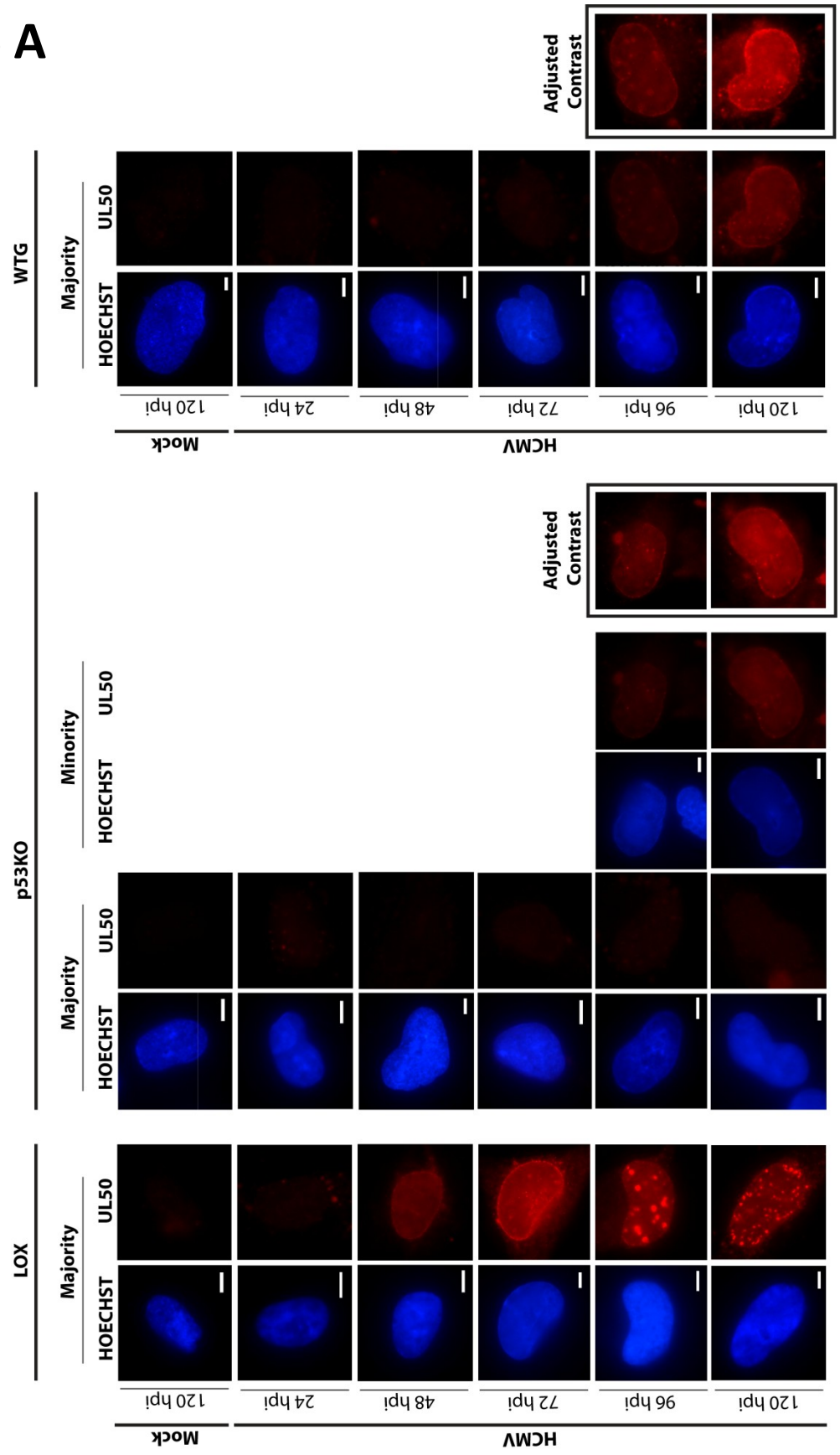


Figure 3.5 Expression of UL53 and localization with its binding partner UL50. (A) Immunofluorescent staining of UL53 at 120 hpi. (B) The percentage of cells expressing UL53 and the presence of IINM patterns in Lox compared to p53KO cells. Percentages represent the average of two separate experiments. (C) Confocal analysis of UL50-UL53 co-localization. See description of layout of images in Figure 3.4E.

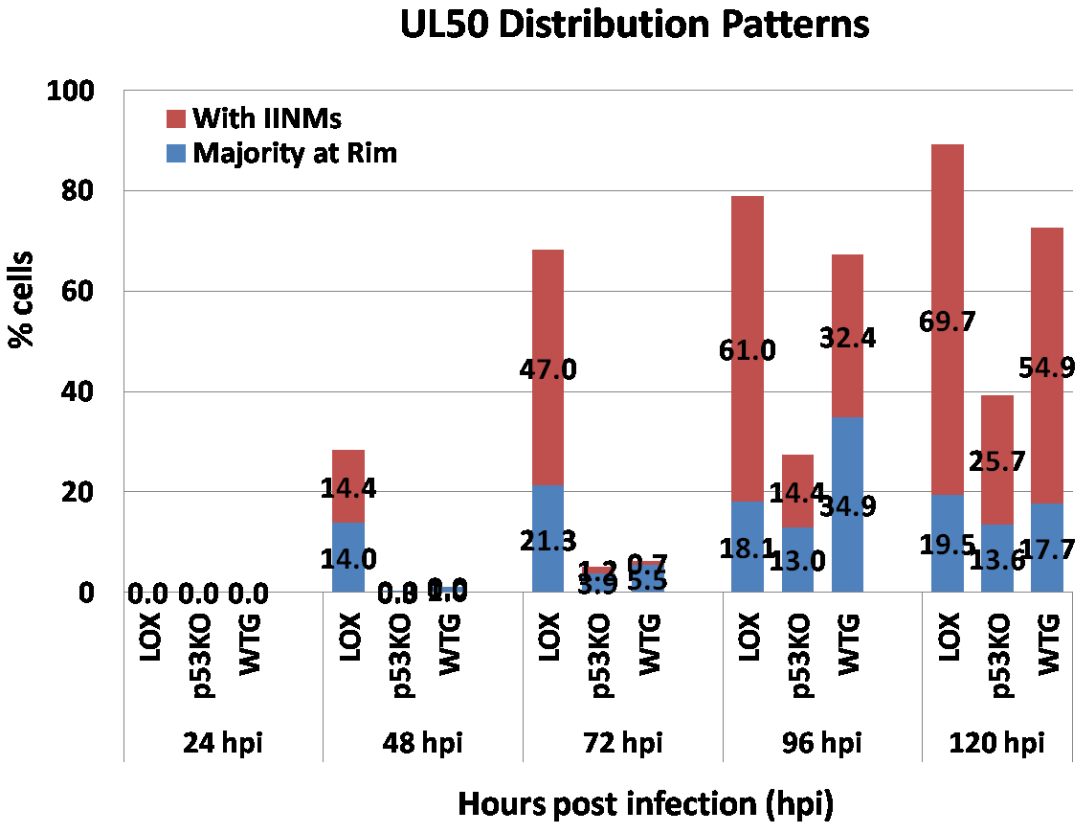
3.4.5 Reintroduction of p53 recovered the accumulation of UL53 and UL50 at the nuclear rim.

The absence of p53 resulted in lower viral titers, abnormal nuclear membrane remodeling and improper re-localization of critical components of the viral nuclear egress machinery. We had previously found that reintroduction of p53 into the KO cells, here stable clone wt G cells (WTGs), recovered virus titers. As described previously, these KO cells were extremely sensitive to p53 expression and could only survive in the presence of vanishingly small amounts of protein, too low to be detected by Western blot. However, p53 transcripts were detectable by reverse-transcription PCR in the re-introduced cells (Casavant, Luo et al. 2006). Somewhat surprisingly even this very small amount of p53 increased virus titers in WTG to within 2-3 fold of full wt virus production, as opposed to the 25 fold differential of the KO cells. Our parallel EM study found an equivalent recovery of virus capsids being trafficked into the cytoplasm in these WTG cells. IF analysis of UL50 INM localization in WTG found it similarly recovered to wt levels in terms of percentage positive cells by 120h pi, but at the approximate expression level of intensity found in the KO cells (Figure 3.6A-B). The percentage of WTG cells with wt-UL50-staining patterns increased by ~100% compared to KO cells (Figure 3.6B). The minimal level of p53 present in the WTGs had recovered UL50 localization, but presumably not expression levels of the protein.

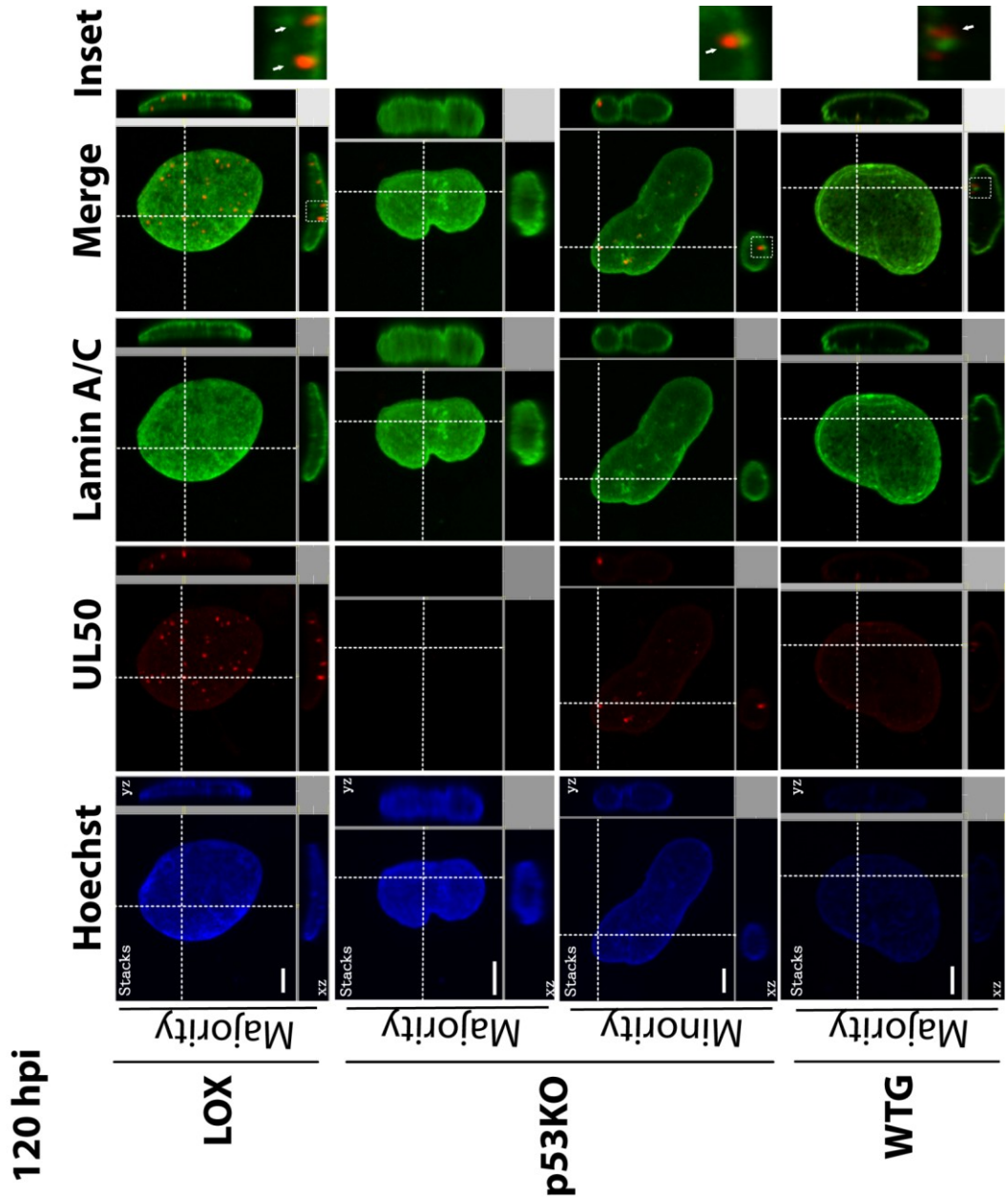
3.6 A


WTG
p53KO
LOX
Majority
Minority
HOECHST
UL50
Adjusted Contrast
Mock
HCMV
120 hpi
24 hpi
48 hpi
72 hpi
96 hpi
120 hpi

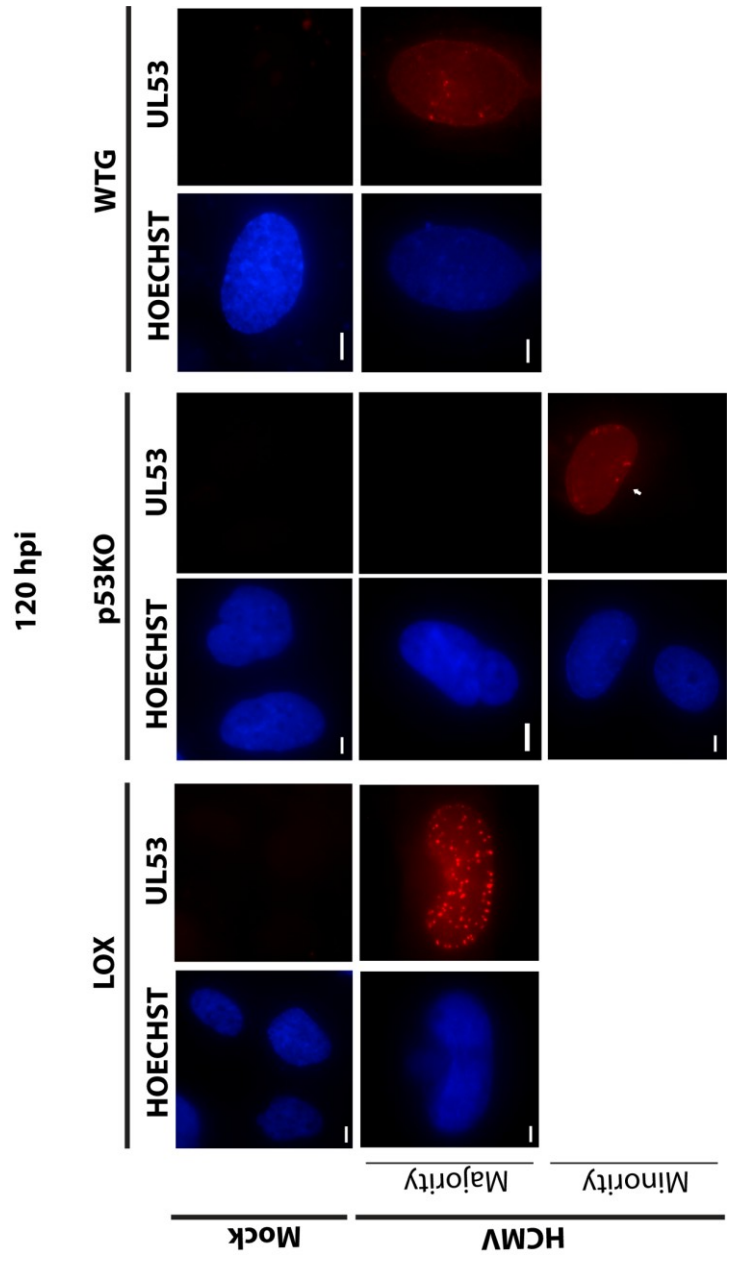
3.6 B


UL50 Distribution Patterns
With IINMs
Majority at Rim
% cells
100
80
60
40
20
0
0.0 0.0 0.0
14.4
14.0
47.0
21.3
61.0
18.1
14.4
13.0
32.4
34.9
69.7
19.5
25.7
13.6
54.9
17.7
LOX
p53KO
WTG
24 hpi
48 hpi
72 hpi
96 hpi
120 hpi
Hours post infection (hpi)

3.6 C


120 hpi
LOX
p53KO
WTG
Majority
Majority
Minority
Majority
Hoechst
UL50
Lamin A/C
Merge
Inset
Stacks
yz
xz

3.6 D


120 hpi
WTG
p53KO
LOX
UL53
HOECHST
Mock
HCMV
Majority
Minority

3.6 E

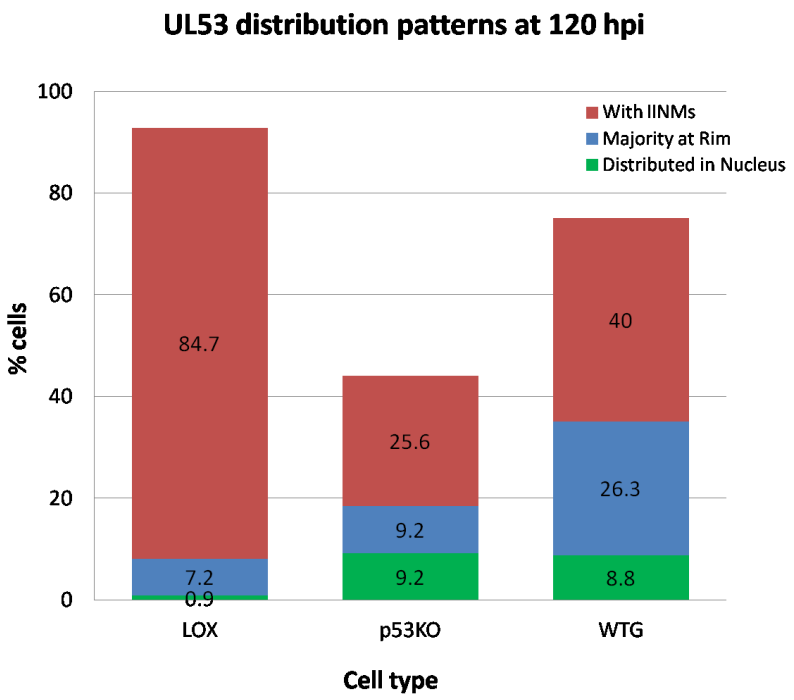


Figure 3.6 Re-introduction of p53 into p53KO cells recovered UL50 localization and UL53 expression. (A) Timecourse of UL50 localization in Lox, p53KO and WTG cells by immunofluorescent staining and (B) the percentage of cells with IINMs in WTG compared to Lox and p53KO cells. Percentages represent the average of two separate experiments. (C) Confocal analysis of IINMs in all three cell types at 120 hpi. (D) UL53 immunofluorescent staining at 120 hpi and (E) the percentage of cells expressing UL53 in WTG in comparison to Lox and p53KO cells. Percentages are derived from a single experiment.

Perhaps in a corresponding manner this recovery was not paralleled by as robust a reconstitution of the IINMs. In the WTG population there were markedly more cells containing infoldings present in comparison to KOs (26 versus 55%) (Figure 3.6B), but short of wt levels. More importantly, these infoldings were only slightly more numerous in the WTG cells compared to their parental KOs. Nor did the infoldings appear as large as in the wt cells. This could be viewed by both epifluorescent and confocal microscopy. The infoldings were found in a range of

sizes, but most were small compared to wt (Figure 3.6C). This suggests that reintroduction of a very limited amount of p53 may not have fully reestablished the wt IINM capsid egress mechanism. Rather, reintroduction permitted an intermediary effect allowing NEC localization to the INM, which increased nuclear capsid egress without large infoldings forming, but in a much less efficient manner than that available to the wt cells.

Since the UL50 nuclear rim and IINM localization was increased in the WTG cells, we next asked if this was due to an increase in the level of its binding partner, UL53, in the nucleus of these cells. Not surprisingly, both the rim/IINM staining of UL53 and the overall percentage of cells positive for the protein were both increased in the WTG cells (Figures 3.6D and E). Parallel to the UL50 nuclear staining, there were increased numbers of WTG cells that had UL53 rim and/or IINM staining, but the numbers of IINM/cell were still lower than that observed in wt cells.

Taken together our results have established that in infected cells the activity of p53 promoted the relocalization of UL50 to the inner nuclear membrane. This may have, in part, been due to p53's promoting expression of UL50's binding partner, UL53. When properly re-localized to the nucleus UL50 was able to complex with UL53 and form the NEC. In turn the NEC was the site of formation of tubule-like infoldings of the inner nuclear membrane. Our data suggests that the observed remodeling, or perhaps more appropriately, rearrangement of the UL97 phosphorylated nuclear lamina, resulted in the formation of the tubules. HCMV

capsids were able to exit the nucleus in the absence of large infoldings/tubules, as observed in the KO and WTG cells, but at a greatly reduced pace than from wt cells.

3.5 Discussion

p53, the “guardian-of-the-genome”, plays many roles within the cell. It is unsurprising that HCMV has evolved to both circumvent and exploit some of this ubiquitous cellular protein’s functions during its lifecycle within the cellular environment. Perhaps the most obvious sign that the virus alters normal p53 function is the near complete association of the protein with the viral RCs (Fortunato and Spector 1998). In unstressed cells the protein is expressed at low levels and present in both the nucleus and cytoplasm. In a stressed cell, which virus infection could easily be considered, p53 is generally stabilized and principally accumulates in the nucleus, although in certain situations, it can be found in the cytoplasm near the mitochondria (Moll, Wolff et al. 2005). The virus-induced removal of p53 from these locations to the site of its own DNA replication suggests that, not only is p53 unavailable to perform its normal cellular functions, but also that the virus may be coopting the protein to its own ends. We have previously shown that p53 can bind specifically to viral DNA and its presence appears to be necessary to transactivate several viral gene products (Rosenke, Samuel et al. 2006; Hannemann, Rosenke et al. 2009). p53’s roles interacting with, and as a transactivator of, the DNA repair machinery suggest the virus may be utilizing this function in the replication and maintenance of fidelity of its own genome. Our earlier finding that the HCMV

genome was selectively repaired in preference to the cellular genome following insult support this supposition (O'Dowd, Zavala et al. 2012).

We have found that deciphering p53's role during infection is easiest through examination of a cellular environment without this critical regulatory protein. To ensure a relevant comparison to wt in these experiments we have utilized the KOs' parental Lox lung fibroblasts. Our earlier work established that in the absence of p53, functional virus production was reduced by 25-fold at late times pi (Casavant, Luo et al. 2006). This study, and its parallel EM analysis, were undertaken to further elucidate particular breakdowns in the viral lifecycle in the absence of p53 to better understand the role of the protein in a functional wt infection.

Our parallel EM study found that in the KO cell environment HCMV was able to produce wt-like quantities and types of virus capsids. However, the capsids generated were significantly less likely to transit into the cytoplasm. Capsid nuclear egress is an envelopment/de-envelopment pathway across the double nuclear membrane. Disruption of the nuclear lamina allows nuclear capsids access to the inner nuclear membrane (INM). Capsids bud through the INM and acquire their primary envelope. This envelope is lost after fusion with the outer nuclear membrane when the capsid enters the cytoplasm. The capsids obtain their final tegument and secondary envelope within the cytoplasm. The reduced number of capsids available in the cytoplasm and a lower rate of secondary envelopment combined to largely reconstitute the substantial drop in titers produced by the KO cells.

Here, the molecular basis of the poor trafficking of capsids was investigated at a number of stages and for accepted molecular markers. The literature indicated that without remodeling of the nuclear lamina capsids were not efficiently trafficked into the cytoplasm (Hamirally, Kamil et al. 2009; Milbradt, Webel et al. 2010; Reim, Kamil et al. 2013; Sharma, Kamil et al. 2014). The nuclear lamina is a dense meshwork that mechanically supports the nuclear architecture. Since HCMV arrests the cell cycle for viral DNA replication at G1/S phase (Bresnahan, Boldogh et al. 1996; Lu and Shenk 1996; Dittmer and Mocarski 1997) as well as G2/M phase (Jault, Jault et al. 1995), HCMV is unable to utilize the nuclear lamina breakdown that occurs at mitosis and is too large to transit through the nuclear pores. It has been demonstrated that HCMV utilizes phosphorylation-dependent lamina remodeling to facilitate exiting the nucleus (Hamirally, Kamil et al. 2009).

The viral protein UL97 plays a critical role in nuclear capsid egress via phosphorylation of lamin A/C and the ensuing lamina dissociation (Krosky, Baek et al. 2003; Hamirally, Kamil et al. 2009; Milbradt, Webel et al. 2010; Reim, Kamil et al. 2013). This phosphorylation is thought to “unzip” the structure and make it more malleable, promoting dissociation. UL97 contributes to various functions during HCMV infection (Krosky, Baek et al. 2003; Marschall, Freitag et al. 2003; Baek, Krosky et al. 2004; Marschall, Marzi et al. 2005; Kamil and Coen 2007; Tran, Mahr et al. 2008; Silva, Strang et al. 2011; Bigley, Reitsma et al. 2013). Absence of UL97 produces a dramatic (up to 1,000-fold) growth defect (Prichard, Gao et al. 1999). Experiments performed here found UL97 was able to incorporate the chain-terminating nucleoside analogue ganciclovir into the growing viral DNA chain (Littler,

Stuart et al. 1992; Sullivan, Talarico et al. 1992) and, in data not shown, phosphorylate Rb protein, another cellular target (Hume, Finkel et al. 2008). Most relevant, virtually all p53KO cells displayed phosphorylated lamin A/C, indicating this UL97-initiated function important to capsid nuclear egress was also being accomplished.

In wt cells large sections of the lamin structure were frequently missing or very fuzzy by comparison with mock-infected cells. In the KO cells only rarely were there large segments of the nuclear rim with similarly fuzzy or missing regions. What was preventing or not causing the rearrangement of the lamins? In order for efficient nuclear capsid egress to occur the nuclear egress complex must function correctly. The NEC has a number of viral and cellular components. In HCMV, this complex contains both viral and cellular proteins. UL50, UL53, UL97, RASCAL, p32, lamin B receptor, emerin, and possibly PKC, are all components of the NEC (Krosky, Baek et al. 2003; Marschall, Marzi et al. 2005; Camozzi, Pignatelli et al. 2008; Hamirally, Kamil et al. 2009; Milbradt, Auerochs et al. 2009; Sam, Evans et al. 2009; Miller, Furlong et al. 2010; Milbradt, Kraut et al. 2014; Sharma, Kamil et al. 2014). Of these components, two proteins conserved across the herpesvirus family are required for efficient primary envelopment and subsequent nuclear egress. In HCMV these two proteins are UL50 and UL53 (Dal Monte, Pignatelli et al. 2002; Milbradt, Auerochs et al. 2007; Camozzi, Pignatelli et al. 2008), UL34 and UL31 in HSV-1 (Reynolds, Ryckman et al. 2001; Reynolds, Wills et al. 2002) and psuedorabies virus (PrV) (Fuchs, Klupp et al. 2002), and BFLF1 and BFRF2 in

Epstein-Barr virus (Lake and Hutt-Fletcher 2004; Farina, Feederle et al. 2005; Gonnella, Farina et al. 2005).

The presence and function of the NEC has been demonstrated as critical to productive CMV infections (Liang and Baines 2005; Granato, Feederle et al. 2008; Sharma, Kamil et al. 2014). UL50 and UL53 null mutations are lethal to replicative HCMV infection (Sharma, Kamil et al. 2014). The temporal recruitment of the NEC building blocks commences with UL53 becoming largely localized in the nucleus by 24 h pi, presumably via its nuclear localization signal (NLS) (Milbradt, Auerochs et al. 2012; Schmeiser, Borst et al. 2013). At this time, UL50 is primarily expressed in the cytoplasm. UL50 then re-locates from the cytoplasm into the nucleus, where, in this study, it was always detected with some degree of nuclear rim signal. From the nuclear rim, UL50 recruits UL53 to the same location (Camozzi, Pignatelli et al. 2008; Sam, Evans et al. 2009; Milbradt, Auerochs et al. 2012; Schmeiser, Borst et al. 2013; Milbradt, Kraut et al. 2014). Transient expression assays have shown that UL50 is required for re-localization of UL53 to the nuclear rim (Camozzi, Pignatelli et al. 2008; Sam, Evans et al. 2009; Milbradt, Auerochs et al. 2012). Our observation confirmed this organizing strategy; in all cases UL50 positive nuclei displayed nuclear rim staining. In addition, when both proteins were present within the nucleus, UL50 and UL53 were co-localized at the rim.

How UL50 is transported to the nucleus is the subject of speculation (Schmeiser, Borst et al. 2013). UL50 lacks its own NLS (Milbradt, Auerochs et al. 2012; Schmeiser, Borst et al. 2013). Interestingly, uninfected cells transiently transfected with UL50 were able to localize UL50 at the nuclear rim in both wt and

KO cells (data not shown). The transfected UL50 also re-localized co-expressed UL53 to the rim. No other viral proteins were necessary. This implied that localization of UL50 into the nucleus was not an inherent problem in the KO cells and could occur in the absence of other viral proteins.

This study has found a new relationship between these proteins. Although UL50 was present in all KO cells, albeit at a lower level, only 30-40% of KO cells displayed the proper localization of this protein at the nuclear membrane rim. In the majority of KOs UL50 remained in the cytoplasm. The level of UL50 protein localized at the rim also appeared to be decreased in magnitude in comparison to wt. Interestingly, in all infected cells with UL50 localized to the rim of the nucleus, UL53, its nuclear egress complex partner, was also there. In the KOs ~50% of cells did not express UL53, or expressed this protein at a level below our IF detection limit. The absence of this important NEC protein from a large number of KO cells would be expected to result in the decreased trafficking observed in the KO cells in our parallel EM analysis. Further, these results suggested that the absence of p53 from the KO cells inhibited UL53 expression. And, in turn, that the absence of UL53 within the nucleus prohibited the entrance of UL50 into that compartment. This indicated that in a wt infection expression of both proteins in concert at a specific moment post infection was required for proper formation of the NEC at the nuclear rim. During infection the inability of UL50 to transit into the nucleus, and on to the nuclear rim, in the absence of UL53 from the nucleus also implied that UL53 was necessary to maintain transport of UL50 into the nucleus.

3.5.1 Formation of tubules and disturbance of the nuclear lamina

Our EM study had identified a reduction in infoldings of the INM in the KO cells. Many ultrastructural studies of CMV-infected cells have found enlargements of the perinuclear space (variously termed intranuclear pseudoinclusions, invaginations or infoldings of the inner nuclear membrane (IINMs)). These structures always contained primary-enveloped capsids (Ruebner, Miyai et al. 1964; Papadimitriou, Shellam et al. 1984; Severi, Landini et al. 1988; Gilloteaux and Nassiri 2000; Dal Monte, Pignatelli et al. 2002; Buser, Walther et al. 2007). Buser et al reported that these infoldings of the INM accounted for approximately 5% of the total INM surface, but the majority of nuclear trafficking capsids (86%) budded into these sites compared to those exiting at the peripheral INM (Buser, Walther et al. 2007). IINMs were conspicuously absent from the KO cells' nuclei by EM analysis. Again, our EM data was too scant to quantify this parameter's significance, but qualitatively in the KO cells that contained them, the tubules were only ~30% the size, capsids per infolding, of their wt counterparts. Interestingly, in both cell types all the tubules were largely full of capsids with very little unoccupied space within them. This implied that the driving force of tubule extension/expansion was the addition of capsids.

In this study, in addition to staining at the rim of the nucleus, we observed a punctate patterning of UL50/UL53 in the nucleus at late times pi. Miller et al indicated that these punctate patterns were IINMs (Miller, Furlong et al. 2010). Confocal analysis revealed that these punctate structures appeared as tubules extending from the inner nuclear membrane. We found that the majority of wt cells

progressed to form abundant IINMs at the late stages of infection. The 30-40% of KO cells that had UL50 and/or UL53 accumulation at the nuclear membrane were also able to progress to IINMs, however, fewer IINMs were present and they were much smaller in size.

Interestingly, these punctate structures were also observed in both wt and KO cells with transiently co-expressed UL50 and UL53 (data not shown), as reported by other groups in HCMV (Camozzi, Pignatelli et al. 2008) as well as with the PrV homologues (Klupp, Granzow et al. 2007). A recent report in HSV-1 showed that UL34-UL31 alone was sufficient to induce membrane deformation in vitro without additional cellular or viral factors (Bigalke, Heuser et al. 2014). In our transfected cells the UL50-UL53 complex also formed puncta, which may deform the nuclear membrane to form tubules without other viral factors. Further experiments will be directed at confocal or electron microscopic imaging of transfected cells to visualize these structures mediated by the UL50-UL53 complex.

Confocal analysis of KO cells revealed that there were minor gaps in the lamina after infection in most cells, but not of the extent observed in the wt cells. This begged the question of how, in the large majority of KO cells lacking the UL50/UL53 complex at the nuclear rim, capsids were transiting through the INM, in the reduced numbers our EM study had identified. The phosphorylation of lamin A/C displayed by all cells may have held the answer. Although studies have found that UL97 is recruited to the NEC and phosphorylates UL50 and UL53 (Sharma, Bender et al. 2014; Sharma, Kamil et al. 2014), our results indicated that it was not necessary for UL97 to be bound to the NEC to phosphorylate the lamina. We had

no indication that UL97 was not necessary for lamin phosphorylation, and accept the literature's record that UL97 is responsible, but phosphorylation occurred with or without the NEC being present. Equally surprising, phosphorylation of the lamina did not induce dramatic remodeling nor lead to large tubule formation. However, phosphorylation did appear to allow capsids to transit through the INM, albeit at a substantially suboptimal rate.

3.5.2 Reintroduction of p53 partially reconstituted capsid egress

The absence of p53 in the KO cell line had contributed to several marked changes in infection, most notably these cells produced ~25-fold less functional virus than their Lox wt parental line (Casavant, Luo et al. 2006). Our experiments had indicated that nuclear egress and, specifically, inhibition of formation of IINMs as the principal cause of the reduction. Would reintroduction of p53 into the KO cells restore formation of IINMs? The KO cells appeared to be extremely sensitive to the presence of reintroduced p53. Only clones expressing extraordinarily small amounts of p53, detectable only by PCR, were viable. Presumably any cells expressing p53 in greater abundance were not viable. Reintroduction had recovered virus titers to ~50% of the wt level in WTG cells. In our EM study the percentage of WTG cells containing IINMs increased compared to KO, almost equivalent to wt levels, but did not recover either the number or size of tubules within the nucleus. In this study, reintroduction of p53 also increased the normal localization of UL50 to nearly wt-levels, presumably by increasing the number of cells expressing UL53. However, it

did not recover expression levels of UL50 on a per cell basis to wt. The WTG cells exhibited a correspondingly lower degree of lamin rearrangement compared to wt, corroborating our supposition that the growth of the IINMs induce the rearrangement of the lamin structure. We hypothesize that the very limited amount of p53 present was sufficient to promote the increased expression of UL53, and the subsequent transport of UL50 to the nucleus, but was inadequate over the timecourse of infection to support the continuing extension/expansion of the IINMs to their fully functional capacity, thereby only recovering a degree of the wt virus production.

3.6 Conclusions

Capsid nuclear egress is not a static process. While we did not fully quantify all of our EM experiments at earlier times pi, we did collect images from one of them. In this experiment in wt cells tubules increased in the population, in number and in size, over the course of infection. Conversely, in KO cells the appearance of tubules was delayed by 24h and they did not increase within the population, in number within the cell, or in size, over the timecourse of infection (data not shown). A steady increase in the number and size of IINMs, which were always full of capsids, would have required a continual supply of UL50, the membrane hook of the NEC, and UL53, the NEC capsid “hand-off” protein. Lacking either one would disrupt continued extension/expansion of the tubules, assuming the tubules’ continuous growth was stimulated by addition of capsids.

UL50 was expressed and present in the cytoplasm of virtually all KOs. UL53 was present in only ~50% of the KO cells and its signal was always confined to the nucleus. However, UL50 was present (at lower than wt levels) in only ~30-40% of KO nuclei, all of which were $UL53^{+}$ cells. We believe the absence of p53 inhibited the continuing virus-induced modification of the nuclear environment necessary for the formation of IINMs. Combining our UL50-53/NEC and EM data led us to believe that in the absence of p53 the NEC was neither established efficiently nor maintained sufficiently to produce the robust IINMs necessary for wt capsid nuclear egress. This study further implicates p53 as an active player in the HCMV lifecycle by contributing to the expression of UL53 and the normal transport and continuous supply of UL50 and UL53 to HCMV's novel tubule-like infoldings of the inner nuclear membrane structures and capsid nuclear egress mechanism.

References

Baek, M. C., P. M. Krosky, et al. (2004). "Phosphorylation of the RNA polymerase II carboxyl-terminal domain in human cytomegalovirus-infected cells and in vitro by the viral UL97 protein kinase." Virology **324**(1): 184-193.

Bigalke, J. M., T. Heuser, et al. (2014). "Membrane deformation and scission by the HSV-1 nuclear egress complex." Nature communications **5**: 4131.

Bigley, T. M., J. M. Reitsma, et al. (2013). "Human cytomegalovirus pUL97 regulates the viral major immediate early promoter by phosphorylation-mediated disruption of histone deacetylase 1 binding." Journal of virology **87**(13): 7393-7408.

Biron, K. K. (2006). "Antiviral drugs for cytomegalovirus diseases." Antiviral research **71**(2-3): 154-163.

Bjerke, S. L. and R. J. Roller (2006). "Roles for herpes simplex virus type 1 UL34 and US3 proteins in disrupting the nuclear lamina during herpes simplex virus type 1 egress." Virology **347**(2): 261-276.

Boppana, S. B., K. B. Fowler, et al. (1999). "Symptomatic congenital cytomegalovirus infection in infants born to mothers with preexisting immunity to cytomegalovirus." Pediatrics **104**(1 Pt 1): 55-60.

Bresnahan, W. A., I. Boldogh, et al. (1996). "Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1." Virology **224**(1): 150-160.

Britt, W., and C. Alford (1996). Cytomegalovirus. Fields Virology. B. N. Fields, Lippincott-Raven Publishers, Philadelphia, Pa.**:** 2493-2523.

Britt, W. J. and L. Vugler (1987). "Structural and immunological characterization of the intracellular forms of an abundant 68,000 Mr human cytomegalovirus protein." The Journal of general virology **68 (Pt 7)**: 1897-1907.

Buchkovich, N. J., T. G. Maguire, et al. (2010). "Role of the endoplasmic reticulum chaperone BiP, SUN domain proteins, and dynein in altering nuclear morphology during human cytomegalovirus infection." Journal of virology **84**(14): 7005-7017.

Bunz, F., A. Dutriaux, et al. (1998). "Requirement for p53 and p21 to sustain G2 arrest after DNA damage." Science **282**(5393): 1497-1501.

Buser, C., P. Walther, et al. (2007). "Cytomegalovirus primary envelopment occurs at large infoldings of the inner nuclear membrane." Journal of virology **81**(6): 3042-3048.

Camozzi, D., S. Pignatelli, et al. (2008). "Remodelling of the nuclear lamina during human cytomegalovirus infection: role of the viral proteins pUL50 and pUL53." The Journal of general virology **89**(Pt 3): 731-740.

Cano-Monreal, G. L., K. M. Wylie, et al. (2009). "Herpes simplex virus 2 UL13 protein kinase disrupts nuclear lamins." Virology **392**(1): 137-147.

Cardoso, M. C., H. Leonhardt, et al. (1993). "Reversal of terminal differentiation and control of DNA replication: cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication." Cell **74**(6): 979-992.

Casavant, N. C., M. H. Luo, et al. (2006). "Potential role for p53 in the permissive life cycle of human cytomegalovirus." Journal of virology **80**(17): 8390-8401.

Cinque, P., R. Marenzi, and D. Ceresa (1997). "Cytomegalovirus infections of the nervous system." Intervirology **40**: 85-97.

Cobbs, C. S., L. Harkins, et al. (2002). "Human cytomegalovirus infection and expression in human malignant glioma." Cancer research **62**(12): 3347-3350.

Dal Monte, P., S. Pignatelli, et al. (2002). "Analysis of intracellular and intraviral localization of the human cytomegalovirus UL53 protein." The Journal of general virology **83**(Pt 5): 1005-1012.

de Bruyn Kops, A. and D. M. Knipe (1988). "Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein." Cell **55**(5): 857-868.

Dechat, T., K. Pfleghaar, et al. (2008). "Nuclear lamins: major factors in the structural organization and function of the nucleus and chromatin." Genes Dev **22**(7): 832-853.

Dittmer, D. and E. S. Mocarski (1997). "Human cytomegalovirus infection inhibits G1/S transition." Journal of virology **71**(2): 1629-1634.

Farina, A., R. Feederle, et al. (2005). "BFRF1 of Epstein-Barr virus is essential for efficient primary viral envelopment and egress." Journal of virology **79**(6): 3703-3712.

Fornara, C., D. Lilleri, et al. (2011). "Kinetics of effector functions and phenotype of virus-specific and gammadelta T lymphocytes in primary human cytomegalovirus infection during pregnancy." Journal of clinical immunology **31**(6): 1054-1064.

Fortunato, E. A., V. Sanchez, et al. (2002). "Infection of cells with human cytomegalovirus during S phase results in a blockade to immediate-early gene expression that can be overcome by inhibition of the proteasome." Journal of virology **76**(11): 5369-5379.

Fortunato, E. A. and D. H. Spector (1998). "p53 and RPA are sequestered in viral replication centers in the nuclei of cells infected with human cytomegalovirus." Journal of virology **72**(3): 2033-2039.

Fowler, K. B., F. P. McCollister, et al. (1997). "Progressive and fluctuating sensorineural hearing loss in children with asymptomatic congenital cytomegalovirus infection." The Journal of pediatrics **130**(4): 624-630.

Fuchs, W., B. G. Klupp, et al. (2002). "The interacting UL31 and UL34 gene products of pseudorabies virus are involved in egress from the host-cell nucleus and represent components of primary enveloped but not mature virions." Journal of virology **76**(1): 364-378.

Gilloteaux, J. and M. R. Nassiri (2000). "Human bone marrow fibroblasts infected by cytomegalovirus: ultrastructural observations." Journal of submicroscopic cytology and pathology **32**(1): 17-45.

Goldman, R. D., A. E. Goldman, et al. (2005). "Nuclear lamins: building blocks of nuclear structure and function." Novartis Found Symp **264**: 3-16; discussion 16-21, 227-230.

Goldman, R. D., Y. Gruenbaum, et al. (2002). "Nuclear lamins: building blocks of nuclear architecture." Genes Dev **16**(5): 533-547.

Gonnella, R., A. Farina, et al. (2005). "Characterization and intracellular localization of the Epstein-Barr virus protein BFLF2: interactions with BFRF1 and with the nuclear lamina." Journal of virology **79**(6): 3713-3727.

Granato, M., R. Feederle, et al. (2008). "Deletion of Epstein-Barr virus BFLF2 leads to impaired viral DNA packaging and primary egress as well as to the production of defective viral particles." Journal of virology **82**(8): 4042-4051.

Hamirally, S., J. P. Kamil, et al. (2009). "Viral mimicry of Cdc2/cyclin-dependent kinase 1 mediates disruption of nuclear lamina during human cytomegalovirus nuclear egress." PLoS pathogens **5**(1): e1000275.

Hannemann, H., K. Rosenke, et al. (2009). "The presence of p53 influences the expression of multiple human cytomegalovirus genes at early times postinfection." Journal of virology **83**(9): 4316-4325.

Harkins, L., A. L. Volk, et al. (2002). "Specific localisation of human cytomegalovirus nucleic acids and proteins in human colorectal cancer." Lancet **360**(9345): 1557-1563.

Hume, A. J., J. S. Finkel, et al. (2008). "Phosphorylation of retinoblastoma protein by viral protein with cyclin-dependent kinase function." Science **320**(5877): 797-799.

Jault, F. M., J. M. Jault, et al. (1995). "Cytomegalovirus infection induces high levels of cyclins, phosphorylated Rb, and p53, leading to cell cycle arrest." Journal of virology **69**(11): 6697-6704.

Johnson, D. C. and J. D. Baines (2011). "Herpesviruses remodel host membranes for virus egress." Nature reviews. Microbiology **9**(5): 382-394.

Kamil, J. P. and D. M. Coen (2007). "Human cytomegalovirus protein kinase UL97 forms a complex with the tegument phosphoprotein pp65." Journal of virology **81**(19): 10659-10668.

Klupp, B. G., H. Granzow, et al. (2007). "Vesicle formation from the nuclear membrane is induced by coexpression of two conserved herpesvirus proteins." Proceedings of the National Academy of Sciences of the United States of America **104**(17): 7241-7246.

Krosky, P. M., M. C. Baek, et al. (2003). "The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress." Journal of virology **77**(2): 905-914.

Krosky, P. M., M. C. Baek, et al. (2003). "The human cytomegalovirus UL44 protein is a substrate for the UL97 protein kinase." Journal of virology **77**(14): 7720-7727.

Lake, C. M. and L. M. Hutt-Fletcher (2004). "The Epstein-Barr virus BFRF1 and BFLF2 proteins interact and coexpression alters their cellular localization." Virology **320**(1): 99-106.

Leach, N. R. and R. J. Roller (2010). "Significance of host cell kinases in herpes simplex virus type 1 egress and lamin-associated protein disassembly from the nuclear lamina." Virology **406**(1): 127-137.

Liang, L. and J. D. Baines (2005). "Identification of an essential domain in the herpes simplex virus 1 UL34 protein that is necessary and sufficient to interact with UL31 protein." Journal of virology **79**(6): 3797-3806.

Liptak, L. M., S. L. Uprichard, et al. (1996). "Functional order of assembly of herpes simplex virus DNA replication proteins into prereplicative site structures." Journal of virology **70**(3): 1759-1767.

Littler, E., A. D. Stuart, et al. (1992). "Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir." Nature **358**(6382): 160-162.

Lombard, D. B. and L. Guarente (2000). "Nijmegen breakage syndrome disease protein and MRE11 at PML nuclear bodies and meiotic telomeres." Cancer research **60**(9): 2331-2334.

Lu, M. and T. Shenk (1996). "Human cytomegalovirus infection inhibits cell cycle progression at multiple points, including the transition from G1 to S." Journal of virology **70**(12): 8850-8857.

Lukonis, C. J., J. Burkham, et al. (1997). "Herpes simplex virus type 1 prereplicative sites are a heterogeneous population: only a subset are likely to be precursors to replication compartments." Journal of virology **71**(6): 4771-4781.

Lukonis, C. J. and S. K. Weller (1996). "Characterization of nuclear structures in cells infected with herpes simplex virus type 1 in the absence of viral DNA replication." Journal of virology **70**(3): 1751-1758.

Marciniak, R. A., D. B. Lombard, et al. (1998). "Nucleolar localization of the Werner syndrome protein in human cells." Proceedings of the National Academy of Sciences of the United States of America **95**(12): 6887-6892.

Marschall, M., M. Freitag, et al. (2003). "The protein kinase pUL97 of human cytomegalovirus interacts with and phosphorylates the DNA polymerase processivity factor pUL44." Virology **311**(1): 60-71.

Marschall, M., A. Marzi, et al. (2005). "Cellular p32 recruits cytomegalovirus kinase pUL97 to redistribute the nuclear lamina." The Journal of biological chemistry **280**(39): 33357-33367.

McGavin, J. K. and K. L. Goa (2001). "Ganciclovir: an update of its use in the prevention of cytomegalovirus infection and disease in transplant recipients." Drugs **61**(8): 1153-1183.

Mettenleiter, T. C., B. G. Klupp, et al. (2009). "Herpesvirus assembly: an update." Virus research **143**(2): 222-234.

Milbradt, J., S. Auerochs, et al. (2007). "Cytomegaloviral proteins pUL50 and pUL53 are associated with the nuclear lamina and interact with cellular protein kinase C." The Journal of general virology **88**(Pt 10): 2642-2650.

Milbradt, J., S. Auerochs, et al. (2012). "Specific residues of a conserved domain in the N terminus of the human cytomegalovirus pUL50 protein determine its intranuclear interaction with pUL53." The Journal of biological chemistry **287**(28): 24004-24016.

Milbradt, J., S. Auerochs, et al. (2009). "Cytomegaloviral proteins that associate with the nuclear lamina: components of a postulated nuclear egress complex." The Journal of general virology **90**(Pt 3): 579-590.

Milbradt, J., A. Kraut, et al. (2014). "Proteomic analysis of the multimeric nuclear egress complex of human cytomegalovirus." Molecular & cellular proteomics : MCP **13**(8): 2132-2146.

Milbradt, J., R. Webel, et al. (2010). "Novel mode of phosphorylation-triggered reorganization of the nuclear lamina during nuclear egress of human cytomegalovirus." The Journal of biological chemistry **285**(18): 13979-13989.

Miller, M. S., W. E. Furlong, et al. (2010). "RASCAL is a new human cytomegalovirus-encoded protein that localizes to the nuclear lamina and in cytoplasmic vesicles at late times postinfection." Journal of virology **84**(13): 6483-6496.

Moll, U. M., S. Wolff, et al. (2005). "Transcription-independent pro-apoptotic functions of p53." Current opinion in cell biology **17**(6): 631-636.

Muranyi, W., J. Haas, et al. (2002). "Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina." Science **297**(5582): 854-857.

O'Dowd, J. M., A. G. Zavala, et al. (2012). "HCMV-infected cells maintain efficient nucleotide excision repair of the viral genome while abrogating repair of the host genome." PLoS pathogens **8**(11): e1003038.

Pante, N. and M. Kann (2002). "Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm." Molecular biology of the cell **13**(2): 425-434.

Papadimitriou, J. M., G. R. Shellam, et al. (1984). "An ultrastructural investigation of cytomegalovirus replication in murine hepatocytes." The Journal of general virology **65 (Pt 11)**: 1979-1990.

Park, R. and J. D. Baines (2006). "Herpes simplex virus type 1 infection induces activation and recruitment of protein kinase C to the nuclear membrane and increased phosphorylation of lamin B." Journal of virology **80**(1): 494-504.

Penfold, M. E. and E. S. Mocarski (1997). "Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis." Virology **239**(1): 46-61.

Prichard, M. N., N. Gao, et al. (1999). "A recombinant human cytomegalovirus with a large deletion in UL97 has a severe replication deficiency." Journal of virology **73**(7): 5663-5670.

Quinlan, M. P., L. B. Chen, et al. (1984). "The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication." Cell **36**(4): 857-868.

Reim, N. I., J. P. Kamil, et al. (2013). "Inactivation of retinoblastoma protein does not overcome the requirement for human cytomegalovirus UL97 in lamina disruption and nuclear egress." Journal of virology **87**(9): 5019-5027.

Reynolds, A. E., B. J. Ryckman, et al. (2001). "U(L)31 and U(L)34 proteins of herpes simplex virus type 1 form a complex that accumulates at the nuclear rim and is required for envelopment of nucleocapsids." Journal of virology **75**(18): 8803-8817.

Reynolds, A. E., E. G. Wills, et al. (2002). "Ultrastructural localization of the herpes simplex virus type 1 UL31, UL34, and US3 proteins suggests specific roles in primary envelopment and egress of nucleocapsids." Journal of virology **76**(17): 8939-8952.

Rixon, F. J., M. A. Atkinson, et al. (1983). "Intranuclear distribution of herpes simplex virus type 2 DNA synthesis: examination by light and electron microscopy." The Journal of general virology **64 (Pt 9)**: 2087-2092.

Rosenke, K., M. A. Samuel, et al. (2006). "An intact sequence-specific DNA-binding domain is required for human cytomegalovirus-mediated sequestration of p53 and may promote in vivo binding to the viral genome during infection." Virology **348**(1): 19-34.

Ruebner, B. H., K. Miyai, et al. (1964). "Mouse Cytomegalovirus Infection. An Electron Microscopic Study of Hepatic Parenchymal Cells." The American journal of pathology **44**: 799-821.

Salvant, B. S., E. A. Fortunato, et al. (1998). "Cell cycle dysregulation by human cytomegalovirus: influence of the cell cycle phase at the time of infection and effects on cyclin transcription." Journal of virology **72**(5): 3729-3741.

Sam, M. D., B. T. Evans, et al. (2009). "Biochemical, biophysical, and mutational analyses of subunit interactions of the human cytomegalovirus nuclear egress complex." Journal of virology **83**(7): 2996-3006.

Samanta, M., L. Harkins, et al. (2003). "High prevalence of human cytomegalovirus in prostatic intraepithelial neoplasia and prostatic carcinoma." The Journal of urology **170**(3): 998-1002.

Sanchez, V., C. L. Clark, et al. (2002). "Viable human cytomegalovirus recombinant virus with an internal deletion of the IE2 86 gene affects late stages of viral replication." Journal of virology **76**(6): 2973-2989.

Sanchez, V., K. D. Greis, et al. (2000). "Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly." Journal of virology **74**(2): 975-986.

Sanchez, V., J. A. Mahr, et al. (2007). "Nuclear export of the human cytomegalovirus tegument protein pp65 requires cyclin-dependent kinase activity and the Crm1 exporter." J Virol **81**(21): 11730-11736.

Sax, J. K. and W. S. El-Deiry (2003). "p53 downstream targets and chemosensitivity." Cell death and differentiation **10**(4): 413-417.

Schmeiser, C., E. Borst, et al. (2013). "The cytomegalovirus egress proteins pUL50 and pUL53 are translocated to the nuclear envelope through two distinct

modes of nuclear import." The Journal of general virology **94**(Pt 9): 2056-2069.

Severi, B., M. P. Landini, et al. (1988). "Human cytomegalovirus morphogenesis: an ultrastructural study of the late cytoplasmic phases." Archives of virology **98**(1-2): 51-64.

Sharma, M., B. J. Bender, et al. (2014). "Human Cytomegalovirus UL97 Phosphorylates the Viral Nuclear Egress Complex." Journal of virology.

Sharma, M., J. P. Kamil, et al. (2014). "Human cytomegalovirus UL50 and UL53 recruit viral protein kinase UL97, not protein kinase C, for disruption of nuclear lamina and nuclear egress in infected cells." Journal of virology **88**(1): 249-262.

Shi, Y., H. Lu, et al. (2011). "Prevalence and clinical management of cytomegalovirus retinitis in AIDS patients in Shanghai, China." BMC infectious diseases **11**: 326.

Silva, L. A., B. L. Strang, et al. (2011). "Sites and roles of phosphorylation of the human cytomegalovirus DNA polymerase subunit UL44." Virology **417**(2): 268-280.

Soderberg-Naucler, C. (2006). "Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer?" Journal of internal medicine **259**(3): 219-246.

Sullivan, V., C. L. Talarico, et al. (1992). "A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells." Nature **358**(6382): 162-164.

Tamashiro, J. C., L. J. Hock, et al. (1982). "Construction of a cloned library of the EcoRI fragments from the human cytomegalovirus genome (strain AD169)." Journal of virology **42**(2): 547-557.

Tran, K., J. A. Mahr, et al. (2008). "Accumulation of substrates of the anaphase-promoting complex (APC) during human cytomegalovirus infection is associated with the phosphorylation of Cdh1 and the dissociation and relocalization of APC subunits." Journal of virology **82**(1): 529-537.

Uprichard, S. L. and D. M. Knipe (1997). "Assembly of herpes simplex virus replication proteins at two distinct intranuclear sites." Virology **229**(1): 113-125.

Wei, W., R. M. Hemmer, et al. (2001). "Role of p14(ARF) in replicative and induced senescence of human fibroblasts." Molecular and cellular biology **21**(20): 6748-6757.

Zhang, L. J., P. Hanff, et al. (1995). "Detection of human cytomegalovirus DNA, RNA, and antibody in normal donor blood." The Journal of infectious diseases **171**(4): 1002-1006.

Chapter 4

Conclusion and future directions

It was striking that p53 was used to contribute to HCMV pathogenesis during nuclear egress by enhancing the expression of UL53, which in turn, directed UL50 to the nucleus, and subsequently both proteins colocalized at the inner nuclear membrane. In HCMV, a proposed Nuclear Egress Complex (NEC) is suggested to facilitate the export of nuclear capsids to the cytoplasm (Milbradt, Auerochs et al. 2009; Milbradt, Kraut et al. 2014). This complex contains both viral and cellular proteins. UL50, UL53, UL97, RASCAL, p32, lamin B receptor, emerin, and potentially PKC, are all components of the NEC (Krosky, Baek et al. 2003; Marschall, Marzi et al. 2005; Camozzi, Pignatelli et al. 2008; Hamirally, Kamil et al. 2009; Milbradt, Auerochs et al. 2009; Sam, Evans et al. 2009; Miller, Furlong et al. 2010; Milbradt, Kraut et al. 2014; Sharma, Kamil et al. 2014), although PCK is recently suggested to be excluded (Sharma and Coen 2014; Sharma, Kamil et al. 2014). UL50 was expressed in the majority of p53KO cells, however, only a subpopulation of these cells localized UL50 at the nuclear membrane. Therefore, we suspected that the NEC might not be forming and localizing properly at the rim of the nucleus in p53KO cells due to the lack of proper UL50 localization. We had previously tried to use the UL50 antibody to co-immunoprecipitate this complex and attempted to determine the relative abundance of each of the components of the NEC by densitometric analysis of Western blots. However, it was unsuccessful to pull down the NEC with the UL50 antibody (data not shown). Future experiments

will be using the UL53 antibody to attempt the same objective. This can determine what is present and what is not of the components of the NEC when p53 is absent.

The absence of p53 inhibited UL53 expression. It was found that UL53's neighboring gene, UL54, carries a p53-binding site (Rosenke, Samuel et al. 2006). This site could potentially be an enhancer for UL53 transcription. Further experiments will be conducted to determine how p53 influences UL53 expression. In addition, the loss of UL53 expression in the absence of p53 prohibited UL50 localization at the inner nuclear membrane. Our data suggests that UL53 was needed to transport UL50 into the nucleus. The mechanisms of how UL53 conducts UL50 to the nucleus, and subsequently colocalizes at the inner nuclear membrane, will further be investigated.

In addition, the absence of p53 distinctly prevented infoldings of the inner nuclear membrane (IINM) formation. The impaired IINM formation was also reflected in the inefficient membrane formation used for the envelopment of capsids in the cytoplasm. Both events need new lipids for new membrane formation. Our preliminary results indicated that lipid biosynthesis was up-regulated in p53KO cells, as seen by another group (Jiang, Du et al. 2011), however, the produced lipids were not being used or not efficiently incorporated into new membrane in these cells during HCMV infection (data not shown). Therefore, it is beneficial to determine the mechanisms of how p53 influences the lipid incorporation into the inner nuclear membrane and the subsequent formation of the infoldings.

A very small amount of p53 re-introduction allowed for UL50-UL53 complex accumulation at the nuclear membrane in WTG cells, however, IINM formation in these cells was still far less than the wt cells. Both UL50 and UL53 alone are sufficient to induce membrane deformation (Bigalke, Heuser et al. 2014), which is likely, a mechanism to incorporate newly synthesized lipids into inner nuclear membrane and to subsequently form IINMs. After a small amount of p53 re-introduction, UL53 expression and UL50 localization were recovered close to the wt level. So why was IINM formation still impaired? One possibility is that guiding lipids into the nucleus as well as incorporation of lipids into deformed inner nuclear membranes may need wt level of p53.

In the previous studies from our lab (Casavant, Luo et al. 2006) and in this study, we consistently observed that a subpopulation of p53KO cells (roughly 30-40% of cells) were capable of localizing the NEC components correctly and trafficking proteins properly from the nucleus into the cytoplasm. We initially hypothesized that this subpopulation might use a p53 homologue, such as p73, in the absence of p53 during infection. We used p73 antibody to determine whether only the subpopulation of p53KO cells that functioned like wt cells expressed p73. However, all infected p53KO cells were expressing p73 (data not shown). Whether p73 is a secondary player in the absence of p53 during HCMV infection is still under investigation.

An alternative strategy is currently used to determine the functional factor(s) that HCMV uses to enhance capsid nuclear trafficking when p53 is absent. These factors can potentially be the target of antivirals. We will subject the infected p53KO

cells to Fluorescence-Activated Cell Sorting (FACS) analysis. $UL53^{+}$ and $UL53^{-}$ cells will be sorted into two groups. After sorting, their proteins will be separated by 2-dimensional (2D) gel electrophoresis to narrow the potential protein candidates differentially expressed between these two groups. Subsequently we will perform a proteomic analysis by mass spectrometry to identify these proteins.

By using p53KO cells, we found that p53 is involved in HCMV nuclear egress. These cells can also be a useful system to investigate the viral components of the NEC, since we were using a fully functional wt HCMV, not a mutant or gene deleted virus. In the absence of p53, UL53 was not expressed and UL50 was not properly localized at the inner nuclear membrane. This situation provides us additional information to study and dissect the NEC components, their sequence of the accumulation at the nuclear membrane and the mechanism of transport to that site.

References

Bigalke, J. M., T. Heuser, et al. (2014). "Membrane deformation and scission by the HSV-1 nuclear egress complex." Nature communications **5**: 4131.

Camozzi, D., S. Pignatelli, et al. (2008). "Remodelling of the nuclear lamina during human cytomegalovirus infection: role of the viral proteins pUL50 and pUL53." The Journal of general virology **89**(Pt 3): 731-740.

Casavant, N. C., M. H. Luo, et al. (2006). "Potential role for p53 in the permissive life cycle of human cytomegalovirus." Journal of virology **80**(17): 8390-8401.

Hamirally, S., J. P. Kamil, et al. (2009). "Viral mimicry of Cdc2/cyclin-dependent kinase 1 mediates disruption of nuclear lamina during human cytomegalovirus nuclear egress." PLoS pathogens **5**(1): e1000275.

Jiang, P., W. Du, et al. (2011). "p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase." Nature cell biology **13**(3): 310-316.

Krosky, P. M., M. C. Baek, et al. (2003). "The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress." Journal of virology **77**(2): 905-914.

Marschall, M., A. Marzi, et al. (2005). "Cellular p32 recruits cytomegalovirus kinase pUL97 to redistribute the nuclear lamina." The Journal of biological chemistry **280**(39): 33357-33367.

Milbradt, J., S. Auerochs, et al. (2009). "Cytomegaloviral proteins that associate with the nuclear lamina: components of a postulated nuclear egress complex." The Journal of general virology **90**(Pt 3): 579-590.

Milbradt, J., A. Kraut, et al. (2014). "Proteomic analysis of the multimeric nuclear egress complex of human cytomegalovirus." Molecular & cellular proteomics : MCP **13**(8): 2132-2146.

Miller, M. S., W. E. Furlong, et al. (2010). "RASCAL is a new human cytomegalovirus-encoded protein that localizes to the nuclear lamina and in cytoplasmic vesicles at late times postinfection." Journal of virology **84**(13): 6483-6496.

Rosenke, K., M. A. Samuel, et al. (2006). "An intact sequence-specific DNA-binding domain is required for human cytomegalovirus-mediated sequestration of

p53 and may promote in vivo binding to the viral genome during infection." Virology **348**(1): 19-34.

Sam, M. D., B. T. Evans, et al. (2009). "Biochemical, biophysical, and mutational analyses of subunit interactions of the human cytomegalovirus nuclear egress complex." Journal of virology **83**(7): 2996-3006.

Sharma, M. and D. M. Coen (2014). "Comparison of effects of inhibitors of viral and cellular protein kinases on human cytomegalovirus disruption of nuclear lamina and nuclear egress." Journal of virology **88**(18): 10982-10985.

Sharma, M., J. P. Kamil, et al. (2014). "Human cytomegalovirus UL50 and UL53 recruit viral protein kinase UL97, not protein kinase C, for disruption of nuclear lamina and nuclear egress in infected cells." Journal of virology **88**(1): 249-262.

Appendices

Appendix A

Copyright permissions from ViralZone, SIB Swiss Institute of Bioinformatics

[help #99315] [Viralzone] Request for permission to use copyrighted figure

Philippe Lemercier via RT <viralzone@isb-sib.ch>

Mon 2/9/2015 1:48 AM
Authorization
To:
Kuan, Man-I (kuan5876@vandals.uidaho.edu);

Dear Mani Kuan,

I hereby grant you permission to use the viralzone herpesvirus picture in your dissertation.

Please cite ViralZone, SIB Swiss Institute of Bioinformatics

best regards

Philippe Le Mercier

On Fri Feb 06 22:04:12 2015, kuan5876@vandals.uidaho.edu wrote:

Sender name: Mani Kuan
Subject: [Viralzone] Request for permission to use copyrighted figure

Dear sir/madam,

I am a graduate student finishing up my dissertation in the University of Idaho. I am writing to ask for permission to use the copyrighted figure published at your website http://viralzone.expasy.org/all_by_species/176.html in my literature review section of my dissertation. My dissertation title is "The Absence of the Cellular Protein p53 Inhibited Efficient Trafficking of HCMV Viral Capsids from the Nucleus." If this is acceptable, may I please to have a written statement from you authorizing use of the copyrighted materials? Thank you for your time.

Sincerely,

Mani Kuan
Ph.D. candidate
University of Idaho
Dept of Biological Sciences
875 Perimeter MS 3051
Moscow, ID 83844-3051
Email: kuan5876@vandals.uidaho.edu
Phone: 208-885-9237

This email message has been generated on the ExPASy WWW server (www.expasy.org).
In case of problems, please contact helpdesk@expasy.org.
--
Message sent from host viralzone.expasy.org.
Referring page: http://viralzone.expasy.org/all_by_species/176.html.
Browser: Mozilla/5.0 (Windows NT 6.1; WOW64) AppleWebKit/537.36 (KHTML, like Gecko) Chrome/40.0.2214.111 Safari/537.36.

Appendix B

Copyright permissions from Caister Academic Press

Re: Response General

noreply@horizonpress.com

Sat 2/7/2015 11:22 AM
Authorization
To:
Kuan, Man-I (kuan5876@vandals.uidaho.edu);

Permission is granted to use the material provided you ALSO obtain permission from the original authors AND full reference is made to the original source.

Please see our permissions policy at http://www.horizonpress.com/help/copyright.html

Horizon Scientific Press/Caister Academic Press, UK

On 7 Feb 2015, at 01:12, kuan5876@vandals.uidaho.edu wrote:

Email: kuan5876@vandals.uidaho.edu

Comments: Request for a written permission to use copyrighted figures

Dear sir/madam,

I am a graduate student finishing up my dissertation in the University of Idaho. I am writing to ask for permission to use two copyrighted figures published at Caister Academic Press in my literature review section of my dissertation. The figures are found from the book Cytomegaloviruses: From Molecular Pathogenesis to Intervention Volume 1 edited by Matthias J. Reddehase and Niels A.W. Lemmermann (ISBN: 978-1-908230-18-8). The two figures of interest are Figure I.13.3 Formation the CMV virion on page 234 and Figure I.13.4 Structure of the HCMV genome on page 236. Both figures are in the chapter I.13 Morphogenesis of the Cytomegalovirus Virion and Subviral Particles by Dr. Gibson and Dr. Bogner. My dissertation title is "The Absence of the Cellular Protein p53 Inhibited Efficient Trafficking of HCMV Viral Capsids from the Nucleus." If this is acceptable, may I please to have a written statement from you authorizing use of the copyrighted materials? Thank you for your time.

Sincerely,

Mani Kuan
Ph.D. candidate
University of Idaho
Dept of Biological Sciences
875 Perimeter MS 3051
Moscow, ID 83844-3051
Email: kuan5876@vandals.uidaho.edu
Phone: 208-885-9237

Sender's IP address: 129.101.244.200

Caister Academic Press
http://www.horizonpress.com

From: "Bogner, Elke" <elke.bogner@charite.de>
Subject: AW: permission
Date: March 10, 2015 at 1:12:08 AM PDT
To: "'Fortunato, Lee (lfort@uidaho.edu)'" <lfort@uidaho.edu>

Hi Lee,

I give you the permission to use the two figures in her thesis. There is no problem for me.

By the way, do you know whether one could book (e.g. via viator) a tour to see bears around Boise?

Best regards from Berlin

Elke

Von: Fortunato, Lee (lfort@uidaho.edu) [mailto:lfort@uidaho.edu]
Gesendet: Montag, 9. März 2015 21:57
An: Bogner, Elke
Betreff: permission

Hi Elke,

Hope you are well. I have a quick favor to ask…. My student Man I Kuan is finishing up her thesis and has used two of your figures from a published article in her thesis. She has asked for permission to use them from Horizon Press (the publisher) and they have granted it. They said, however, that she should obtain permission from you as well.

The two figures come from Gibson, W. and E. Bogner (2013). Morphogenesis of the Cytomegalovirus Virion and Subviral Particles. Cytomegaloviruses: From Molecular Pathogenesis to Intervention. M. J. Reddehase and N. A. W. Lemmermann. Norfolk, UK, Caister Academic Press. **1:** 230-246.

Can we have permission to use these in her thesis?

Thanks,

Lee

Appendix C

Copyright permissions from the Journal of Virology

2/9/2015

To Whom It May Concern,

This letter confirms that Mani Kuan has the American Society for Microbiology's permission to reuse the following material in a dissertation:

Figure 2 and 5 from "Cytomegalovirus Primary Envelopment Occurs at Large Infoldings of the Inner Nuclear Membrane" Christopher Buser, Paul Walther, Thomas Mertens, and Detlef Michel
J. Virol. March 15, 2007 81:3042-3048; Accepted manuscript posted online 27 December 2006, doi:10.1128/JVI.01564-06

Figure 1 from "Cytomegalovirus Capsid Protease: Biological Substrates Are Cleaved More Efficiently by Full-Length Enzyme (pUL80a) than by the Catalytic Domain (Assemblin)" Steve M. Fernandes, Edward J. Brignole, and Wade Gibson
J. Virol. April 1, 2011 85:3526-3534; Accepted manuscript posted online 26 January 2011, doi:10.1128/JVI.02663-10

Please contact us if you have any questions.

Thank you.
ASM Journals
journals@asmusa.org

PERMISSION GRANTED
CONTINGENT ON AUTHOR PERMISSION (which you must obtain)
AND APPROPRIATE CREDIT
American Society for Microbiology
Journals Department
Date Feb 9, 2015

Appendix D

Copyright permissions from Elsevier

ELSEVIER LICENSE
TERMS AND CONDITIONS

Mar 03, 2015

This is a License Agreement between MANI KUAN ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	MANI KUAN
Customer address	University of Idaho MOSCOW, ID 83844
License number	3565500945038
License date	Feb 10, 2015
Licensed content publisher	Elsevier
Licensed content publication	Trends in Microbiology
Licensed content title	Viral and host control of cytomegalovirus maturation
Licensed content author	Ritesh Tandon,Edward S. Mocarski
Licensed content date	August 2012
Licensed content volume number	20
Licensed content issue number	8
Number of pages	10
Start Page	392
End Page	401
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	3
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No

Original figure numbers	Table 2
Title of your thesis/dissertation	The Absence of the Cellular Protein p53 Inhibited Efficient Trafficking of HCMV Viral Capsids from the Nucleus.
Expected completion date	Mar 2015
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the

amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- after the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

Appendix E

Copyright permissions from Springer Vienna

Copyright Clearance Center

Confirmation Number: 11298992
Order Date: 02/16/2015

Customer Information

Customer: MANI KUAN
Account Number: 3000887831
Organization: MANI KUAN
Email: kuan5876@vandals.uidaho.edu
Phone: +1 (208)8859237
Payment Method: Invoice

This is not an invoice

Order Details

Congenital Cytomegalovirus Infection : Epidemiology, Diagnosis, Therapy

Billing Status: **N/A**

Order detail ID: 66162863
ISBN: 978-3-7091-0208-4
Publication Type: Book
Publisher: Springer Vienna

Permission Status: **Granted**
Permission type: Republish or display content
Type of use: Thesis/Dissertation
Order License Id: 3570960681840

Requestor type	Not-for-profit entity
Format	Print, Electronic
Portion	cartoon
Number of cartoons	1
Title or numeric reference of the portion (s)	Fig. 2.3 Replication cycle of cytomegalovirus on page 15
Title of the article or chapter the portion is from	Chapter 2 Virus-host interaction for defence and transmission (Sub-chapter 2.1 The virus)
Editor of portion(s)	N/A
Author of portion(s)	N/A
Volume of serial or monograph	N/A
Issue, if republishing an article from a serial	N/A
Page range of portion	15
Publication date of portion	2011
Rights for	Main product
Duration of use	Life of current and all future editions
Creation of copies for the disabled	no
With minor editing privileges	yes
For distribution to	Worldwide
In the following language(s)	Original language of publication
With incidental promotional use	no
	Up to 499

Lifetime unit quantity of new product	
Made available in the following markets	professional
The requesting person/organization	MANI KUAN / University of Idaho
Order reference number	
Author/Editor	MANI KUAN
The standard identifier	N/A
Title	The Absence of the Cellular Protein p53 Inhibited Efficient Trafficking of HCMV Viral Capsids from the Nucleus
Publisher	University of Idaho
Expected publication date	Apr 2015
Estimated size (pages)	200

Note: This item was invoiced separately through our **RightsLink service.** More info **$ 0.00**

Total order items: 1 **Order Total: $0.00**

Appendix F

Copyright permissions from the Journal of Biological Chemistry

RE: Request for permission to use a copyrighted figure (JBC Feedback Form)

Ed Marklin <emarklin@asbmb.org>

Sat 2/7/2015 6:44 AM
Authorization
To:
Kuan, Man-I (kuan5876@vandals.uidaho.edu);

Hi Dr. Kuan,

According to the Journal of Biological Chemistry copyright permission policy below and online:http://www.jbc.org/site/misc/Copyright_Permission.xhtml you are allowed to use the article for your thesis and dissertation. Please be sure to cite the manuscript in your dissertation.

If you have any additional questions please let me know.

Regards,
Ed Marklin

These guidelines apply to the reuse of articles, figures, charts and photos in the Journal of Biological Chemistry, Molecular & Cellular Proteomics and the Journal of Lipid Research.
For authors reusing their own material:

Authors need NOT contact the journal to obtain rights to reuse their own material. They are automatically granted permission to do the following:
•Reuse the article in print collections of their own writing.
•Present a work orally in its entirety.
•Use an article in a thesis and/or dissertation.
•Reproduce an article for use in the author's courses. (If the author is employed by an academic institution, that institution also may reproduce the article for teaching purposes.) •Reuse a figure, photo and/or table in future commercial and noncommercial works.
•Post a copy of the paper in PDF that you submitted via BenchPress.

◦Only authors who published their papers under the "Author's Choice" option may post the final edited PDFs created by the publisher to their own/departmental/university Web sites.
◦All authors may link to the journal site containing the final edited PDFs created by the publisher.

Please note that authors must include the following citation when using material that appeared in an ASBMB journal:

"This research was originally published in Journal Name. Author(s). Title. Journal Name. Year; Vol:pp-

pp. © the American Society for Biochemistry and Molecular Biology."

For other parties using material for noncommercial use:

Other parties are welcome to copy, distribute, transmit and adapt the work — at no cost and without permission — for noncommercial use as long as they attribute the work to the original source using the citation above.

Examples of noncommercial use include:

•Reproducing a figure for educational purposes, such as schoolwork or lecture presentations, with attribution.
•Appending a reprinted article to a Ph.D. dissertation, with attribution.

For other parties using material for commercial use:

Navigate to the article of interest and click the "Request Permissions" button on the middle navigation bar. (See diagram at right.) It will walk you through the steps for obtaining permission for reuse.

Examples of commercial use by parties other than authors include:
•Reproducing a figure in a book published by a commercial publisher.
•Reproducing a figure in a journal article published by a commercial publisher.

_____
From: MANI KUAN [kuan5876@vandals.uidaho.edu]
Sent: Friday, February 06, 2015 9:58 PM
To: jbc-feedback@highwire.stanford.edu
Cc: kuan5876@vandals.uidaho.edu
Subject: Request for permission to use a copyrighted figure (JBC Feedback Form)

Comments sent via JBC Feedback Page

TO: jbc-feedback@highwire.stanford.edu
NAME: MANI KUAN USER NAME: hslic
EMAIL: kuan5876@vandals.uidaho.edu
IP ADDRESS: 128.95.104.66
HOSTNAME: dir1.lib.washington.edu
PREVIOUS
PAGE: http://www.jbc.org.offcampus.lib.washington.edu/content/285/18/13979.full.pdf+html?frame=sidebar
BROWSER: Mozilla/5.0 (Windows NT 6.1; WOW64) AppleWebKit/534.57.2 (KHTML, like Gecko) Version/5.1.7 Safari/534.57.2, JBC
PROMOTIONAL USE: Not granted
SESSION ID: tbeNg6iXjOOUJwqpwtT5Jg

COMMENTS:
Dear sir/madam,

I am a graduate student finishing up my dissertation in the University of Idaho. I am writing to ask for permission to use a copyrighted figure published at your Journal in my literature review section of my dissertation.

The figure of my interest is “FIGURE 8. Hypothetical model of the course of events triggering the formation of nuclear lamina-depleted areas in HCMV-infected cells” originated from the article "Novel mode of phosphorylation-triggered reorganization of the nuclear lamina during nuclear egress of human cytomegalovirus" by Milbradt, J., R. Webel, et al. (2010) in the Journal of biological chemistry 285(18): 13979-13989.

My dissertation title is “The Absence of the Cellular Protein p53 Inhibited Efficient Trafficking of HCMV Viral Capsids from the Nucleus.” If this is acceptable, may I please to have a written statement from you authorizing use of the copyrighted material? Thank you for your time.

Sincerely,

Mani Kuan
Ph.D. candidate
University of Idaho
Dept of Biological Sciences
875 Perimeter MS 3051
Moscow, ID 83844-3051
Email: kuan5876@vandals.uidaho.edu
Phone: 208-885-9237

Appendix G

Copyright permissions from the Journal of Cell Science

RE: Request for permission to use a copyrighted figure

permissions <permissions@biologists.com>

Wed 2/11/2015 12:02 AM
Authorization
To:
Kuan, Man-I (kuan5876@vandals.uidaho.edu);

Dear Mani

Permission is granted with no charge.

The acknowledgement should state "reproduced / adapted with permission" and give the source journal name - the acknowledgement should either provide full citation details or refer to the relevant citation in the article reference list - the full citation details should include authors, journal, year, volume, issue and page citation.

Where appearing online or in other electronic media, a link should be provided to the original article (e.g. via DOI).

Journal of Cell Science: jcs.biologists.org

Best wishes,
Jenny Ostler

Mrs J Ostler
Senior Administrator
Development
The Company of Biologists, Bidder Building, Station Road, Cambridge, CB24 9LF, UK
www.biologists.com
T: +44 (0) 1223 632863 | jenny.ostler@biologists.com | http://dev.biologists.org
Follow us on Twitter: @Dev_journal
Like us on Facebook: /developmentjournal

From: Kuan, Man-I (kuan5876@vandals.uidaho.edu) [mailto:kuan5876@vandals.uidaho.edu]
Sent: 07 February 2015 03:12
To: permissions
Subject: Request for permission to use a copyrighted figure

Dear sir/madam,

I am a graduate student finishing up my dissertation in the University of Idaho. I am writing to ask for permission to use a copyrighted figure published at your Journal in my literature review section of my dissertation.

The figure of my interest is titled “p53 protein domain structure” originated from the article "p53 at a glance” by Brady, C. A. and L. D. Attardi (2010) in Journal of cell science **123**(Pt 15): 2527-2532.

My dissertation title is “The Absence of the Cellular Protein p53 Inhibited Efficient Trafficking of HCMV Viral Capsids from the Nucleus.” If this is acceptable, may I please to have a written statement from you authorizing use of the copyrighted material? Thank you for your time.

Sincerely,

Mani Kuan
Ph.D. candidate
University of Idaho
Dept of Biological Sciences
875 Perimeter MS 3051
Moscow, ID 83844-3051
Email: kuan5876@vandals.uidaho.edu
Phone: 208-885-9237

Copyright Clearance Center

Confirmation Number: 11297408
Order Date: 02/10/2015

Customer Information

Customer: MANI KUAN
Account Number: 3000887831
Organization: MANI KUAN
Email: kuan5876@vandals.uidaho.edu
Phone: +1 (208)8859237
Payment Method: Invoice

This is not an invoice

Order Details

Journal of cell science

Billing Status: **N/A**

Order detail ID: 66134262
ISSN: 0021-9533
Publication Type: Journal
Volume:
Issue:
Start page:
Publisher: Company of Biologists Ltd.
Author/Editor: COMPANY OF BIOLOGISTS

Permission Status: Granted
Permission type: Republish or display content
Type of use: Republish in a thesis/dissertation
Order License Id: 3565530596070

Requestor type	Not-for-profit entity
Format	Print, Electronic
Portion	chart/graph/table/figure
Number of charts/graphs/tables/figures	1
Title or numeric reference of the portion (s)	One of the poster sections: p53 protein domain structure
Title of the article or chapter the portion is from	p53 at a glance
Editor of portion(s)	N/A
Author of portion(s)	Colleen A. Brady and Laura D. Attardi
Volume of serial or monograph	123
Issue, if republishing an article from a serial	15
Page range of portion	2527-2532
Publication date of portion	2010/8/1
Rights for	Main product
Duration of use	Life of current and all future editions
Creation of copies for the disabled	no
With minor editing privileges	yes
For distribution to	Worldwide
In the following language(s)	Original language of publication
With incidental promotional use	no

Lifetime unit quantity of new product	Up to 499
Made available in the following markets	Professional
The requesting person/organization	University of Idaho
Order reference number	
Author/Editor	MANI KUAN
The standard identifier	N/A
Title	Ms
Publisher	University of Idaho
Expected publication date	Apr 2015
Estimated size (pages)	200

Note: This item was invoiced separately through our **RightsLink service.** More info **$ 0.00**

Total order items: 1 **Order Total: $0.00**

Appendix H

Copyright permissions from John Wiley and Sons

RE: Request for permission to use a copyrighted figure Your case 00246210 [ref:_00Dd0eeku._500d0P0TxS:ref]

Wiley Global Permissions <permissions@wiley.com>

Wed 2/11/2015 12:23 PM
Authorization
To:
Kuan, Man-I (kuan5876@vandals.uidaho.edu);

Dear Mani Kuan:

Thank you for your request.

Permission is granted for you to use the material requested for your thesis/dissertation subject to the usual acknowledgements and on the understanding that you will reapply for permission if you wish to distribute or publish your thesis/dissertation commercially.

Permission is granted solely for use in conjunction with the thesis, and the material may not be posted online separately.

Any third party material is expressly excluded from this permission. If any material appears within the article with credit to another source, authorization from that source must be obtained.

Sincerely,

Paulette Goldweber
Associate Manager, Permissions
Wiley

pgoldweb@wiley.com
T +1 201-748-8765
F +1 201-748-6008

111 River Street, MS 4-02
Hoboken, NJ 07030-5774
U.S.
permissions@wiley.com

---------- Original Message ----------
From: Kuan, Man-I [kuan5876@vandals.uidaho.edu]
Sent: 2/7/2015 11:25 AM
To: cs-journals@wiley.com
Subject: Request for permission to use a copyrighted figure {500d000000P0TxSAAV.003d0000028dztqAAA}

Dear sir/madam,

I am a graduate student finishing up my dissertation in the University of Idaho. I am writing to ask for permission to use a copyrighted figure published at your Journal in my literature review section of my dissertation.

The figure of my interest is “Figure 1. Interaction between p53 and Hdm2 and their autoregulatory loop” originated from the article "Interference with p53 functions in human viral infections, a target for novel antiviral strategies?" by Lazo, P. A. and C. R. Santos (2011) in Reviews in medical virology 21: 285–300.

My dissertation title is “The Absence of the Cellular Protein p53 Inhibited Efficient Trafficking of HCMV Viral Capsids from the Nucleus.” If this is acceptable, may I please to have a written statement from you authorizing use of the copyrighted material? Thank you for your time.

Sincerely,

Mani Kuan
Ph.D. candidate
University of Idaho
Dept of Biological Sciences
875 Perimeter MS 3051
Moscow, ID 83844-3051
Email: kuan5876@vandals.uidaho.edu
Phone: 208-885-9237

ref:_00Dd0eeku._500d0P0TxS:ref

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Mar 03, 2015

This Agreement between MANI KUAN ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	3565491494544
License date	Feb 10, 2015
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Reviews in Medical Virology
Licensed Content Title	Interference with p53 functions in human viral infections, a target for novel antiviral strategies?
Licensed Content Author	Pedro A. Lazo,Claudio R. Santos
Licensed Content Date	Jul 1, 2011
Pages	16
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 1
Will you be translating?	No
Title of your thesis / dissertation	The Absence of the Cellular Protein p53 Inhibited Efficient Trafficking of HCMV Viral Capsids from the Nucleus.
Expected completion date	Mar 2015
Expected size (number of pages)	200
Requestor Location	MANI KUAN University of Idaho Dept of Biological Sciences 875 Perimeter MS 3051 MOSCOW, ID 83844 United States Attn: MANI KUAN
Billing Type	Invoice
Billing Address	MANI KUAN University of Idaho Dept of Biological Sciences 875 Perimeter MS 3051 MOSCOW, ID 83844 United States Attn: MANI KUAN

Total 0.00 USD

Terms and Conditions

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking □accept□ in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.
- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this licence must be completed within two years of the date of the grant of this licence (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.
- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.
- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of

and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.

- These terms and conditions together with CCC□s Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC□s Billing and Payment terms and conditions, these terms and conditions shall prevail.

- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC□s Billing and Payment terms and conditions.

- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state□s conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish

properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by non-commercial users

For non-commercial and non-promotional purposes, individual users may access, download, copy, display and redistribute to colleagues Wiley Open Access articles, as well as adapt, translate, text- and data-mine the content subject to the following conditions:

- The authors' moral rights are not compromised. These rights include the right of "paternity" (also known as "attribution" - the right for the author to be identified as such) and "integrity" (the right for the author not to have the work altered in such a way that the author's reputation or integrity may be impugned).
- Where content in the article is identified as belonging to a third party, it is the obligation of the user to ensure that any reuse complies with the copyright policies of the owner of that content.
- If article content is copied, downloaded or otherwise reused for non-commercial research and education purposes, a link to the appropriate bibliographic citation (authors, journal, article title, volume, issue, page numbers, DOI and the link to the definitive published version on **Wiley Online Library**) should be maintained. Copyright notices and disclaimers must not be deleted.
- Any translations, for which a prior translation agreement with Wiley has not been agreed, must prominently display the statement: "This is an unofficial translation of an article that appeared in a Wiley publication. The publisher has not endorsed this translation."

Use by commercial "for-profit" organisations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee. Commercial purposes include:

- Copying or downloading of articles, or linking to such articles for further redistribution, sale or licensing;
- Copying, downloading or posting by a site or service that incorporates advertising with such content;
- The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee (for example, a compilation produced for marketing purposes, inclusion in a sales pack)
- Use of article content (other than normal quotations with appropriate citation) by for-profit organisations for promotional purposes