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Role of emerin and protein kinase C in herpes simplex nuclear egress

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ROLE OF EMERIN AND PROTEIN KINASE C IN HERPES SIMPLEX NUCLEAR

EGRESS

by

Natalie Leach

<u>An Abstract</u>

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
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Thesis Supervisor: Professor Richard J. Roller

The nuclear lamina consists of a mesh-like network of lamin proteins anchored to the inner nuclear membrane by interactions with integral membrane proteins such as emerin. Emerin binding to lamin A/C is one of the interactions that connect the inner nuclear membrane to the lamina. Infection by herpesviruses results in changes in the organization of the nuclear lamina, perhaps in order to facilitate envelopment of capsids at the inner nuclear membrane. In HSV-1 infected cells, alterations to the lamin proteins have been shown to involve pUL34, pUL31, and pUS3 proteins, which are also required for normal nuclear envelopment. We tested hypotheses about the mechanism and significance of lamina disruption. This thesis presents the following data. Infection of multiple cell types induced emerin hyperphosphorylation that was dependent on the presence of pUL34 and kinase active pUS3 proteins. The pUL34-dependent component was also sensitive to Rottlerin treatment suggesting that cellular kinases sensitive to Rottlerin were involved in emerin modification. LAP2 (another lamin associated protein) was de-modified (perhaps de-phosphorylated) in a pUS3 and pUL34 independent manner. Emerin was not required for growth of HSV-1. Hyperphosphorylation of emerin was required for its disassociation from the lamina.

PKC family members have been implicated in the disruption of the nuclear lamina during herpesvirus nuclear egress. To test the hypothesis that PKC activity was required for viral replication, PKC activity was blocked with PKC inhibitors and dominate negative PKC constructs. Chemical inhibition of all PKC isoforms reduced viral growth five-fold and inhibited capsid egress from the nucleus. However, specific inhibition of either conventional PKCs or PKC delta did not inhibit viral growth.

In addition to lamin associated proteins, lamin localization is also disrupted during herpesvirus infections. Emerin and lamin A/C are binding partners and the localization of both proteins is disrupted by pUS3 and cellular kinase mediated phosphorylation. To test the hypothesis that HSV-1-induced lamin A/C disruption is mediated via a mechanism similar to emerin's, pUS3 and Rottlerin Sensitive Kinases

were inhibited and lamin A/C localization was observed. Unlike emerin, HSV-1-induced disruption of lamin A/C was not altered by Rottlerin Sensitive Kinase inhibition suggesting that HSV has multiple mechanisms for disruption of the lamina.

Phosphorylation of lamina components, by Rottlerin Sensitive Kinases, may be a required event prior to primary envelopment. To test this hypothesis, growth of HSV-1 was tested in Rottlerin treated infected cells. Although the inhibitor Rottlerin, did reduce viral growth, it was also was also associated with severe depression of viral late-gene expression. TEM analysis suggested that Rottlerin Sensitive Kinases(s) were required for: (i) nuclear egress and (ii) capsid accumulation or formation supporting the hypothesis that the capsids were made in the presence of Rottlerin were unable to leave the nucleus.

pUS3 is a multi-functional protein in alpha-herpesviruses. It has been implicated in lamina disruption, protecting the infected cell from apoptosis, and de-envelopment at the outer nuclear membrane. In BT-549 cells, a breast cancer cell line with low PKC delta expression, the hypothesis was tested that in the absence of cellular lamina disrupting kinases, an US3-null virus would be blocked at the lamina disruption step. In BT-549 cells, the US3-null (vRR1202) virus was 10-fold decreased above the typical 10-fold decrease, compared to WT virus, to produce a 100-fold decrease in infectious PFU yet apoptosis was not increased. Lamin A/C disruption occurred via similar mechanism in both breast cancer cell lines: BT-549 and MCF-7. Interestingly, in the BT-549 cells, emerin was extensively hyperphosphorylated in an US3-null infection, yet was not redistributed along the NE. These data support a model that one or more specific residue(s) must be phosphorylated for emerin disconnection from lamina.

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Thesis Supervisor: Professor Richard J. Roller

Graduate College
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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To my father, my favorite biology and math teacher.

How far you go in life depends on your being tender with the young, compassionate with the aged, sympathetic with the striving and tolerant of the weak and strong, because someday you will have been all of these.

George Washington Carver

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And last but not least, I would not have completed this thesis without the comforting purrs of my cats.

ABSTRACT

The nuclear lamina consists of a mesh-like network of lamin proteins anchored to the inner nuclear membrane by interactions with integral membrane proteins such as emerin. Emerin binding to lamin A/C is one of the interactions that connect the inner nuclear membrane to the lamina. Infection by herpesviruses results in changes in the organization of the nuclear lamina, perhaps in order to facilitate envelopment of capsids at the inner nuclear membrane. In HSV-1 infected cells, alterations to the lamin proteins have been shown to involve pUL34, pUL31, and pUS3 proteins, which are also required for normal nuclear envelopment. We tested hypotheses about the mechanism and significance of lamina disruption. This thesis presents the following data. Infection of multiple cell types induced emerin hyperphosphorylation that was dependent on the presence of pUL34 and kinase active pUS3 proteins. The pUL34-dependent component was also sensitive to Rottlerin treatment suggesting that cellular kinases sensitive to Rottlerin were involved in emerin modification. LAP2 (another lamin associated protein) was de-modified (perhaps de-phosphorylated) in a pUS3 and pUL34 independent manner. Emerin was not required for growth of HSV-1. Hyperphosphorylation of emerin was required for its disassociation from the lamina.

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phosphorylation. To test the hypothesis that HSV-1-induced lamin A/C disruption is mediated via a mechanism similar to emerin's, pUS3 and Rottlerin Sensitive Kinases were inhibited and lamin A/C localization was observed. Unlike emerin, HSV-1-induced disruption of lamin A/C was not altered by Rottlerin Sensitive Kinase inhibition suggesting that HSV has multiple mechanisms for disruption of the lamina.

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LIST OF ABBREVIATIONS

4E-BP1	eukaryotic initiation factor 4E Binding Protein 1
AD-EDMD	Autosomal dominant Emery-Dreifuss Muscular Dystrophy
AGC	c<u>A</u>MP-dependent, c<u>G</u>MP-dependent, and protein kinase <u>C</u>
aPKC	Atypical PKC
BAF	Barrier to auto-integration factor
BIM I	Bisindolylmaleimide I
CA	Capsid protein
Cdk1	Cyclin dependent kinase 1
CHPK	Conserved Herpes Protein Kinase
CIAP	Calf Intestinal Alkaline Phosphatase
cPKC	Conventional PKC
DAG	Diacylglycerol
ds	double stranded
E	Early
EBV	Epstein-Barr Virus
EDMD	Emery-Dreifuss Muscular Dystrophy
EGFP	Enhanced Green Fluorescent Protein
eIF4E	eukaryotic Initiation Factor 4E
FRAP	Fluorescence Recovery After Photobleaching
GCL	Germ Cell Less
GCV	Gancyclovir
HCMV	Human Cytomegalovirus
hpi	hours post infection
hr	hour
HSV-1	Herpes Simplex Virus type 1
HSV-2	Herpes Simplex Virus type 2

IE	Immediate Early
IN	Integrase
INM	Inner Nuclear Membrane
Kapa1	Karyopherin alpha 1
kB	kilobase
kD	kilodalton
KSHV	Kaposi's Sarcoma Herpes Virus
L	Late
LAP	Lamin Associated Protein
LBR	Lamin B Receptor
LEM	LAP2, Emerin, MAN1
LMNA	Lamin A
LMNB	Lamin B
LMNC	Lamin C
MA	Matrix
MAPK	Mitogen-Activated Protein Kinase
MCMV	Mouse Cytomegalovirus
MEF	Mouse Embryonic Fibroblasts
min	minutes
MLV	Murine Leukemia Virus
MOI	Multiplicity of Infection
mTOR	Mechanistic Target of Rapamycin
MVM	Minute Virus of Mice
NE	Nuclear Envelope
NLS	Nuclear Localization Signal
NM	Nuclear Membrane
NPC	Nuclear Pore Complex

nPKC	Novel PKC
ONM	Outer Nuclear Membrane
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
PFU	Plaque Forming Unit
PK	Protein Kinase
PIC	Pre-Integration Complex
PKA	Protein Kinase A
PKC	Protein Kinase C
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2 A
PP4	Protein Phosphatase 4
PRV	Pseudorabies Virus
pRB	phosphorylated Retinoblastoma
RSK2	Ribosomal S6 Protein Kinase
RttSK	Rottlerin Sensitive Kinase
S6K	p70 Ribosomal S6 Protein Kinase
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SRPK1	Serine/Theronine Protein Kinase 1
ss	single stranded
Strep	Streptactin-Binding
SUN	Sad1 and UNC-84 homology
TM	Transmembrane domain
TSC2	Tuberous Sclerosis 2 protein
UL	Unique Long
US	Unique Short
vhs	viron host shutoff

VSV	Vesicular Stomatitis Virus
VZV	Varicella Zoster Virus
X-EDMD	X-linked Emery-Dreifuss Muscular Dystrophy

CHAPTER I

INTRODUCTION TO HSV-1 NUCLEAR EGRESS AND

NUCLEAR LAMINA

<u>Herpesvirus</u>

Herpes public health. Herpes simplex virus type 1 (HSV-1) are enveloped, large, double stranded (ds) DNA viruses that infect most vertebrates. There are three subfamilies within *Herpesviridae*: alpha, beta, and gamma (Table 1.1). The subfamilies are divided based on sequence similarity, tropism, length of replication cycle, and efficiency of dissemination in tissue culture. Alpha-herpesviruses, such as HSV-1, have a wide tropism, short replication cycle, and are able to efficiently spread in tissue culture (reviewed in (286)). In the US adult human population, it is estimated that 80% of individuals are latently infected with HSV-1, the causative agent of "cold sores" (61, 286). HSV-1 can also induce genital lesions, encephalitis in the immunocompromised, conjunctivitis and keratisis that can lead to blindness, and disseminated infection in neonates. Due to establishment of latent infection in sensory neurons, herpesviruses are nearly impossible to eradicate from the human population (61, 286). During latency, limited viral transcription and translation occurs but no virus particles are produced. The transition from latency to lytic replication is field of intense research but the mechanism of reactivation has yet to be completely elucidated (286).

Treatment. At this time, there are a variety of treatment options for herpesvirus infections. Acyclovir and derivatives of this compound are effective at inhibiting replication of alpha-herpesviruses, but can lead to selection of resistant virus growth in the immunocompromised (61, 286). Acyclovir is a guanine analogue that preferentially inhibits the viral polymerase (65). Gancyclovir (GCV) and other similar pharmaceuticals are available for treatment of beta- and gamma-herpes infections but have toxic side effects such as bone marrow and kidney damage (250). When GCV treatment is used for

long term in the in the immunosuppressed due to co-infection with HIV or organ transplant, selection of GCV-resistant herpes infections can arise (250). This makes treatment of herpes infections in the already compromised individuals especially difficult. These data indicated that there is high need for development of pharmaceuticals that can target a conserved process in the herpesvirus life cycle to block replication of all herpesviruses. There is also need for development of a pharmaceutical that targets a specific viral life cycle event and not a cellular process to decrease the possibility of toxic side effects.

Life cycle. The HSV-1 genome is approximately 150 kilobases (kB) long and is packaged into an icosahedral, enveloped capsid. The dsDNA genome contains two unique segments, unique long (UL) and unique short (US). Each unique segment is flanked by segment specific repeat elements. The icosahedral capsid is composed of six different proteins, surrounded by an amorphous structure of tegument proteins (Figure 1.1). The tegument is composed of at least fifteen proteins (179). Surrounding the tegument is the glycoprotein-studded lipid-membrane derived from the infected cell Golgi apparatus (Figure 1.1) (178, 179, 286).

A herpesvirus enters an uninfected cell by binding of the viral glycoproteins, which stud the lipid envelope, to hepran sulfate (259). Entry of HSV can differ between cell types. One mechanism for entry involves direct fusion between the viral envelope and the cell plasma membrane. The second involves endocytosis of the enveloped particle and fusion of the envelope with the endosomal membrane (259). Both mechanisms result in deposition of the nucleocapsid and tegument proteins directly into the cytoplasm of the cell. Once the capsid is inside the cell, some of the tegument proteins are thought to have immediate functions in the cell. Examples of tegument proteins are vhs, VP1/2, and VP16. The tegument protein viron host shutoff protein (vhs) is involved in inhibiting cellular protein synthesis, which is thought to aid in blocking the host immune response (174).

VP16, another tegument protein, is involved in activation of viral gene expression, and is trafficked to the nucleus immediately following entry and uncoating (179, 286). After uncoating, the capsid is also trafficked to the nucleus via the microtubule network, docks at the nuclear pore, and injects the genomic dsDNA into the nucleus. One of VP1/2 many functions is to mediate capsid-nuclear pore attachment (47). After the genome circularizes inside the nucleus, viral gene expression proceeds in a regulated cascade (179). There are three rounds of gene expression: alpha, beta, and gamma also called immediate-early (IE), early (E), and late (L). IE licenses E gene expression. Many E genes are involved in genome replication, which requires E gene expression and precedes L gene expression. Many L genes are involved in capsid assembly or egress, but L gene expression requires genome replication (286). Once new genomes and capsids are formed, the viral DNA is packaged into the empty capsids and the process of primary envelopment, or maturation of the capsid from inside the nucleus to the cytoplasm, must occur (178, 179, 286).

Herpes primary envelopment. Nuclear egress of all herpesviruses occurs via (i) envelopment of the capsid at the inner nuclear membrane (INM) and (ii) fusion of the perinuclear-enveloped viron with the outer nuclear membrane (ONM) (Figure 1.2). This process of envelopment/de-envelopment at the nuclear membrane (NM) deposits a naked capsid into the cytoplasm, which is transported via cellular machinery to the Golgi apparatus for secondary envelopment (178, 179). Nuclear egress is common to all herpesviruses, utilizes homologous proteins and mechanisms, and is essential for replication (178). The process of budding something out of the nucleus is not performed by an uninfected cell, making primary envelopment an attractive therapeutic target. It is reasonable to hypothesize that a compound that specifically inhibits primary envelopment of an alpha-herpesvirus would not alter normal cellular functions, because they do not normally perform this process. This hypothetical compound might also inhibit primary

envelopment (and therefore growth) of beta- and gamma- herpesviruses because primary envelopment is a conserved and essential process in the life-cycle of all herpesviruses.

UL34. Herpesviruses encode several conserved proteins involved in the essential process of primary envelopment. The protein product of the gene UL34 encodes a 34 kilodalton (kD) polypeptide with a predicted type II transmembrane topology (215, 216). The UL34 gene is conserved across *Herpesviradae* (51, 58, 268). Deletion of this gene in HSV-1 leads to accumulation of DNA containing capsids in nucleus but few capsids outside the nucleus and infectious particles are formed (232). The nuclear egress function of pUL34 is conserved in the alpha-herpesvirus peudorabies virus (PRV), equine herpesvirus, and the gamma-herpesvirus Epstine-Barr virus (EBV) (71, 128, 195).

UL31. Together with pUL34, pUL31 are called the envelopment complex proteins. The protein product of the gene UL31 encodes a 31 kD polypeptide that localizes in the nucleoplasm of infected cells (37). pUL31 and pUL34 are sufficient for nuclear targeting in the absence of other viral proteins (224, 225). Recently, an additional interaction domain between pUL34 and pUL31 was identified (228). pUL31 is also conserved across *Herpesviradae* but HSV pUL31 is not essential for nuclear egress in all cell lines (90, 156, 238, 294). The literature supports a model that pUL34 and pUL31 function as a complex for nuclear egress, and this function has been conserved in their homologs in other herepesviruses (137, 225).

<center>Lamina disruption by Herpesviridae</center>

The size of the herpesvirus capsid is problematic for direct movement of the capsid from the nucleus to the cytoplasm (Figure 1.3). Herpesviruses are considered large viruses because a ~120 nm capsid is required to accommodate their large genome. After genome replication and late gene expression, new capsids assemble in the nucleus and are subsequently packaged with genomic dsDNA (178). The capsid must exit the nucleus through an envelopment/de-envelopment process at the INM or nuclear egress

(Figure 1.2). This process and the proteins involved are conserved across alpha, beta and gamma herpesviruses. The openings in the lamin meshwork, 50-80 nm, are too small for direct capsid movement though the lamin mesh to the INM (179). LAPs, which anchor the lamin network to the INM, may also pose as a structural barrier to the capsid. It has therefore been hypothesized that the lamina, both lamins and LAPs, is a physical obstacle that herpesviruses must overcome.

Lamin disruption by HSV-1. In support of the hypothesis that the lamina is a physical barrier to a herpes capsid, it has been demonstrated that infection results in increased nucleus size and irregular shape (20, 217, 249). Endogenous lamin A/C, lamin B1, and lamin B2 are redistributed during infection starting at eight hours post infection (hpi) (20, 207, 242, 248, 249). GFP-lamin A and GFP-lamin B2 are redistributed as well (242). While both are disrupted, lamin A and B may be targeted for disruption in distinct manners. Lamin B shows cytoplasmic aggregation while lamin A does not (207). Lamin disruption has also been observed in HSV-2 (36). These data suggest that alpha-herpesviruses are specifically disrupting the two types of lamins (A/C and B) via different mechanisms. These data lead to the hypothesis that lamina disruption is a key functional event in HSV-1 nuclear egress, not a by-product of infection. However, it is unknown if lamina disruption is required for nuclear egress.

The viral envelopment complex proteins, pUL31 and pUL34, appear to have functions in lamina disruption. pUL31 and pUL34 can directly bind to lamin A/C (223). pUL34 also appeared necessary for altering lamin A/C protein tertiary confirmation resulting in antibody epitope masking (223). *Simpson-Holley et al* confirmed that GFP-lamin A was redistributed during HSV-1 infection in a pUL34/pUL31 dependent manner, and went on to demonstrate that even in the absence of nuclear expansion, GFP-lamin A was disrupted (249). It has been demonstrated that pUL34 was sufficient and necessary for lamin disruption while pUS3 regulates the degree of disruption (20). These data suggest that disruption of the lamina was coupled with nuclear egress.

One alternative hypothesis to lamin redistribution observed by immunofluorescence could be a decrease in total lamin content and/or an indication of apoptosis. This alternative hypothesis appears only to be valid in certain cell types. Total cell lysates from COS-1 and HEp-2 cells showed a decrease for lamin A/C and B (20, 242). However, in Vero cells, there appears to be no change in lamin levels although there is disruption in localization (20). Together with the data that HSV-1 infection disrupts the localization of lamin B into cytoplasmic puncta but not lamin A/C suggests that alpha-herpesviruses are specifically disrupting the two types of lamins (A/C and B) via different mechanisms (206). The differing mechanisms may be due to differential tissue expression of lamins or function.

Lamin disruption in other herpesviruses. There are similar examples of infection- and envelopment complex-dependent reorganization of the nuclear lamina in beta- and gamma- herpesviruses.

Beta. The MCMV pUL34 homologue, M50/p35, was sufficient to disrupt localization of lamin A/C and lamin B without activating apoptosis (191). The HCMV pUL34 homologue, pUL50, colocalized with and disrupted lamin A/C (34, 98, 180, 181, 217). Active pUL97, the HCMV protein kinase involved in egress, was also sufficient to redistribute endogenous lamin A/C (170). These data suggest that the lamina disruption is a conserved process and is mediated via conserved proteins.

Gamma. BFRF1 is the pUL34 homologue and BFLF2 is the pUL31 homologue in EBV (137). Similar to HSV, these proteins require the expression of the other for nuclear rim localization (89, 137). As is pUL34, BFRF1 is also required for nuclear egress (71). When BFRF1/BFLF2 or BFRF1 was expressed, the nuclear membrane was disrupted (89). The BFLF2-BFRF1 complex colocalized and co-immunoprecipitated with lamin B (89). These data suggest that envelopment complex protein function in lamina disruption and this function is conserved across the herpesvirus family. The EBV viral kinase, BGLF4, has also been implicated in lamina disruption (145).

LAP disruption by HSV. In addition to disruption of the lamins, herpesviruses induce disruption of LAPs as well.

LBR. Lamin B Receptor (LBR) was the first LAP to be observed to be effected by infection. The GFP-LBR fusion protein was redistributed from the nuclear rim to a blebby nuclear and ER distribution in COS-1 cells infected with HSV-1 (242). Fluorescence Recovery After Photobleaching (FRAP) analysis showed GFP-LBR was more mobile upon infection (242). This was also true in beta-herpesviruses infections such as HCMV. The HCMV envelopment complex protein, pUL50, and kinase, pUL97, colocalized with and disrupted LBR (170, 180).

LAP2beta. LAP2beta was also redistributed along the NE during HSV-1 infection in a pUL34- and pUL31- dependent manner suggesting yet again a linkage between envelopment and nuclear lamina disruption (249). Beta-herpesvirus envelopment complex protein, M50/p35, was sufficient to disrupt LAP2beta localization suggesting a conserved mechanism of disruption (191).

Emerin. During HSV-2 infection, emerin is phosphorylated as early as four hpi (186). Inhibition of PKC alpha and cdc2, reduced but did not eliminate modification of emerin in HEp-2 cells (186). Emerin also became more extractable and more mobile in FRAP after infection suggesting that it has lost its connections to the lamina (142, 186). Thus, it seems clear that in addition to lamins, LAPs are targets of the entire family of herpesvirus and that one conserved function of the envelopment complex proteins might be lamina disruption prior the envelopment of the capsid at the INM.

<div align="center">The Lamina and other viruses</div>

Herpesviruses and many other viruses must assemble newly formed capsids inside the nucleus. However, for the newly formed herpesvirus capsid to escape the nucleus and complete maturation in the cytoplasm, the capsid must undergo the envelopment/de-envelopment process described in (Figure 1.2). The INM is not freely accessible to the

capsid for primary envelopment due to size restrictions (capsid ~120 nm). Access to the INM is in fact blocked for a large macromolecular complex such as a herpesvirus capsid by the nuclear lamina that supports the INM (Figure 1.3). Some viruses, including herpesviruses, have developed mechanisms to deal with the different lamina components in different ways, presumably due to differing functions and tissue expression profiles.

Barrier: The lamina could also serve as a barrier for things trying to obtain entry into the nucleus. Parvoviruses, linear single stranded (ss) DNA viruses, must replicate in the nucleus, but how they gain entry is unclear. Some viruses use normal cellular nuclear-transport machinery or NPCs to gain access to the nucleus (116, 251, 287). Until 2005, it was widely assumed that parvoviruses used an NPC-mediated entry mechanism. The Pante group in 2005 first demonstrated that minute virus of mice (MVM), a parvovirus, may induce breaks or create holes in the NE to gain entry (46). Lamin A/C staining of MVM infected mouse fibroblasts, showed irregular staining and invaginations of the NE (45). Interestingly, the virus accumulated at areas of the NE where lamin A/C staining was absent (45). EM analysis also showed chromatin redistribution and breaks in the ONM (45). *Cohen et al* hypothesized that the virus encodes its own mechanism of lamina disruption due to the phospholipase A2 activity encoded in the capsid of MVM (45, 298). The mechanism of the NPC-independent MVM entry remains unknown as well as if this is common to all parvoviruses.

Viruses are obligate parasites. Even the most complex virus is too simple to support all the required processes required for production of a new virus particle or "life." Therefore, all viruses must replicate inside a cell and take advantage of host cell functions. Viruses with dsDNA genomes or RNA viruses, like influenza, that replicate inside the nucleus of the host cell likely at some time in their life cycle interact with the nuclear lamina. The lamina could also be a structural barrier to DNA viruses such as adeno- and polyoma- viruses prior to release. Both adeno- and polyoma-viruses induce lysis of the host cell for release (42, 62), so although these viruses may interact with the

nuclear lamina in an entirely different manner than a herpesvirus, this cellular structure could still be a barrier to egress.

Influenza is a negative sense RNA virus that is unique because it replicates in the nucleus (24). After genome replication, vRNPs are assembled. vRNPs contain one negative sense viral genome segment, each of polymerase subunits (PA, PB1 and PB2), and NP. These RNA-protein complexes must leave the nucleus for packaging into virions (24). The RNA-protein complexes are thought to egress the nucleus via NPC. Influenza inhibited cellular mRNA export factors and induced the degradation of mNup98 (239). Export of cellular factors was inhibited while viral factors that do not rely on these particular export factors is not affected (239). Other viruses interact with NPCs by inhibiting or altering cellular nuclear export. Ebola viruses and severe acute respiratory syndrome coronavirus (SARS-CoV) sequester karyopherin alpha1 (Kapa1) and Kapa2 to block the antiviral response (77, 219). Other viruses including, poliovirus, rhinovirus, vesicular stomatitis virus (VSV) also targets Nups for degradation to skew preference of viral molecules for export (95). While this thesis will focus on the effects of herpesviruses on LAPs, it should be appreciated that many viruses have developed mechanisms of interacting with different components of the multifunction structure that is the nuclear envelope.

Co-factor: It is unclear if retroviruses specially induce lamina disruption or if the only role of the nuclear lamina for retroviruses is as a co-factor for facilitating integration of their genomes. HIV is a retrovirus in the genus lentivirus and can infect both dividing and non- cells (72, 208). For persistent infection retroviruses must integrate their genomes into the host genome. This requires entry of the pre-integration complex (PIC) into the nucleus, possibly via host cell nuclear transport mechanisms (72, 243). Conversely, gamma-retroviruses such as murine leukemia virus (MLV) require active mitosis for integration (154, 227). The mechanism of HIV PIC entry into non-dividing cells has been widely debated. Both nuclear localization signal (NLS)- dependent and

independent pathways have been proposed (72). The HIV PIC is composed of the newly

synthesized vDNA (discontinuous, triple stranded), capsid protein (CA), structural

protein p6, Vpr, integrase (IN), and matrix protein (MA) (31). MA, IN, Vpr, and vDNA

(discontinuous, triple stranded at the central polypurine tract) all have proposed roles in

PIC nuclear entry (25, 31, 52, 83-85, 96, 102, 209, 210, 299). Although it seems

apparent that some of these viral factors are essential for replication, it is unclear, how

these viral factors exactly mediate nuclear entry. Conflicting results about the nuclear

import function of these factors have arisen and it is still unknown if PIC entry is

mediated via the NPC or not (30, 60, 75, 76, 159, 295, 296). Vpr is also associated with

lamina disruption. It has been hypothesized that Vpr is required to induce lamina or NE

disruption to allow PIC entry into non-dividing nuclei since this complex is too large for

NPC transport (52, 208, 243). When Vpr is expressed in HeLa cells, herniations (blebs)

where found in the NE (52). These herniations contain cellular DNA and the nuclear

protein involved in cell-cycle control, Wee1 (52). Vpr expression also induced

translocation of cdc25c and cyclin B1 into the nucleus from the cytoplasm (52). Lamin C

formed gaps around the NE herniations and lamin A and B staining changed from smooth

to wrinkled in the presence of Vpr (52). Vpr does have a NLS sequence and from these

data the authors proposed a model in which Vpr enters the nucleus via a CRM1

dependent pathway (244), then from the interior of the nucleus induces a break in the NE,

and allows the PIC to enter for integration (52, 243). However, the authors have not

subsequently demonstrated this mechanistically. Regardless of the mechanism, it seems

possible that in non-dividing cells if the NPCs are not used, the lamina may pose as a

structural barrier to the retroviral PIC.

After genome replication, retroviruses must integrate their genome into the host

genome for persistent infection. At first it was not understood how MLV and HIV

integrate *inter*molecularly rather than *intra*molecularly to prevent auto-integration. Using

MLV as a model retrovirus, it was demonstrated that an unknown cellular nuclear protein

could block MLV autointegration (148). The cellular factor responsible for blocking

MLV *intra*molecular integration was later identified and the 89AA protein was named

barrier-to-autointegration factor (BAF) (147). Subsequently, it was shown that

recombinant BAF restored HIV intermolecular integration activity (38). The mechanism

of BAF activity in retrovirus integration was later determined. BAF only binds dsDNA,

an activity required for HIV-1 integration (101). Additionally, misfolded BAF mutants

could not support HIV integration (101). This suggested that DNA binding activity and

structure are essential for BAF function in HIV integration.

BAF. BAF appears to be an important component of MLV and HIV PICs and

retrovirus particles. BAF is part of the mature MLV and HIV virus particle (160, 165,

263). Viral DNA in the PIC is "compacted" by BAF, which also enhances association of

PIC with target cellular DNA (263). BAF binds directly to p55 gag and the matrix

subunit, suggesting to *Mansharamani et al* that BAF serves as a bridge between the PIC

and chromatin by simultaneously binding to the matrix protein and cellular DNA (165).

Thus, BAF can be viewed as protector from auto-integration and helper of association

with cellular chromatin. In the host cell, BAF binds to the LEM domain of many LAPs

and is thought to help organize heterochromatin to the nuclear periphery (167).

LAP2alpha. LAPs interact with BAF via their conserved LEM domain. The

hypothesis that retroviruses might utilized this interaction to assist in integration has been

examined for two LAPs: LAP2alpha and emerin. In addition to BAF, LAP2alpha is part

of the MLV PIC and depletion of LAP2alpha inhibited viral replication (263). Domains

of LAP2alpha that are both common and unique to all LAP2 isoforms increased and

stabilized BAF association with DNA (263).

Emerin. Emerin's role in HIV replication remains controversial. In siRNA

transfected primary macrophages, it was demonstrated that BAF and emerin were

required for productive HIV infection yet had no effect on MLV replication (118). Viral

entry into the nucleus was unchanged by siRNA knockdown yet did cause relocation of

viral DNA from the soluble chromatin to the insoluble nuclear-matrix fraction. This suggested that BAF and emerin are required for viral DNA-chromatin association (118). BAF and emerin knockdown caused integration to be negligible but genome replication was unaltered (118). The authors proposed a model that suggests BAF and emerin are guiding vDNA to chromatin (118).

In contradiction to *Jacque et al*, another group found that in HeLa-P4 siRNA transfected cells, emerin, BAF, and LAP2alpha were not required for HIV or MLV replication (247). Viral growth was also unaffected in emerin null mouse embryonic fibroblasts (177, 247). *Shun et al* also demonstrated that the siRNA used in *Jacque et al* did not affect emerin protein levels in HeLa-P4 cells (247). The conflicting data could be due to different cell types yet, it remains to be determined if emerin is required for HIV replication. It is possible that it is really BAF that is required and the loss of emerin prevents BAF-dependent viral association with chromatin. Emerin might just be guiding the PIC to the chromatin via its interaction with BAF. These data suggest a model where the nuclear lamina is a multifunctional structure to both the host and the pathogen.

The Nuclear Lamina

Lamin associated proteins. The lamin filament network is anchored to the INM via interactions with lamin associated proteins (LAPs) (Figures 1.3, 1.4, and 1.5). In additional to lamins, these proteins are potential viral targets because of their functions in cellular processes such as nuclear integrity, nuclear assembly, cell cycle regulation, and gene expression (92, 291). Prototypic LAPs include LAP2, emerin, MAN1, and lamin B receptor (LBR) while new LAPs are added to this list continuously (Figure 1.4). Many LAP proteins contain a conserved LEM (LAP2, Emerin, MAN1) domain capable of binding the dsDNA binding protein barrier-to-auto-integration factor (BAF) (92). The work described in this thesis will focus on LAP2beta and emerin. The focus was selected due to the availability of reagents and the finding that herpes simplex has apparently

developed different mechanisms of dislodging these two proteins from the NE although they share many structural and functional similarities.

LAP2. The LAP2 gene is alternatively spliced to generate a number of isoforms (100). LAP2 alpha/beta share a common N-terminal domain yet LAPbeta contains a TM domain that LAP2alpha does not (Figure 1.5 A and 1.4) (82, 100, 276). Similar to emerin and MAN1, LAP2 proteins bind BAF through the LEM domain (246). The N-terminus is common to all isoforms of LAP2 and can bind directly to DNA (33).

LAP2beta has important roles in maintaining the structure of the nuclear envelope and promoting proper localization of transcription factors within the nucleus for proper gene expression. LAP2beta contains a TM domain in its C-terminus, which anchors it at the INM (79, 82). LAP2beta functions in maintaining nuclear integrity by binding to lamin A/C and lamin B (Figure 1.5 A) (139). The N-terminus of HA95, a chromatin-associated protein, binds LAP2beta and while the C-terminus binds within the lamin B binding domain (171, 172). Germ cell less (GCL) is a binding partner of LAP2beta and emerin (125). LAP2beta is important in regulating gene expression by: interactions with the N-terminus of HA95 to regulate transcription initiation by preventing degradation of Cdc6, repression the transcription activity of E2F, and interactions with HDAC3 (59, 87, 92, 172, 173, 204, 246, 254). All of these interactions are predicted to cause transcriptional repression. LAP2beta also has an important role in chromatin structure and organization by binding to BAF (87, 92, 246). LAP2beta is key in nuclear envelope breakdown and reassembly during mitosis due to its a reversible phosphorylation-dependent binding to lamin B (53).

LAP2alpha, unlike LAP2beta, does not have a structural role in the nuclear envelope. LAP2alpha is a non-integral membrane protein but has a nuclear localization signal, which helps retain it in the nucleus (79, 82). LAP2alpha plays important roles within the nucleus by regulating phosphorylated retinoblastoma (pRB) function (Figure 1.4) (193, 204). Similar to LAP2beta but via different mechanisms, LAP2alpha also is

involved in gene expression regulation. LAP2alpha regulates gene expression by interfering with cell cycle progression by binding directly to Rb and inhibiting expression of E2F/Rb-dependent target genes (59, 193).

Emerin. Emerin is another LAP that is structurally quite similar to LAP2beta. Emerin is a 254 amino acid type II transmembrane protein that has a LEM domain at the amino terminus that mediates binding BAF (Figure 1.5 B) (16, 66). The LEM-domain is necessary for recruitment of emerin to the newly forming nuclear envelope following mitosis (146). Emerin also contains a lamin A/C-binding domain that helps retain it in the interphase nuclear envelope (146). Emerin is ubiquitously expressed, but is not essential for the viability of cells in culture. Mice with deletions in the gene encoding emerin develop apparently normally, but show defects in muscle regeneration and subtle defects in motor coordination (177). Failure to express or properly localize emerin in humans, however, is one cause of Emery-Dreifuss Muscular Dystrophy (EDMD) (69). Emerin has many known binding-partners including: transcriptional regulating proteins GCL and Btf, splicing regulatory factor YT521-B, actin, nesprin, Lmo7, and MAN1 (Figure 1.5 B) (99, 107-109, 166, 288). There are two serine/theronine rich regions that flank the lamin A/C binding domains.

Other LAPs. MAN1 is another LAP known to regulate linkage of the lamin network to the INM and gene-expression. MAN1 has two TM domains with both the N- and C- terminal domains located in the nucleoplasm (Figure 1.4) (111). MAN1 can bind to lamins and also to emerin, another LAP, to regulate integrity of the nucleus (166). MAN1 and emerin are thought to have some functional redundancy since in *C. elegans* single deletions are not deadly but double deletions are (161). Lamin B Receptor (LBR) is an essential LAP with 8 predicted TM domains with functions in nuclear integrity, chromatin organization, lipid modification, and nuclear assembly (Figure 1.4) (291, 292). As its name implies, LBR can bind lamin B. Additionally, LBR is a lipid-modifying

enzyme. Its C-terminal domain is a C-14 sterol reductase, which is involved in ergosterol biosynthesis (110, 111).

Nesprins: Connecting inside to outside. Nesprins are another family of proteins that function to coordinate signals to the nuclear lamina by connecting the actin cytoskeleton network to the INM. Nesprins are anchored to the INM by interacting with Sad1 and UNC-84 homology (SUN)-domain proteins via their KASH-domains in the perinuclear space (Figure 1.4) (92, 284). This interaction helps regulate the regular spacing between the two nuclear membranes, which herpesviruses expand by approximately 10-fold each time they exist as an enveloped perinuclear particle (48). It is likely that in addition to disruption of the lamina, herpesvirus must also disrupt protein-protein interactions within the perinuclear space that mediate normal spacing of the two nuclear membranes. Nesprins are transcribed from two separate genes, which are all subject to alternative splicing, generating many isoforms with a variety of sizes and conserved domains (302). Smaller nesprin isoforms interact with both emerin and lamin A/C creating an additional link the lamina (301).   Although this thesis will not directly examine the effects of herpesvirus on nesprin interactions, it is possible that emerin-nesprin and/or lamin A/C-nesprin interactions are abolished during primary envelopment.

Meshwork. The main component of the nuclear lamina is the type V filament proteins called lamins (Figure 1.4) (27, 92). The lamin protein meshwork is primarily composed of A-type and B-type lamin proteins. These proteins are involved in maintaining nuclear mechanical integrity and sensing various intra- and extra- cellular signals, making the lamina a signal integrator (27, 92, 113). A-type lamins include lamin A and C, which are alternatively spliced transcripts from the same gene while the two B-types are transcribed from two separate genes (Figure 1.6 A) (92). A-type lamins are expressed only in differentiated tissues while B-type are expressed in all cells. A and B -type lamins are farnesylated at a CaaX motif but this portion of A-type lamin proteins is post-translationally cleaved (Figure 1.6 A) (27). All lamins have three domains: head,

rod, and tail (Figure 1.6 B). These three domains are important in formation of dimers (hetero- or homo-) and interactions with other proteins (Figure 1.3 C). Lamin dimers are incorporated into filaments that assemble into the meshwork that underlies the inner most part of the nucleus (92). The lamin meshwork is anchored to the INM by directly binding to integral proteins of the INM such as emerin and LAP2beta (Figure 1.4). Therefore, a virus capsid inside the nucleus that does not utilize the nuclear pores to exit from the nucleus must interact with the nuclear lamina first prior to obtaining access to the INM or cytoplasm. This "problem" facing all nuclear replicating viruses can easily be observed when the nuclear lamina is modeled as a cross-section in (Figure 1.3).

Lamin A/C. As mentioned above, lamins are the main component of the nuclear lamina. The *LMNA* gene encodes A-type lamins (A and C) (23, 293). The *LMNA* gene can be alternatively spliced to produce the lamin C protein (Figure 1.3 A). Due to the splicing, lamin A and C are identical for the first 566 residues but differ at the C terminus (39). Pre-lamin A is post-translationally modified but this farnesylated 15 C-terminal residue portion, including the CaaX box, is removed to produce mature lamin A (6). In addition to creating structural integrity, lamin A/C appears important in regulation of transcription, chromatin organization, and cell signaling pathways (6). Lamin A-null mice are not viable and die eight weeks after birth (39).

Lamin B. Lamin B is found in all mammalian tissues (39). There are two lamin B genes: B1 and B2 and until recently, no disease was attributed to the loss of lamin B. Loss of lamin B2 in mice results in brain development defects such as lissencephaly (44). Lissencephaly, or defective layering of neurons in the cerebral cortex and crebellum, is likely due to defective migration of cells in the developing lamin B2null mice (44). Lamin B1 null mice die soon after birth and have bone, and lung defects (274). These data suggest that the B type lamins may have non-redundant functions in development (39).

Laminopathies. Mutations in lamina components (lamins and LAPs) can lead to genetic diseases termed laminopathies. By understanding how viruses disrupt lamina components, a greater understanding may result about how genetic mutations lead to lamina dysfunction (66, 92, 255). The phenotype of this class of diseases can range from premature aging (progeria), cardiac and skeletal muscle wasting, dilated cardiomyopathy, to partial lipodystrophy (255). Mutations in MAN1 are linked to Osteopoikilosis, Buschke–Ollendorff syndrome and melorheostosis (15, 103). Autosomal dominant Emery-Dreifuss Muscular Dystrophy (AD-EDMD) results from loss of lamin A/C at the INM due to mutant lamin A/C (185). Lamin A/C null mice are viable for a short time but exhibit some of these same defects as AD-EDMD patients (16, 138). In addition to lamin A/C linked laminopathies, there are different mutations that cause loss of emerin or results in expression of mutant emerin that cause of X-linked Emery-Dreifuss Muscular Dystrophy (X-EDMD) (16). Mutations in LAP2 can also result in dilated cardiomyopathy (267).

Both X-linked and AD-EDMD are laminopathies in which patients exhibit slow, progressive skeletal muscle wasting and cardiac conductive system defects (91, 92). Although emerin is ubiquitously expressed, this disease primarily affects skeletal and/or cardiac muscle. The muscle tissue on the back and backs of the arms are often extremely atrophied (55). The atrophied muscles of the back and backs of the arms can result in restricted mobility but the cardiac conduction defects are ultimately the most life-threatening symptom of this disease (237). EDMD-associated heart disease is characterized by atrial fibrillation, arrhythmias, various forms of cardiomyopathies, and atrioventricular conduction defects due to sinus node dysfunction, all of which can induce sudden cardiac death (68).

The relationship between abnormalities in emerin and the pathogenesis of EDMD is not clear (66, 68). Emerin-null mice are viable but also have cardiac defects and exhibit hind-limb problems (177, 202). Studies on fibroblasts from emerin-null mice

have provided valuable information about emerin function and specific interactions important for its functions. Lamin A/C is mislocalized in emerin-null and EDMD mutant cells suggesting that emerin-lamin interactions are important for proper linkage of the lamin mesh to the INM (Figures 1.5 B and 1.4) (13, 112, 138, 177, 218, 260). Understanding emerin function and functional interactions may lead to elucidation of the molecular mechanism responsible for EDMD symptoms.

There are two proposed hypotheses for emerin involvement in EDMD pathogenesis, yet both imply that emerin function is tightly coupled with its ability to make functional protein-protein interactions (16, 66). The first hypothesis suggests that EDMD results from nuclei being unable to withstand physical force when mutant emerin cannot connect to its binding partners. The second suggests that emerin is critical for functional localization of lamin A/C, an emerin binding partner. Thus EDMD pathology may be the result of aberrant emerin-protein interactions due to the lack of or expression of mutant emerin. It also seems apparent from several lines of evidence that emerin interaction with lamin A/C is especially critical for lamina function. One example is the observation that AD-EDMD (autosomal dominant) mutations in lamin A/C that are located in the tail region which interacts with emerin, result in a loss of emerin-lamin A/C co-localization suggesting that they no longer interact. Mutations outside of the emerin interaction domain of lamin A/C had no effect (112). Another finding supporting that emerin interactions are critical for its function demonstrated that retention of emerin at the INM requires lamin A/C and nesprin-1-alpha (200, 260). Various X-linked EDMD mutants are more extractable suggesting a loss in emerin binding to other lamina components (67). EDMD pathology might also arise from improper emerin retention at the NM when it can no longer interact with lamina components. This hypothesis is supported by evidence that X-linked EDMD mutant emerin does not properly localize at the INM (16). A G82K mutation in lamin A/C, from an AD-EDMD patient with dilated cardiomyopathy, resulted in emerin mislocalization again suggesting that lamin A/C-

emerin interactions are key for proper lamina organization and function and the avoidance of EDMD (282). Additionally, because mutations in either lamin A/C or emerin result in similar clinical phenotypes it seems reasonable to hypothesize that a functional lamin A/C-emerin complex is critical to cardiac muscle function. It is not understood how emerin protein-protein interactions are regulated and there is much to learn about normal emerin function and the molecular mechanism of EDMD pathology.

Mitosis. Mitosis requires breakdown of the NE. This dynamic process might be mimicked for lamina structural or functional disruption during viral infection. Mitosis requires disruption of the nuclear lamina (reviewed in (169) which is mediated by phosphorylation of lamins and LAPs by cellular kinases including cyclin dependent kinase 1 (Cdk1), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), protein kinase A (PKA), casein kinase II, and AKT (91, 92, 169). Phosphorylation disrupts the lamina protein-protein and protein-DNA interactions. For division of a cell to occur, the nuclear membrane, lamins, and NPC disassemble between prophase and prometaphase, which allows chromatin to associate with the mitotic spindle (168).

Phosphorylation of nuclear proteins by cdk1, which is regulated by cyclinB1, reverses the interactions between proteins and allows the NE to be broken down for completion of mitosis (169). The N-terminal domain of LBR is phosphorylated by serine/theronine protein kinase 1 (SRPK1) and cdk1 kinases to terminate chromatin binding (196, 197, 265). LAP2beta is phosphorylated during mitosis, which disrupts binding to lamin B (53). In Xenopus, MAN1 binding to BAF is terminated by mitotic phosphorylation of serine 402 (5). Phosphorylation of serine four of BAF in Xenopus disrupts emerin-BAF binding in mitosis as well (105).

Reassembly of the nuclear lamina is accomplished by targeting of nuclear structural proteins to the reforming nuclear envelope. The proteins are dephosphorylated so that they may once again interact with their interphase binding-partners (169). Phosphate removal is mediated by phosphatases such as cdc25, cdc14, protein

phosphatase 1 (PP1), PP2A, and PP4 (22). First, LBR and emerin begin to associate with the reforming nuclear envelope and then LAP2beta (169). Then the membrane reforms and NPC reassemble. Lamins are then transported via the NPC and the lamin network reforms and the LAP-lamin interactions are reestablished. Lamin B and LBR are known to interact with importin beta for nuclear envelope proper reassembly (163). Corruption or mimicking of mitotic events is an example of a possible viral target for disruption of nuclear lamina structure and/or function.

<div align="center">The Kinases</div>

The mechanism of lamina disruption during herpesvirus nuclear egress is poorly understood. During mitosis, the lamina is disrupted via phosphorylation of lamins and LAPs. Phosphorylation disrupts the protein-protein and protein-DNA interactions within the lamina (169). One hypothesis in the field suggests that herpesviruses may encode a viral and/or recruit a cellular kinase(s) to phosphorylate lamina components to create flexibility within the lamina necessary for capsid access to the INM for primary envelopment.

CHPK or pUL97. All herpesviruses encode a conserved protein kinase. CHPK (Conserved Herpes Protein Kinase) or pUL97 in HCMV (pUL13 in HSV) has many similarities to the cellular kinase cdk1 in function and substrate specificity (114, 121, 122). The beta- and gamma- herpes CHPK has been implicated in nuclear lamina disruption and function of CHPK in nuclear egress has been established (134, 145). The HCMV CHPK phosphorylates lamins on previously defined cdk1 sites (98). HCMV pUL97 interacts with p32, an INM protein, at the nuclear rim (170) and both p32 and lamins were phosphorylated by pUL97 (170). The HSV-1 CHPK, pUL13, apparently does not directly phosphorylate nuclear lamina proteins, but the HSV-2 pUL13 homolog has the ability to disrupt lamin localization in transiently transfected cells and to directly phosphorylate lamins *in vitro* (36).

US3. Alpha-herpesviruses encode a second serine/threonine protein kinase designated US3 in HSV. pUS3 mediates phosphorylation of lamina components, including lamin A/C, lamin B1, and emerin (Chapter II), and regulates the degree of lamina disruption (20, 142, 190). Between alpha-herpesviruses and cellular kinases, the kinase domain of US3 is highly conserved (175). The apparent molecular mass of pUS3 is 66 kD (49). The pUS3 consensus sequence is RRR X (S/T) but is a promiscuous kinase *in vitro* (49, 188). Some of the viral and cellular pUS3 substrates have been identified and include: pUL34, pUL31, US9, gB, emerin, lamin A/C, and lamin B1 (49, 119, 142, 188, 190, 236, 290). HSV-1 pUS3 (and its homologes) is thought to function in: (i) blocking apoptosis, (ii) de-envelopment at the ONM, (iii) redistribution and phosphorylation of nuclear lamina components including the envelopment complex (pUL34 and pUL31), (iv) downregulation of gB expression, (v) re-organization of the actin cytoskeleton (review (184)). Deletion of US3 in HSV-1, HSV-2, or PRV leads to significant impairment of viral PFU production (10^4–fold) and virulence (ID_{50} down 10^5-fold) in mice, however, in tissue culture replication of mutant viruses lacking US3 or expressing catalytic mutant versions of US3, have only modest growth defects (10-20-fold or 1-2 log decrease in PFU) (7, 9, 124, 213). This creates a disparity about pUS3 function between *in vivo* and tissue culture models of infection. It also suggests that pUS3 anti-apoptotic function could be crucial *in vivo* but dispensable in cultured cells.

PKC. The protein kinase C (PKC) family is composed of ten isoforms encoded by nine genes of cellular serine threeonine kinases (review (220)). Although yeast only have one PKC isoform, PKCs are conserved from humans to yeast and share sequence and functionally similarity with other AGC (c<u>A</u>MP-dependent, c<u>G</u>MP-dependent, and protein kinase <u>C</u>) kinases. AGC kinases are kinases that have a catalytic core that is allosterically regulated by another domain in the same molecule. Other AGC kinases include protein kinase A (PKA) and PKB/AKT and like PKC, they also have regulatory and catalytic domains. PKCs are divided into three subclasses based on activation or

second messenger requirements. The conventional isoforms (cPKCs) include alpha, beta I, beta II, and gamma and require Ca^{+2} plus diacylglycerol (DAG) for activation. Novel isoforms (nPKCs) include delta, epsilon, eta, and theta, however, only require DAG. Atypical PKCs (aPKC) include iota and lambda. All PKC isoforms contain a pseudosubstrate sequence, which in the absence of stimuli, is *intra*molecularly bound by the catalytic subunit. Upon stimulation such as binding of a growth factor to a growth factor receptor, DAG is released from the hydrolysis of PIP2. Activation of PKC is thought to follow a two-step process. First, the increased DAG concentration at membranes in the stimulated cell attracts C1 and increases C1 affinity for membranes. Interaction of C1 with the acidic membrane lipids generates the energy necessary for expulsion of the pseudosubstrate and complete activation of the kinase (review(220)).

PKC alpha. PKC alpha is a conventional PKC isoform, which requires Ca^{+2} and DAG for activation (220). PKC alpha promotes the cell cycle progression by up-regulating cdk1 inhibitors. This isoforms also regulates cell survival though paradoxical pro- and anti- apoptotic signals (220). Ro-31-7549 is a small molecule inhibitor often used to inhibit PKC alpha, along with all the other conventional PKCs (289) (Figure 1.6).

PKC delta. PKC delta is a novel PKC (nPKC) isoform only requiring DAG for activation (220). It can be knocked out of mice but results in autoimmune disorders (222). PKC delta has been implicated in many cellular functions such as, apoptosis, DNA damage, differentiation, migration, and development (56, 57). Its mis-regulation is implicated in diabetes and cancer.

PKCs and herpesvirus associated lamina disruption. MCMV infection resulted in conventional PKC (cPKC) isoform recruitment to the nuclear rim and lamin A/C phosphorylation (191). cPKCs colocalized with MCMV envelopment complex proteins p52/p38 and M50/p35 (191). This was blocked by treatment with Ro-31-7549, which inhibits cPKCs including PKC alpha (191). The cellular kinases PKC delta and alpha, but not the aPKC, PKC zeta, were recruited to the nuclear rim in a pUL34-dependent

manner during HSV-1 infection (207). pUL34/pUL31 association was required but pUS3 was not (207). Rottlerin treatment of HEp-2 HSV-1 infected cells lead to a decrease in lamin B phosphorylation suggesting that PKC delta was involved in lamin B phosphorylation for lamina disruption (207). HCMV pUL50 interacted with PKC alpha and co-expression with pUL53 resulted in PKC alpha recruitment to the nuclear rim (180). Together these data suggest that a conserved mechanism *Herpesviridae* lamina disruption might be mediated by the cellular PKC kinases. This thesis addresses the many unanswered questions concerning the mechanism of lamina disruption by viral and cellular kinases.

<div align="center">Kinase inhibitors</div>

BIM I. Bisindolylmaleimide I (BIM I) or GF-109203X was introduced as a PKC kinase inhibitor in 1991 (272). This was generated to be an alterative to stauroporine (Figure 1.6 A). BIM I is used to inhibit the conventional PKC isoforms at 1 uM or the entire PKC family at 10 uM. BIM I competes with ATP to bind to the ATP binding site to exert competitive inhibition (272). It has been previously observed that BIM I can inhibit cellular protein translation, potentially by inhibiting the ribosomal S6 protein kinase (RSK2) and the p70 ribosomal S6 protein kinase (S6K) (283). Although BIM I was originally designed to solve the problems of the off target effects of staurosporine, another PKC inhibitor with off target effects, it may have created more.

Ro-31-7549. After determining that BIM I was able to inhibit the function of PKCs, a series of bisindolylmaleimide compounds with differing side chains were evaluated for their PKC activity inhibitory functions. It was determined that BIM VIII (Ro-31-7549) (Figure 1.6 B) could inhibit conventional PKCs, including PKC alpha, but was a poor inhibitor of PKC epsilon, a novel PKC (289). Subsequently, Ro-31-7549 is often used to inhibit cPKC isoforms at 1 uM (191, 201, 273, 289).

Rottlerin. Rottlerin is marketed as a specific PKC delta inhibitor because at 10 uM it does not inhibit other PKC isoforms (Figure 1.6) (94). It does, however, inhibit other cellular kinases such as calmodulin kinase II, AKT, PKA, and GSK3 beta (253). Rottlerin is thought to compete with ATP for binding to the ATP binding site to exert competitive inhibition (94). It has been previously observed that Rottlerin treatment of HEK293 cells caused the inactivation of the S6 kinase (S6K), eEF2 kinase, and the eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1) protein resulting in protein translation inhibition (93, 187, 253, 283). Rottlerin can also induce autophagy in a PKC delta-independent manner (256). Taken together with other data presented in this thesis, Rottlerin treatment of intact cells inhibits cellular and viral protein translation and its use in intact cells should be avoided.

<u>Thesis objectives</u>

1. To determine the role of the envelopment complex in LAP (emerin and LAP2) phosphorylation.

2. To determine the role of pUS3 and PKC family in lamina disruption and if this disruption is essential for herpes virus growth.

3. To elucidate the molecular mechanism of the US3-null virus growth defect in BT-549 cells.

Table 1.1. Herpesviruses and public health implications. *Herpesviridae* is divided into three subfamilies based on tropism, length of replication cycle, and the ability to replicate in tissue culture. Alpha-herpesviruses have a shorter replication cycle than the other two sub-families. This is an incomplete list of all known herpesviruses but rather describes the prevalence of common herpesviruses in humans.

Subfamily		Seroprevalence	Common symptoms	Ref.
Alpha-	HSV-1	80%	Oral lesions, encephalitis, keratisis	1
	HSV-2	25-80%	Genital lesions, encephalitis, neonatal disseminated infections	1, 2
	VZV	90%	Chicken pox, shingles	1
Beta-	HCMV	70-90%	Mononucleosis, infant deafness and retardation	1, 2
Gamma-	EBV	90%	Mononucleosis, B-cell lymphoproliferative diseases, Burkett's lymphoma	2, 3
	KSHV or HHV-8	5-50%	Kaposi's sarcoma	2, 3

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Figure 1.1. Diagram of a herpesvirus. The dsDNA genome is packaged inside the icosahedral capsid. The HSV-1 capsid is composed of six different viral proteins (purple). Surrounding the capsid is an amorphous structure of viral and cellular proteins called the tegument (yellow). Encasing the tegument and the capsid, is the lipid bilayer or envelope (dark blue) which contains the viral glycoproteins (light blue).

Glycoproteins

Lipid bilayer

Tegument
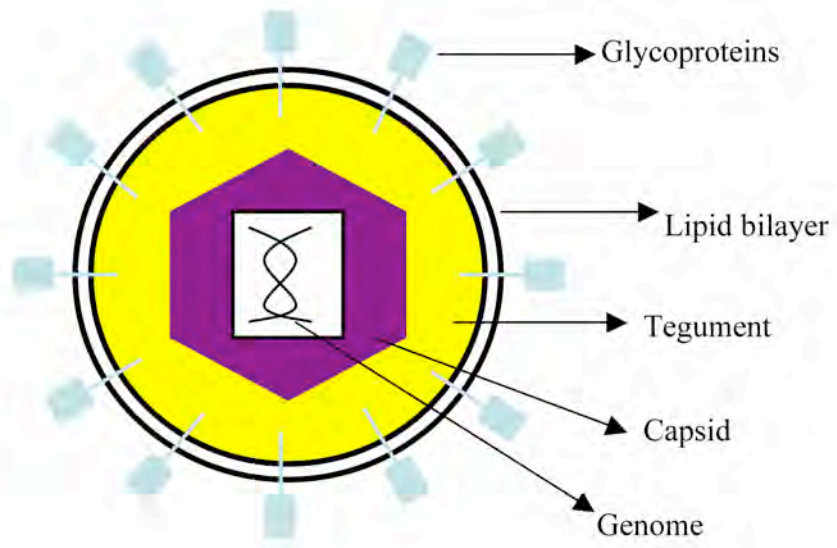
Capsid

Genome

Figure 1.2. Model of nuclear egress via envelopment at the inner nuclear membrane. Shown is a schematic of nuclear egress. 1) Empty, or capsids with out DNA, are rarely observed outside the nucleus. 2) Once genomes are packaged into assembled capsids they are complete or C capsids. 3) These capsids are trafficked to the INM via an unknown mechanism, where in a pUL34/pUL31 dependent manner, they undergo an envelopment event at the INM. 4) The capsid is enveloped and part of the peri-nuclear space. 5) The envelope (lipids from the INM) of the peri-nuclear viron then fuses with the ONM. 6) An un-enveloped or naked capsid is released in the cytoplasm.

ONM

INM

6

5

4

3

2

1
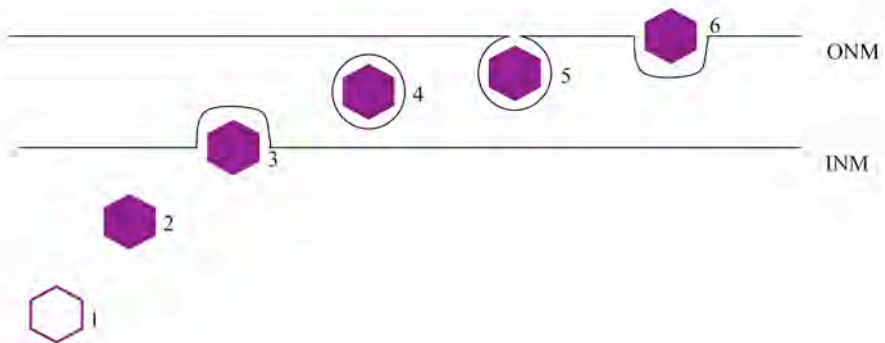
Figure 1.3. Cross-sectional view of the nuclear lamina. The ONM is shown in black. The INM in red. The NPCs are represented in blue are important in nuclear transport. The perinuclear space is shown in gray and the nuclear lamina in teal. A HSV-1 capsid (purple) is modeled inside the nucleus. This demonstrates the problem facing newly formed herpesviruses and any virus formed inside the nucleus. A herpesvirus capsid is between 120-200 nm in diameter while the holes in the lamina meshwork are 50-80 nm in diameter. The NPC can transport small molecules but is limited to things smaller than 70 nm.
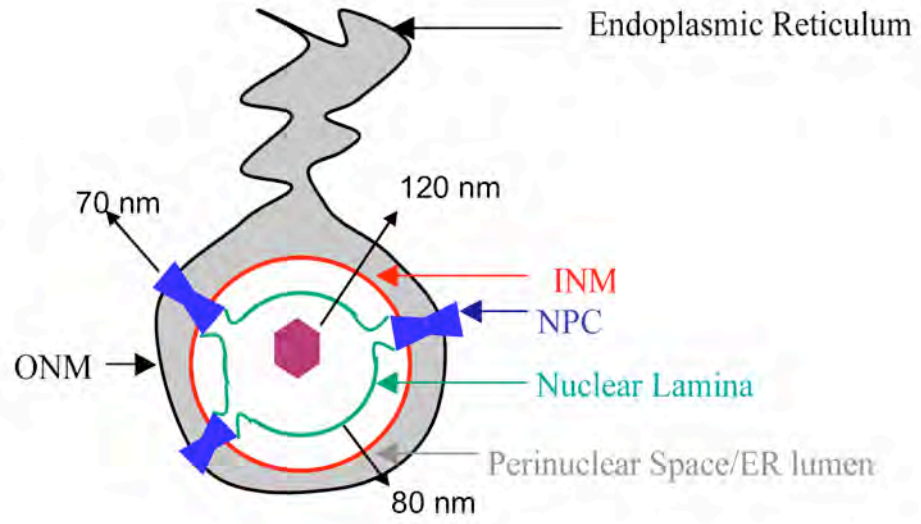
Figure 1.4. Diagram of the nuclear lamina components, side view. LAP2 isoforms are shown in red. LAP2beta has a TM domain, which anchors it at the INM. LBR is shown in orange with eight TM domains and both the N- and C-terminal domains in the nucleoplasm. MAN1 (green), an emerin-binding partner, has two TM domains and like LBR both the N- and C-terminal domains are in the nucleoplasm. Emerin (light blue) has one TM domain, which retains it at the INM. It interacts with BAF (purple cross) via the N-terminal LEM domain and emerin's C-terminal domain is in the peri-nuclear space. Emerin, as well as other LAPs, bind the lamin meshwork (black lines). BAF (purple) can bind non-specifically to DNA anchoring chromatin to the nuclear periphery (black hashed line). Sun (bright blue) proteins interact with nesprins (yellow) via a KASH domain in the peri-nuclear space to regulate the spacing of the nuclear membranes.

Cytoplasm

ER lumen

Nesprins

ONM

NPC

INM

Emerin

Lamins:
A, B, C

BAF

Emerin   MAN1

LBR

LAP2beta

Sun

LAP2 alpha

Nucleoplasm

Chromatin
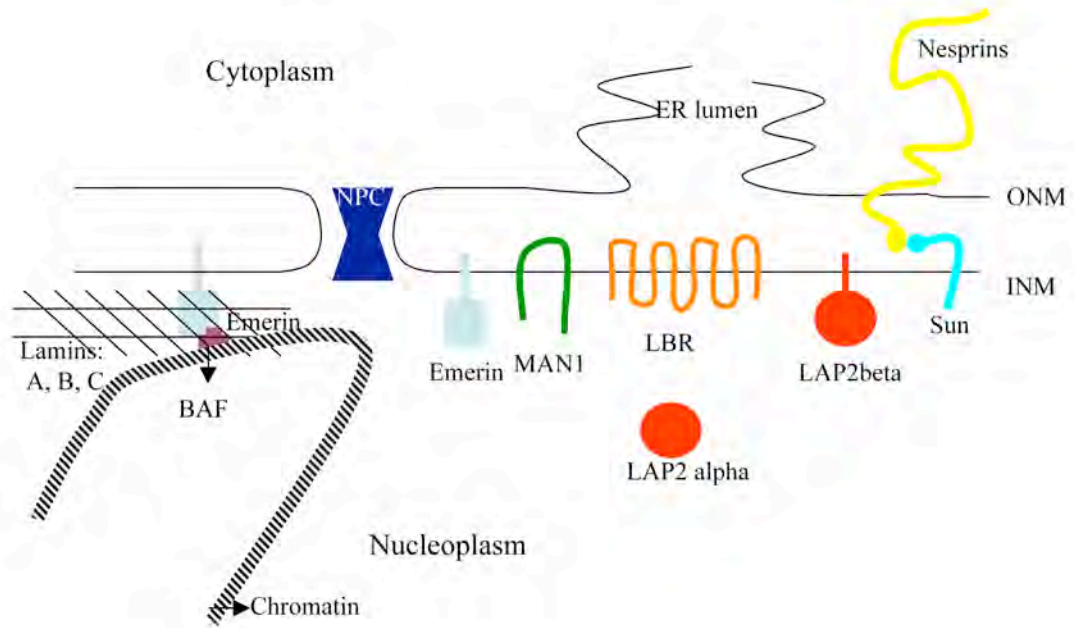
Figure 1.5. Diagram of LAP2beta and emerin. A) The N-terminus is common to all isoforms of LAP2 and can bind DNA. LAP2 proteins bind BAF through the LEM domain (AA 111-152) is which is common to all lamin associated proteins. BAF functions as a trimer but is drawn as a monomer. The N-terminus of HA95, a chromatin-associated protein, binds LAP2 beta at residues 137-242 and while the C-terminus binds within the lamin B binding domain (AA 299-373). Germ-cell-less (GCL) is a binding partner of LAP2beta (binds AA 219-328). LAP2 beta contains a TM domain in its C-terminus. **B)** Emerin is also an integral membrane protein with a TM domain and a LEM domain that interacts with BAF. There are two serine/theronine rich regions that flank the lamin A/C binding domains in emerin. Lamin A/C is drawn to represent its rod and tail domains. Emerin binds the tail of lamin A (AA 384-566). Emerin binds to transcriptional regulating proteins GCL and Btf. Emerin also associates with splicing regulatory factor YT521-B and the viral protein pUL34. Emerin is predicted to have a structural role though interactions with actin, nesprin, and MAN1. The LEM domain of emerin is also thought to interact with trimers of BAF.

A.   LAP2beta 454 a.a.

TM

Lamin B-binding domain

GCL

HA95

BAF   LEM

DNA-binding domain

B.   EMERIN - 254 a.a.

TM

S/T-rich

Lamin-binding domain

GCL
Btf
YT521-B
Actin
MAN1
LUMA
Nesprin
UL34

Lamin A/C

S/T-rich   US3 consensus

LEM

BAF
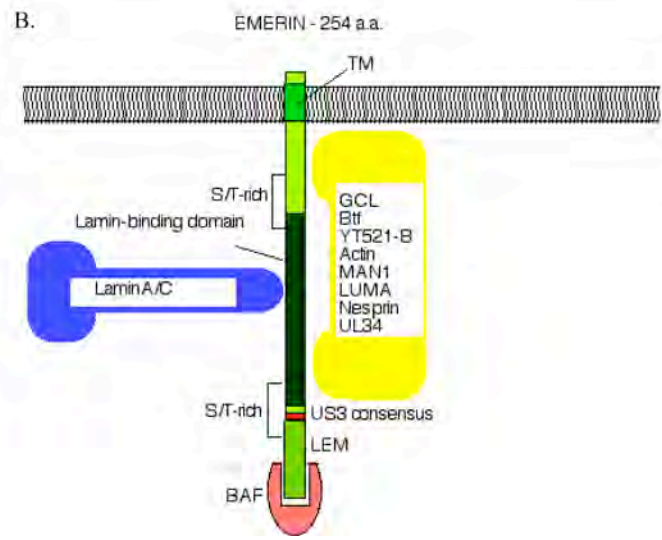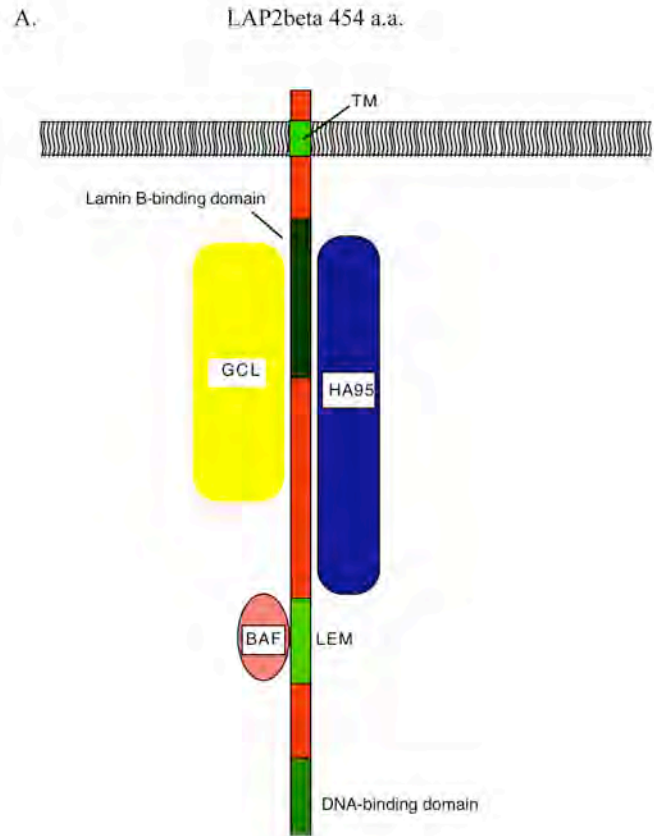
Figure 1.6. Schematic of lamin proteins. **A)** A-type lamins include lamin A and C, which are alternatively spliced transcripts from the same gene while the two B-types are transcribed from two separate genes. A and B -type lamins are farnesylated at a CaaX motif but this portion of A-type lamin proteins is post-translationally cleaved. **B)** All lamins have three domains: head, rod, and tail. One important function of the domains is in the formation of dimers (hetero- or homo-) and interactions with other proteins such as LAPs. **C)** The lamin dimers are assembled into filaments that assemble into the meshwork that surrounds the nucleus.

A.
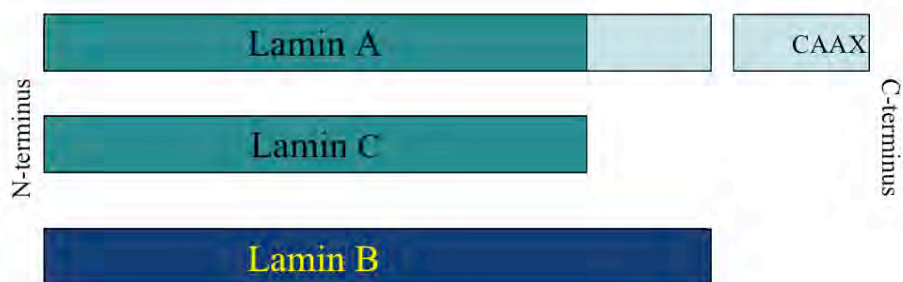
B.
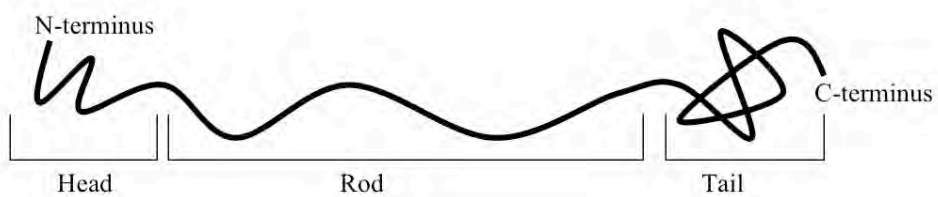
C.
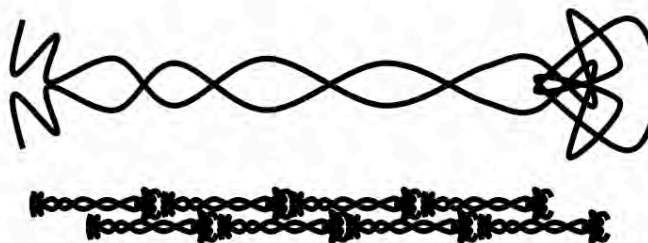
Figure 1.7. Chemical structure comparison of PKC inhibitors. A)
Bisindolylmaleimide I or BIM I was introduced as a specific PKC protein kinase
inhibitor. **B)** Variations of the BIM I side chain produced BIM VIII as a compound that
had preference for inhibition of cPKCs. **C)** Rottlerin was developed as a specific PKC
delta inhibitor as it does not inhibit the kinase activity of other PKC isoforms. This
compound does inhibit other non-PKC cellular kinases.

A.
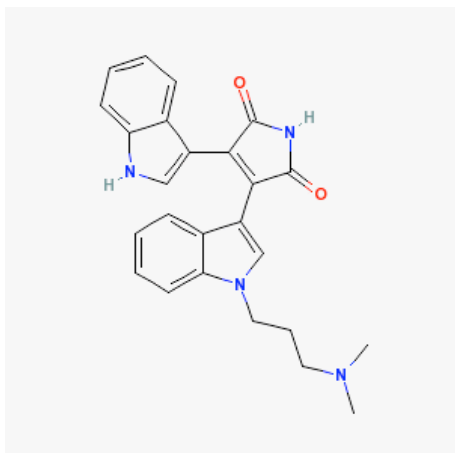
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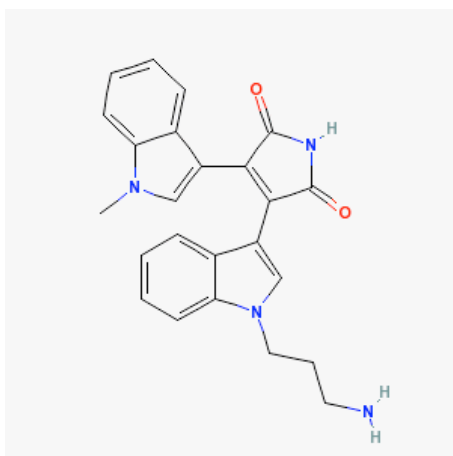
C.
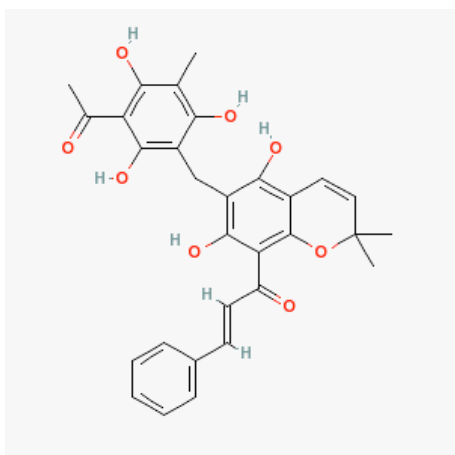
CHAPTER II

CHANGES IN POST-TRANSLATION MODIFICATION

OF THE LAMIN ASSOCIATED PROTEINS EMERIN

AND LAP2 DURING HERPES SIMPLEX TYPE 1

INFECTION

Abstract

The nuclear lamina consists of a mesh-like network of lamin proteins anchored to the inner nuclear membrane by interactions with integral membrane proteins such as emerin. Emerin is a member of the LEM domain family, members of which interact with BAF. LEM domain protein-BAF interactions create a bridge between the INM and chromatin, which aids in reassembly of the nuclear envelope after mitosis. Emerin binding to lamin A/C is one of the interactions that connect the INM to the lamina. Infection by herpesviruses results in changes in the organization of the nuclear lamina, perhaps in order to facilitate envelopment of capsids at the inner nuclear membrane. In HSV-1 infected cells, alterations to the lamin proteins have been shown to involve pUL34, pUL31, and pUS3 proteins, which are also required for normal nuclear envelopment. This chapter presents the following data. (i) Infection of multiple cell types induces emerin hyper-phosphorylation that is dependent on the presence of pUL34 and kinase active pUS3 protein. (ii) The pUL34 dependent component is also sensitive to Rottlerin treatment suggesting that PKC delta or RttSK might be involved in emerin modification. (iii) LAP2 is de-modified in a pUS3 and pUL34 independent manner during infection. (iv) Emerin is not required for growth of WT or US3-null virus.

Introduction

All herpesviruses assemble newly formed capsids in the nucleus of the host cell. To escape the nucleus, the capsids must traverse the inner and outer nuclear membranes via an envelopment/de-envelopment process at the NE to release un-enveloped capsids into the cytoplasm (178). Access to the INM however, is obstructed by the nuclear lamina (1, 92, 291). The periphery of the nucleus is organized around a fibrous mesh called the nuclear lamina that lies underneath the inner nuclear membrane (reviewed in (92). The lamina is composed of a meshwork of intermediate filaments related proteins called lamins. These lamins are linked to the INM via interactions with lamin associated proteins (LAPs). LAPs such as emerin, LAP2 beta, and lamin B receptor, are integral membrane proteins of the INM. This meshwork of proteins provides stability as well as signal integration to the nucleus (92).

Infection with wild-type herpesviruses results in changes in nuclear architecture consistent with disruption of the nuclear lamina. These changes include enlargement of the nucleus as demonstrated for HSV-1 (19, 249). The nucleus changes shape from smooth-ovoid to raisin-like in contour in HSV and HCMV infected cells (19, 98, 248, 249). Both A- and B- type lamin proteins change localization upon infection with HSV-1 and HSV-2. The lamin protein localization changes from a smooth, even lining of the INM to an uneven distribution showing gross thickening of the lamin layer at some sites and small perforations in the layer at other sites (19, 35, 36, 98, 145, 223, 248, 249). HSV-1 infection induces masking and unmasking of monoclonal antibody epitopes on the lamin proteins that indicate a change in the conformation or associations of the lamin proteins (223). Herpes simplex infections cause redistribution of LAPs including LBR, LAP2beta, and

emerin (20, 186, 242, 248). All of these data suggest that disruption of lamina components is a conserved feature of the herpesvirus lifecycle.

Division of the host-cell during mitosis requires disruption of the nuclear lamina (reviewed in (169) mediated by phosphorylation of lamins and LAPs by cellular kinases including Cdk1, PKC, MAPK, PKA, casein kinase II, and AKT (91, 92, 169). A growing body of evidence suggests that herpesviruses adapt this mechanism and induce phosphorylation of nuclear lamina components during infection. HSV-1, HSV-2 and HCMV infections induce phosphorylation of all three types of lamins (36, 170, 188, 190, 207)). MCMV infections induce phosphorylation of at least lamin A/C (145, 191).

Phosphorylation of lamina components during infection is mediated by both viral and cellular kinases. Herpesviruses encode a conserved protein kinase called CHPK (Conserved Herpesvirus Protein Kinase), which shows some similarities to Cdk1 in function and substrate specificity (115, 121). Involvement of the beta-and gamma-herpesvirus CHPKs in nuclear lamina disruption and nuclear egress has been established (135, 145). Alpha-herpesviruses encode a second serine/threonine protein kinase designated pUS3 in HSV. pUS3 regulates the degree of lamina disruption (20, 190).

During HSV-1, MCMV, and HCMV infections, PKC isoforms are recruited to the nuclear rim by viral proteins that are required for lamina disruption, suggesting that PKC activity may contribute to lamina-disrupting phosphorylation events (191, 207). There are ten PKC isoforms divided into three groups that differ in their activation mechanisms and all may be involved in herpesvirus-mediated lamina disruption. Conventional PKC isoforms (cPKCs), such as protein kinase c alpha (PKC alpha), require an efflux of calcium and DAG for activation. Novel PKC (nPKC) family members, such as PKC delta (encoded by the *PRKCD* gene), are activated by diacylglycerol (DAG) in a calcium-independent manner. Atypical PKCs

(aPKC) do not require either for activation (221). Both PKC alpha and PKC delta are recruited to the nuclear rim upon HSV-1 infection, and treatment with Rottlerin, a widely used putative PKC delta inhibitor, blocks lamin B phosphorylation (207). Cdk1, also known as cdc2, has also been implicated in HSV-2 lamina disruption via its potential role in emerin phosphorylation (186).

pUL34, pUL31, and pUS3 are part of the envelopment complex in HSV-1 (224). pUL34 and pUL31 are conserved across *Herpesviridae* (78, 89, 238). All envelopment complex proteins co-localize at the INM in infected cells, and each is necessary for normal localization of the other (137). pUL34 and pUL31, however, are sufficient to target each other to the INM in the absence other viral proteins (155, 297). pUL31 and pUL34 and their homologs are necessary for efficient nuclear egress (156, 225, 232). pUS3 is not required for primary envelopment, however in the absence of pUS3 efficient de-envelopment of the perinuclear particle is reduced and the overall rate of nuclear egress is decreased (236). pUS3 is also key in protecting the infected cell from apoptosis. (8, 153, 266)

Recruitment of PKCs to the nuclear rim in herpesvirus infections requires expression of the conserved proteins of the virus nuclear egress complex. In HSV, these proteins are called pUL31 and pUL34, and they form a complex that is required for events in lamina disruption including redistribution of lamin proteins, masking and unmasking of lamin epitopes during infection and full hyperphosphorylation and redistribution of emerin (223). In HSV-1 infection, recruitment of both PKC alpha and delta depends on pUL34 expression (207). In MCMV, M50/p38, the pUL34 homolog, is required to recruit cPKCs to the nuclear rim (191).

Emerin is an integral membrane protein containing a TM domain and a LEM domain that interacts with BAF. There are two serine/theronine rich regions that flank the lamin A/C binding domains. Emerin has many known binding-partners

including: transcriptional regulating proteins germ-cell-less (GCL) and Btf, splicing regulatory factor YT521-B, actin, nesprin, and MAN1 (99, 107, 166, 288).

LAP2 is another LEM domain protein (Figure 1.4 A). It is alternatively spliced to produce multiple isoforms (17). The N-terminus is common to all isoforms of LAP2 and can bind DNA (33, 54, 80, 81, 146, 277). LAP2 proteins bind BAF through the LEM domain (33, 246). The N-terminus of HA95, a chromatin-associated protein binds LAP2beta and while the C-terminus binds within its lamin B binding domain (171, 172). GCL, transcription factor that binds emerin, is also a binding partner of LAP2beta (107, 125). LAP2beta contains a TM domain in its C-terminus (17). LAP2beta is important in regulating gene expression by: interaction with the N-terminus of HA95 to regulate transcription initiation by blocking the degradation of Cdc6, inhibits the transcription activity of E2F, and interactions with HDAC3 that are predicted to cause transcriptional repression (59, 172, 173, 204, 254). It also has am important role in chromatin structure and organization by binding to BAF (87, 92, 246). LAP2beta is also important in nuclear envelope breakdown and reassembly during mitosis due to reversible phosphorylation-dependent binding to lamin B (53). LAP2alpha is a non-integral membrane protein but has a nuclear localization signal (Figure 1.6) (193, 204). LAP2alpha also is involved in regulating gene expression blocking cell cycle progression and inhibiting expression of E2F/Rb-dependent target genes (59, 193). It is possible that the virus may interact with different LAPs in different way due to their different functions and localizations.

This chapter presents data that suggests that HSV-1 induced emerin phosphorylation is dependent upon both pUS3 kinase activity and pUL34 expression, in multiple cell types. The pUL34-dependent component of emerin hyperphosphorylation was not sensitive to inhibition by Rottlerin suggesting that PKC delta might mediate the cellular component of emerin hyperphosphorylation

(142). In contrast to emerin, LAP2 was not modified upon infection, but rather de-modified. Due to the shift in mobility in SDS-PAGE and the type of protein the post-translational modification observed is likely de-phosphorylation but this will have to be confirmed with future experiments. This de-modification was not dependent on expression of pUL34 or pUS3. This was interesting since LAP2beta was redistributed to lamin A/C containing areas during infection (Bjerke, unpublished). Emerin was not required for efficient replication of WT or US3-null viruses.

Materials and Methods

Cells and viruses. Parental (MEF+/y) and emerin-null (EM-/y) mouse embryonic fibroblasts were kindly provided by Colin Stewart (138) and maintained in Dulbecco's modified Eagles' medium supplemented with in 10% fetal calf serum. HEp-2 and Vero cells were maintained as previously described (232). SNB19 cells were kindly provided by Wendy Maury and maintained in Dulbecco's modified Eagles' medium supplemented with in 10% fetal calf serum. The properties of HSV-1(F), vRR1072(tk+) (UL34-null mutant virus), vRR1202 (US3-null virus), vRR1204 (US3 kinase-dead virus K220A), and repair viruses for vRR1072(tk+) (vRR1072Rep) and vRR1202 (vRR1202Rep) were previously described and characterized.

Chemicals. Rottlerin (Santa Cruz Biotechnology) was diluted in DMSO to a stock concentration of 10 mM and used to treat cells at 10 uM. Ro-31-7539 (Calbiochem) was diluted in DMSO to a stock concentration of 1 mM and used to treat cells at 1 uM. Protease inhibitors were used in all steps of lamina preparations (Sigma).

Plasmids and cell lines. The enhanced green fluorescent protein (EGFP)-emerin fusion protein was provided by Y. Hiraoka. To create the Strep-His-EGFP-emerin plasmid, PCR amplification of EGFP from the EGFP-emerin plasmid from Y. Hiraoka with the following primers: 5'

CACAGCTAGCCATGGCGTGGAGCCACCCCCAGTTCGAAAAGGCGCACCAT
CACCATCACCATCACCATATGGTGAGCAAGGGCGAGGA 3' and 5'
AGTTGTCCATGGATCTGAGTCCGGA 3'. The 0.8kb PCR product and EGPF-
emerin-C1 were digested with Nhe1 and BspE1 and the PCR product was ligated into
the 4.6kb product of the EGFP-emerin-C1 digestion to create pRR1315. To create
the retroviral expression plasmid for Strep-His-GFP-emerin-C1, Strep-His-EGFP-
emerin was PCR amplified from pRR1315 using the following primers: 5'-
CACAGTTAACGCTAGCCATGGCGTGGAGC-3' and 5'-
CACAGCTCGAGATCCTAGAAGGGGTTGC-3'. This 1.6kb PCR product and
5.9kb pLXSN vector were digested with Xho1 and Hpa1 and ligated to create
pRR1319. pLXSN was kindly provided by A. Klingelhutz. The University of Iowa
Vector Core used pRR1319 to generate a MoMLV pseudotyped retroviral
transduction particle. These particles were used to transduce HEp-2 and Vero cells.
48 hours (hr) post transduction, cells were placed under G-418 selection for two
weeks. Colonies were then scraped from the plate, transferred to individual cultures,
and amplified to create the HEp-2 and Vero Strep-His-EFGP-emerin cell lines. HEp-
2 stable cell lines include: A4, B2, C4, D2, and D5. Vero stable lines include: F, G,
H, I, K, L, and M. All cell lines were tested for proper emerin localization.

     Preparation of nuclear fractions. Nuclear-lamina fractions were prepared
similar to the procedure described by Krohne with a few modifications (133).
Confluent 100 mm (1.2 x10^7 cells) cultures of HEp-2, Vero, or SNB19 cells were
infected with five PFU (PFU)/cell for 16 hr. The infected cells were washed twice
with PBS, then scraped into 5 mL of PBS, and pelleted at low speed. A clinical
centrifuge was used to pellet the cells by spinning at 600 rpm for 10 minutes (min).
The cells were then resuspended in 5 mL of 10 mM Tris pH 7.5 with protease
inhibitors. After 10 min on ice, cells were lysed with 10 strokes of a Dounce
homogenizer. The nuclei were separated from the cytoplasm by spinning at 3000 x g

for 10 min. The nuclear fraction was then resuspended in DNase/RNase Buffer I (10

mM Tris pH 8.5, 2 mM $MgCl_2$, 50 U/mL DNase I, 50 mg/mL RNase, and protease

inhibitors) and incubated at room temperature with intermittent agitation for 20-40

min. The nuclear fraction was then pelleted at 3000 x g again. Washed once by

resuspension in 2 mL of 10 mM Tris pH 7.5 with protease inhibitors followed by a 10

min 3000 x g spin. The nuclei were then resuspended in 2 mL of DNase/RNase

Buffer II (10 mM Tris pH 7.5, 2 mM $MgCl_2$, 50 U/mL DNase I, 50 mg/mL RNase,

and protease inhibitors) and incubated at room temperature with intermittent agitation

for 20-40 min. The nuclear fraction was then pelleted at 3000 x g again. To extract

soluble proteins, the pellet was resuspended in high-salt extraction buffer (10 mM

Tris pH 7.5, 500 mM NaCl, 1 mM dithiotheitol, and protease inhibitors) and left on

ice for 10 min. The extracted nuclear lamina proteins were pelleted by spinning at

10,000 x g for 10 min and resuspended in SDS-PAGE buffer.

Western blots. Nitrocellulose sheets bearing proteins of interest were

blocked with 10% non-fat milk T-TBS (0.2% Tween-20, Tris buffered saline) for at

least one hr. The membrane was then incubated with mouse monoclonal anti-emerin

antibody (1:500, Lab Vision Corp) or LAP2 (1:1000) diluted in 3% gelatin-TBS for at

least one hour. After three five min washes with T-TBS, the membrane was then

incubated with anti-mouse secondary AP conjugated antibody diluted in 3% gelatin –

TBS for 30 min (Sigma, mouse A3562).

Virus Growth. Single-step growth analysis was performed as previously

described (225, 232). Briefly, replicate cultures of cells were infected at an MOI of

five, and residual virus was removed or inactivated with a low-pH-buffer wash. Cells

were untreated or treated with DMSO, Rottlerin (10 uM) or Ro-31-7539 (1 uM) at

five hpi. At the indicated times, virus was harvested and total culture PFU were

calculated by titration on Vero cells.

Inhibitor toxicity assays. 2×10^5 HEp-2, Vero, MEF, EM-/y, or SNB19 cells were plated in a 48 well dish in triplicate. 12 hr later, cells were treated with vehicle (DMSO), 10 uM Rottlerin, 10 uM BIM I, and 1uM Ro-31-3549. 24 hr post-treatment, the ATPLite assay (Perkin-Elmer, #6016739) was followed per manufactures directions. This was repeated three independent times to determine that at the indicated dosage, the kinase inhibitors did not induce significant toxicity compared to vehicle (DMSO). Since the ATPLite assay measures the amount of ATP in the cell, this assay also controls for any reduction in the amount of PO_4 for phosphorylation of proteins. This could be an issue in Rottlerin treated cells where mitochondrial uncoupling had been previously observed (253). MEF and EM-/y cells did not tolerate Rottlerin treatment, thus were not used in any inhibitor studies, however Vero, SNB19, and HEp-2 cells did tolerate treatment. ATPLite data was not included since at the concentrations used in these experiments, no toxicity was observed (data not shown).

Confocal Microscopy. Briefly, cells were fixed with 4% formaldehyde for 10 min, washed with phosphate-buffered saline (PBS), and then permeabilized and blocked in the same step with IF buffer (0.5% Triton X-100, 0.5% Sodium Deoxycholate, 1% BSA, 0.05% Sodium Azide diluted in PBS). Slo-fade II (Molecular Probes) was used to mount cover slips on glass slides. All confocal microscopy work was done with a Zeiss 510 microscope.

<div align="center">Results</div>

To test the hypothesis that emerin is hyperphosphorylated during HSV-1(F) infection in both Vero and HEp-2 cells, lamina fractions from infected and uninfected cells were treated with calf intestinal alkaline phosphatase (CIAP). The untreated, infected samples show many slowly migrating emerin species that become one species upon CIAP treatment (Roller data, (142). This demonstrated that

phosphorylation was responsible for the multiple, slower-migrating species of emerin at 16 hr post infection (hpi). To determine when in the course of infection emerin becomes hyperphosphorylated, Vero cells were infected with five PFU/cell of HSV-1(F) for four, eight, and twelve hr or left uninfected. The cells were then fractioned and the lamina fraction was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for emerin (Figure 2.1 A). Emerin modification appears to occur in two distinct phases. The first phase was apparent at four hpi when two species of emerin are easily discernable (compare lane 2 to 1). The phase of limited modification continues to at least eight hpi (lane 4). The second phase was apparent by twelve hpi where extensive hyperphosphorylation was present (lane 6).

Emerin hyperphosphorylation requires the envelopment complex. Two separate kinases have been implicated in herpesvirus mediated lamina disruption: pUS3 and PKC. One untested function of the envelopment complex (pUL34, pUL31, and pUS3) may be to phosphorylate directly (pUS3) or recruit a kinase(s) to phosphorylate emerin. pUL34 has been shown to recruit PKC isoforms to the nuclear envelope presumably to phosphorylate lamina components (207). To test the hypothesis that HSV-1(F) envelopment complex proteins, pUL34 and pUS3, are required for phosphorylation of emerin, lamina fractions were prepared from uninfected Vero (and HEp-2) cells and cells infected with five PFU/cell of wild-type HSV-1(F) and recombinant mutant viruses that fail to express pUS3 (vRR1202) or pUL34 (vRR1072(TK+)) at 16 hpi. The lamina fractions were separated by SDS-PAGE gel, transferred to nitrocellulose, and then immunoblotted with a monoclonal antibody to emerin (Figure 2.1B). There are many species of emerin from HSV-1(F) infected cells while only one from mock-infected lamina fractions (Figure 2.1 B compare lanes 2,7 to lanes 1,6). Infection with the US3-null virus or the catalytically dead pUS3 (K220A) virus reduces but does not completely inhibit hyperphosphorylation of emerin (lanes 3 and 5). Similarly infection with UL34-null

virus results in the reduction of emerin hyper-phosphorylation (lane 8). Repair of the US3 or UL34 genes results in wild-type levels of emerin hyper-phosphorylation (lanes 4 and 9). Note that the phosphorylation patterns seen in US3-null and UL34-null infected cells are different (compare lanes 3 and 8). These results suggest that there are both US3 and UL34 dependent components to emerin hyperphosphorylation.

Hyperphosphorylation of emerin in HEp-2 cells, while less extensive than in Vero cells, shows a similar dependence on pUS3 and pUL34 expression (Figure 2.1B HEp-2). Deletion of pUL34 or pUS3 or expression of catalytically dead pUS3 results in appearance of more rapidly-migrating emerin species than those seen in wild-type infected cells (compare lane 2 with 3, 5, and 6). However, neither results in emerin migration equivalent to that seen in uninfected cells (compare 1 with 3, 5, and 6).

Vero and HEp-2 cells are common tissue culture models of lytic epithelial replication (235, 240). HSV-1 and other herpesviruses also cause disease in neurons (12, 286). To determine if emerin hyperphosphorylation occurs in neuronal cells, SNB-19 cells were obtained. SNB-19s were derived from a glioblastoma (97). To test the hypothesis that HSV-1(F) envelopment complex proteins, pUL34 and pUS3, are required for phosphorylation of emerin, lamina fractions were prepared from uninfected SNB-19 cells and cells infected with five PFU/cell of wild-type HSV-1(F) and recombinant mutant viruses that fail to express pUS3 (vRR1202) or pUL34 (vRR1072(tk+)) at 16hpi. The lamina fractions were separated by SDS-PAGE gel, transferred to nitrocellulose, and then immunoblotted with monoclonal antibody to emerin (Figure 2.1 B SNB19). While also a human cell line, the phenotype of SNB-19 emerin modification appears to be a hybrid of HEp-2 and Vero. Infection with WT HSV-1(F) created two prominent bands (like HEp-2 cells) with many hyperphospho species (like Vero cells) while mock infected cells have one major

species (compare lanes 2 to 1). The requirement of pUS3 catalytic activity (lanes 3 and 5) and pUL34 did not change (lane 6).

LAP2 is de-modified by HSV-1 infection but independent of pUL34 and pUS3 expression. Phosphorylation is one mechanism of generating flexibility within the nuclear lamina. LAP2 is another LAP whose interactions within the nuclear envelope is regulated by phosphorylation (53). To test the hypothesis that HSV-1(F) envelopment complex proteins are required for phosphorylation of LAP2 alpha and beta, lamina fractions were prepared from uninfected HEp-2 (Figure 2.2 A) and Vero (Figure 2.2 B) cells and cells infected with five PFU/cell of wild-type HSV-1(F) and recombinant mutant viruses that fail to express pUS3 (vRR1202) or pUL34 (vRR1072(tk+)) at 16 hpi. The lamina fractions were separated by SDS-PAGE gel, transferred to nitrocellulose, and then immunoblotted with monoclonal antibody to LAP2 (Figure 2.2). Infection both Vero and HEp-2 cells with WT HSV-1(F) lead to a decrease in both LAP2 alpha and beta species compared to mock infection (compare lanes 2 with 1). LAP2 de-modification however, was not dependent on the expression of pUL34 (lane 6 and 7) or pUS3 (lanes 3, 5, and 4). These results suggest that LAP2 was de-modified upon infection but that (viral) proteins other than pUL34 and pUS3 are required for this yet uncharacterized post-translational modification.

Emerin phosphorylation may require PKC delta. pUL34 is known to recruit the PKC isoforms alpha and delta to the nuclear envelope during infection (207). The pUS3-independent emerin hyperphosphorylation events could be dependent on the activities of PKC alpha and/or delta. In order to test the hypothesis that PKC isoforms are responsible for pUS3 independent phosphorylation of emerin in infected cells, Vero and HEp-2 cells were infected and treated with vehicle (DMSO), Rottlerin, or Ro-31-7549 (BIM VIII) beginning at 5 hpi or 13 hpi respectively. Rottlerin is a small molecule inhibitor often used to inhibit PKC delta, since at the concentrations it inhibits delta activity, it does not inhibit other PKC

isoforms (94). Ro-31-7549 is a cPKC inhibitor and inhibits the kinase activity of PKC alpha, beta I, beta II, and gamma (289). Lamina fractions were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for emerin (Figure 2.3). These time points were chosen because they represented the greatest inhibition of phosphorylation (data not shown). Treatment of Vero cells with vehicle did not inhibit emerin hyper-phosphorylation induced by wild-type HSV-1(F) infection (Figure 2.3, compare lanes 2 and 11 to 1 and 10), nor did it change the reduction hyperphosphorylation of emerin seen in US3-null infected cells (lanes 3 and 12). Rottlerin treatment of HSV-1(F) or US3-null infected cells results in significant reduction but not elimination in the hyperphosphorylation of emerin (compare lanes 2 and 3 with lanes 5 and 6). Ro-31-7549 treatment of HSV-1(F) and US3-null infected cells resulted in a minor reduction in hyperphosphorylation of emerin (compare 11 and 12 to 14 and 15). This suggested that cPKCs contribute little to emerin modification. Treatment with both inhibitors of US3-null infected cells caused the complete inhibition of emerin hyperphosphorylation (compare lane 9 with 10). Treatment with both inhibitors of wild-type HSV-1(F) infected cells does not eliminate hyper-phosphorylation since emerin from these cells migrates more slowly than emerin from uninfected cells. This retardation in migration is presumably due to pUS3 phosphorylation of emerin. Similar effects are seen in inhibitor treated HEp-2 cells (Figure 2.3B).

pUL34 dependent emerin phosphorylation is also Rottlerin sensitive emerin phosphorylation. Emerin hyperphosphorylation appears to require pUL34 (Figure 2.1) yet pUL34 has not intrinsic kinase activity. Inhibition of cellular kinases (mainly PKC delta or "Rottlerin sensitive kinases") eliminated the pUS3-independent phosphorylation (Figure 2.3), yet it remained unknown if the pUL34-depedent and the Rottlerin sensitive component are the same. To test this hypothesis, Vero cells were mock infected or infected with five PFU/cell of WT, pUL34-null, or pUL34-

null-Repair virus. At five hpi, the cells began treatment with Rottlerin or vehicle (DMSO) until 16 hpi, when lamina fractions were prepared, separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for emerin (Figure 2.4). Un-infected and pUL34-null infected cells treated with Rottlerin had emerin hyperphosphorylation motilities that did not differ (compare lanes 5 and 6). The pUS3-dependent phosphorylation event was difficult to discern in this western for emerin due to the curve of the dye front, however this does not detract from the ability to conclude that the Rottlerin sensitive component was also the pUL34 sensitive component (Figure 2.4).

Emerin is not required for viral growth. Disruption of the lamina may be an essential step in nuclear egress. LAPs such as emerin have important functions in addition to their structural roles in the NE. It has also been demonstrated that emerin is a binding partner of pUL34 (142). To test the hypothesis that emerin was required for viral growth, parallel cultures of mouse embryonic fibroblasts (MEFs), derived from wild-type (MEF+/y) and emerin null mice (EM-/y), were infected with five PFU/cell of HSV-1(F) and production of infectivity was measured over time. When compared to parental cells, WT HSV-1(F) growth from EM-/y cells is not significantly different (Figure 2.5). This indicates that the expression of emerin was not essential for WT viral growth. To test the hypothesis that in the absence of emerin, the US3-null virus would have a larger growth defect due to pUS3 function in lamina disruption, the previous experiment was repeated with the US3-null and US3-null-Repair virus (20). No significant difference was observed in growth of any virus in the absence of emerin.

Purification of emerin and emerin-protein complexes with S-His-Emerin. Emerin hyperphosphorylation appeared to correlate with increased fluidity within the INM suggesting that emerin was no longer binding to some of its binding partners following infection (142). Data presented in Figures 2.1, 2.3, and 2.4 suggested that

emerin is hyperphosphorylated upon infection in a pUS3- and pUL34- dependent manner. The pUL34-dependent component is sensitive to Rottlerin treatment suggesting PKC delta is involved in phosphorylation of emerin. The residues on emerin that become phosphorylated however are not known, nor are the changes in interactions due to phosphorylation. To test which emerin residues are modified upon infection, tandem epitope tagged emerin fusion protein, S-His-EGFP-emerin was created (Figure 2.6 A). The S stands for the Strep tag, derived from the streptactin binding protein. This epitope is useful in protein purification due its properties of binding to streptactin coated beads and can be eluted by competitive binding with biotin. The 8X-His repeat allows for purification on nickel or cobalt resins while the EGFP moiety would allow for antibody purification of emerin or emerin-binding partner complexes (Figure 2.6B). HEp-2 and Vero cell lines were created that stably express SHis-emerin. To determine if the tagged emerin re-localizes as previously demonstrated, SHis-emerin expressing HEp-2 cells were mock or HSV-1(F) infected. At 16 hpi, SHis-EGFP-emerin localization was observed (Figure 2.6 C). In mock infected cells, the EGFP-emerin was at the NE with no or few wrinkles. All the cells in the field expressed similar levels and had similar localizations of SHis-EGFP-emerin (Figure 2.6 Ci). As previously demonstrated, infection with HSV-1(F) induces SHis-EGFP-emerin localization to a wrinkled, blebby distribution (Figure 2.6 Cii) (142).

Although many attempts were made to purify emerin and emerin-binding partner complexes, none were successful. The Strep- and His- tags appear to be not assessable in the native folding of the protein for binding to the resin. Even under denaturing conditions, SHis-EGFP-emerin purification is extremely inefficient. Additionally, a non-specific protein that binds cobalt resin co-migrates with the SHis-EGFP-emerin band in Commassee stained SDS-PAGE gels (Data not shown).

Discussion

Emerin phosphorylation can be linked to its function in many ways. In the uninfected cell, emerin is phosphorylated in a cell-cycle dependent manner. Although emerin phosphorylation is maximal at M phase, phosphorylation can occur at any time (70). These data suggest that emerin phosphorylation is responsible for its disconnection from the lamina during disassembly of the nuclear envelope prior to mitosis. In nuclei from patients with EDMD, emerin is aberrantly hyper-phosphorylated suggesting that this is one possible mechanism for emerin to contribute to EMDM pathology (67).

Emerin is hyperphosphorylated in HSV-1 infected cells as demonstrated by the decrease in emerin mobility in SDS-PAGE that is reversible by phosphatase treatment (Roller data, (142). Emerin modification was apparent as early as four hpi but dramatic hyperphosphorylation was not apparent until 12 hpi (Figure 2.1 A). It also seems clear that all of the emerin in the infected cell is modified (Figure 2.1 B). This is different from lamins, which are also phosphorylated during herpesvirus infections but only a small percentage of the total lamin content appears to be modified (34, 188, 190). Moreover, all of the emerin appeared to be phosphorylated and relocalized while only a small portion of the lamins are modified and relocalized during lamina disruption (20, 142). This would suggest the virus has developed multiple mechanisms for disrupting the different lamina components.

There are cell type differences in the degree of emerin hyperphosphorylation, although in each case all the emerin in each cell was modified and the same viral genes were required (Figure 2.1 B and C). Vero cells demonstrated extensive hyperphosphorylation of emerin with upwards of 20 emerin species in WT infected cells (Figure 2.1 B). Interestingly, in HEp-2 cells WT infection resulted in two or three emerin hyperphosphorylated species and the similar degree of emerin relocalization or disconnection from lamina binding-partners (Figure 2.1 B) (142).

Also, during mitosis, emerin is massively hyperphosphorylated, which was once considered necessary for emerin disconnection, but data from WT infected HEp-2 cells would suggest one or two *critical* phosphorylation events are sufficient to disconnect emerin (66, 67, 69, 70, 142). Together, these data suggest a hypothesis that a critical phosphorylation event is required to dislodge emerin from the lamina, not overall hyperphosphorylation levels. Our strategy with the S-His-emerin sought to discover those critical residues and the interactions disrupted by phosphorylation, however technical difficulties prevented success of this strategy.

Data presented in Chapter II demonstrated that pUL34 and pUS3 are essential in WT levels of emerin hyperphosphorylation during infection (Figure 2.1). It seems clear that both viral proteins contribute to emerin modification. We have not demonstrated that pUS3 directly phosphorylates emerin however, there is a pUS3 consensus sequence in emerin. This site, serine 49, has previously been demonstrated to be key in emerin dissociation from BAF (226). However, it remains to be determined if, pUS3 phosphorylation of serine 49 of emerin is an essential event in the disruption of the nuclear lamina prior to nuclear egress. It is possible that the pUL34-recruited kinase phosphorylates (i) the same site or (ii) a different site on emerin.

Envelopment complex proteins across *Herpesviridae* have been implicated in the recruitment of PKC isoforms to the nuclear envelope. The sensitivity of pUL34-depenent emerin phosphorylation to Rottlerin treatment suggests that a cellular kinase sensitive to Rottlerin is responsible for emerin phosphorylation. At its conception, Rottlerin was considered a specific inhibitor of PKC delta, however, new data suggests non-specific cellular kinases (non-PKC isoforms) are also inhibited by Rottlerin (253). Thus, we cannot conclude that PKC delta is cellular kinase recruited by pUL34 to phosphorylated emerin, but the suggestion is there. More work should

be done to determine which of the 53 emerin serine/theronine residues are modified upon infection and which kinases mediate those post-translational events.

We began these studies guided by the hypothesis that emerin as a component of the nuclear lamina, represented a problem for HSV-1 replication. Our results, however, are consistent with other interesting possibilities. The interaction of emerin with pUL34 and the co-localization of emerin with pUL34 in US3-null infected cells suggest the hypothesis that emerin, like pUL34, may also be part of the perinuclear viron (142). However unlike pUL34, emerin may not be an essential co-factor for virus assembly (Figure 2.5). It is possible that HSV-mediated hyperphosphorylation of emerin regulates emerin function in lamina, actin organization, and gene regulation.

LAP2 alpha and beta were both de-modified during WT HSV-1 infection, however, unlike emerin and lamins, this de-modification did not required the expression of pUL34 or pUS3 (Figure 2.2). While the exact post-translational modification is unknown, it is likely de-phosphorylation due to the size shift in SDS-PAGE and type of protein. Both LAP2 alpha and beta are reversibly phosphorylated during mitosis in a manner that regulates their association with lamins and chromosomes (53, 73, 275). This novel discovery that infection induces de-modification creates a third class of lamina disruption phenotypes. The third class is separated from emerin (the first) and lamins (the second) in that it is de-modified and its association with the lamina is actually enhanced upon infection (Figure 2.2 and Bjerke, unpublished). As described previously, LAPs, especially LAP2alpha, have non-structural functions such as regulating gene expression though pRB and controlling the cell cycle (193, 198). It is possible that herpesvirus have developed a mechanism of co-opting LAP2alpha function control of the cell cycle (193). This could also explain why the envelopment complex proteins were not involved in de-modification. In the future, the role of HSV-1 proteins such as ICP0 and ICP27

should be examined in LAP2 de-modification since they have previously been

implicated in arresting the infected cell-cycle at G1/S and G2/M (63, 64, 106, 162).

Table 2.1. List of mutant HSV-1 viruses used in this thesis. Listed in the table are the official mutant HSV-1 virus name and the corresponding genetic change. This list includes all viruses used in the entire thesis.

Virus Name	Genetic name
HSV-1(F)	WT No Change
vRR1072(TK+)	UL34-null
vRR1202	US3-null
vRR1204	US3-K220A-Kinase dead

Table 2.2. List of molecular weights of important proteins in this thesis.
This table includes the apparent molecular weight in SDS-PAGE of proteins
important to this thesis.

Protein	Molecular Weight
emerin	34 kD
PKC delta	78 kD
pUL34	34 kD
VP5	150 kD
US11	23 kD
actin	43 kD
LAP2alpha	80 kD
LAP2beta	55 kD

Figure 2.1. Post-translational modification of emerin in HSV mutants.

Digital images of western blots from lamina fractions of infected cells were immunoblotted with monoclonal antibodies to emerin. Cells were infected at an MOI of 5 for 16 hr. Lamina fractions were then isolated, run on SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with monoclonal antibody to emerin. A preliminary immunoblot for emerin from each lamina preparation was always performed to allow for equal emerin loading. Emerin is a highly phosphorylated protein due to the 53 serine and theronines. **A)** Vero cells were infected for four, eight, or 12 hr prior to lamina fractioning. **B)** Vero, HEp-2, or SNB-19 cells were infected with WT, US3-null, US3-catalytically dead (K220A), or pUL34-null viruses or their repairs (US3-null-Repair or UL34-null-Repair) prior to fractionation at 16 hpi. Base emerin=emerin species with fastest mobility in SDS-PAGE
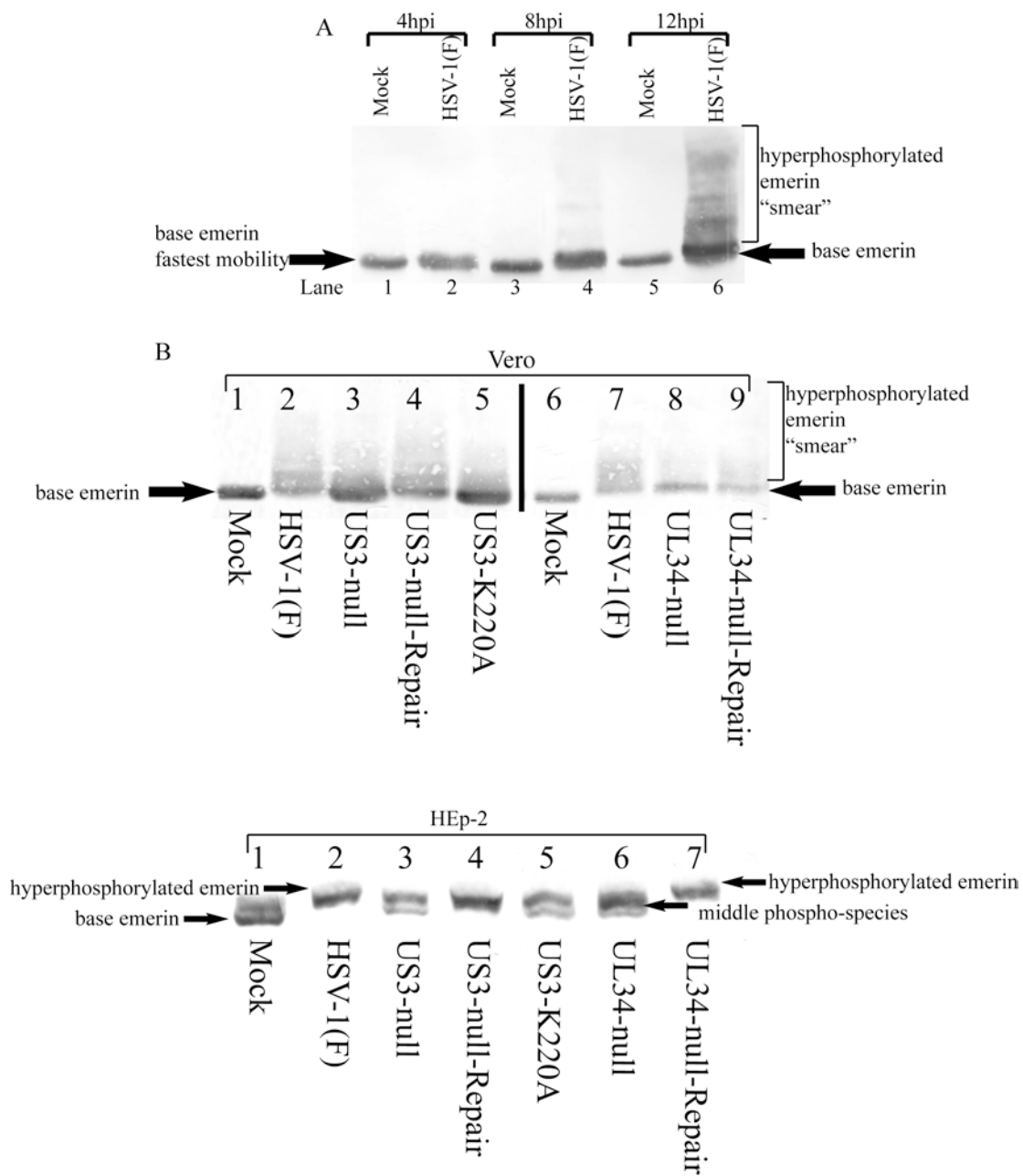
B. cont.
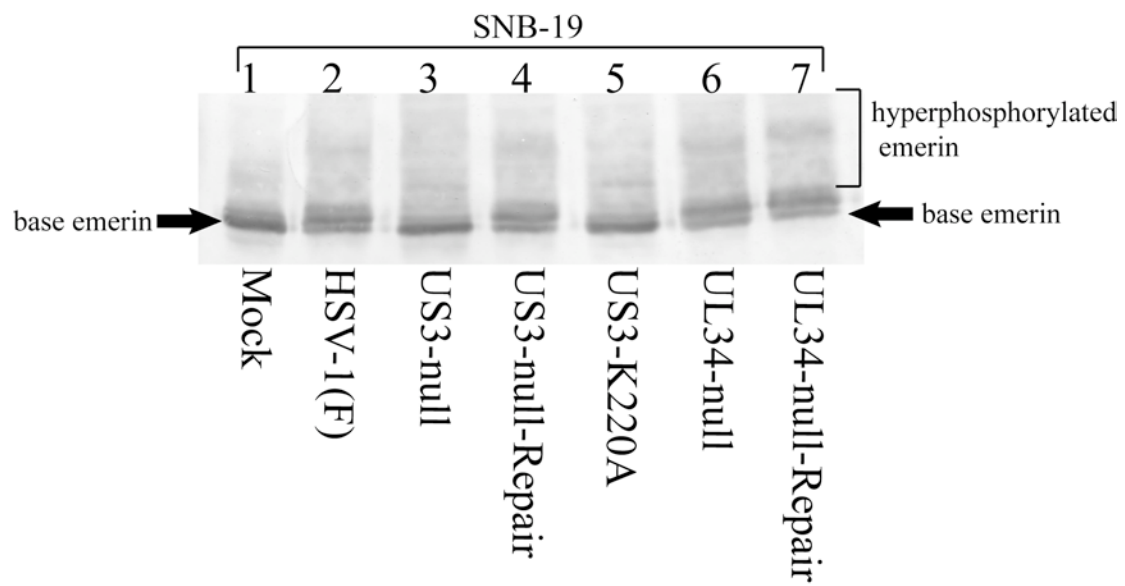
Figure 2.2. LAP2 is de-modified during HSV-1(F) infection but in a pUS3 and pUL34 independent mechanism. Digital images of western blots from lamina fractions of infected cells were immunoblotted with monoclonal antibodies to LAP2. A preliminary immunoblot for LAP2 from each lamina preparation was performed to allow for equal LAP2 loading **A)** HEp-2 or **B)** Vero cells were infected at an MOI of five with WT, US3-null, US3-catalytically dead (K220A), or UL34-null viruses or their repairs (US3-null-Repair or UL34-null-Repair) for 16 hr. The box in lane 6 indicates that this band was digitally spliced from another gel. Lamina fractions were then isolated, run on SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with monoclonal antibody to LAP2, which will detect LAP2alpha and LAP2beta.

A   HEp-2

LAP2alpha →

LAP2alpha mobility increased ←

LAP2beta 3 forms →

← LAP2beta 2 forms

Lanes: 1 2 3 4 5 6 7

Mock | HSV-1(F) | US3-null | US3-null-Repair | US3-K220A | UL34-null | UL34-null-Repair

B   Vero

LAP2alpha →

LAP2beta 3 forms →

← LAP2beta 2 forms

Lanes: 1 2 3 4 5 6 7

Mock | HSV-1(F) | US3-null | US3-null-Repair | US3-K220A | UL34-null | UL34-null-Repair
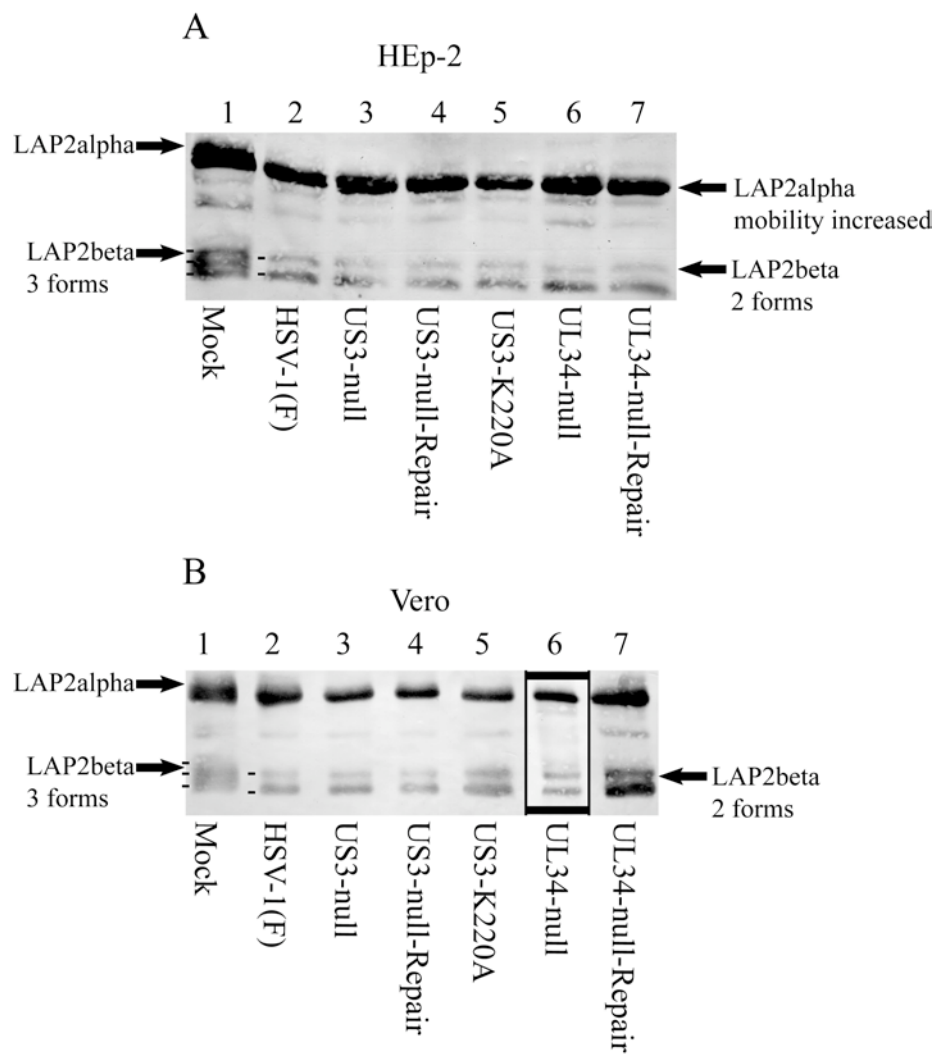
Lane 6 is spliced from another gel

Figure 2.3. Role of PKC in emerin modification. Digital images of western blots from lamina fractions of mock, HSV-1(F), or US3-null infected Vero **(A)** or HEp-2 **(B)** cells immunoblotted for emerin. A preliminary immunoblot for emerin from each lamina preparation was always performed to allow for equal emerin loading. Cells were treated with the PKC delta isoform inhibitor Rottlerin at five hpi (lanes four-nine), cPKC isoform inhibitor Ro-31-7549 at 13 hpi (lanes seven-nine and 13-15), or vehicle (DMSO) (lanes one-three and ten-12). Cells were maintained in the presence of inhibitors until 16 hr after infection, when cells were harvested and nuclear lamina fractions were prepared. Lamina fractions were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for emerin. Emerin is a highly phosphorylated protein due to the 53 serine and theronines.

A **Vero**

Mock	HSV-1(F)	vRR1202	Mock	HSV-1(F)	vRR1202	Mock	HSV-1(F)	vRR1202	Mock	HSV-1(F)	vRR1202	Mock	HSV-1(F)	vRR1202
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

DMSO — Rottlerin — Rottlerin + Ro-31-7549 — DMSO — Ro-31-7549

B **HEp-2**

Mock	HSV-1(F)	vRR1202	Mock	HSV-1(F)	vRR1202	Mock	HSV-1(F)	vRR1202	Mock	HSV-1(F)	vRR1202	Mock	HSV-1(F)	vRR1202
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

DMSO — Rottlerin — Rottlerin + Ro-31-7549 — DMSO — Ro-31-7549
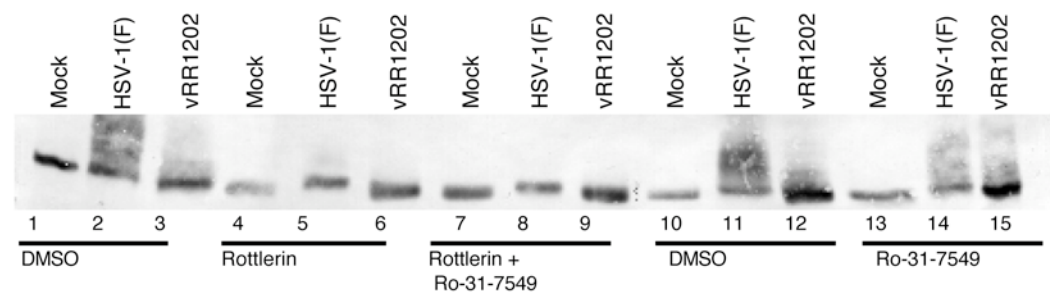
Virus Name	Genetic name
HSV-1(F)	WT No Change
vRR1202	US3-null

Figure 2.4. pUL34 is required for Rottlerin sensitivity. Digital images of western blots from lamina fractions of mock, HSV-1(F), pUL34-null, or pUL34-null-Repair infected Vero cells immunoblotted for emerin. Cells were treated with the PKC delta isoform inhibitor Rottlerin at five hpi (lanes five-eight) or vehicle (DMSO) (lanes one-four). Cells were maintained in the presence of inhibitors until 16 hr after infection, when cells were harvested and nuclear lamina fractions were prepared. Lamina fractions were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for emerin. A preliminary immunoblot for emerin from each lamina preparation was always performed to allow for equal emerin loading . The pUS3-dependent phosphorylation events were difficult to discern in this western for emerin due to the curve of the dye front. Emerin is a highly phosphorylated protein due to the 53 serine and theronines. Base emerin=emerin species with fastest mobility in SDS-PAGE
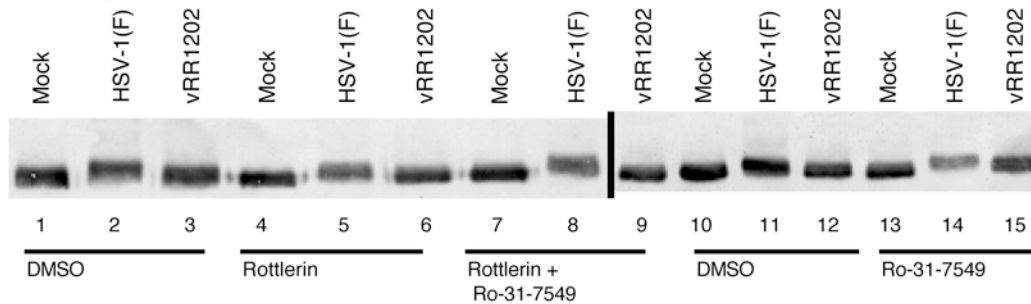
Lane 1 2 3 4 5 6 7 8

hyperphosphoryted emerin

base emerin

base emerin

Mock
UL34-null
UL34-Rep
HSV-1(F)
Mock
UL34-null
UL34-Rep
HSV-1(F)

DMSO-5 hpi

Rottlerin-5 hpi
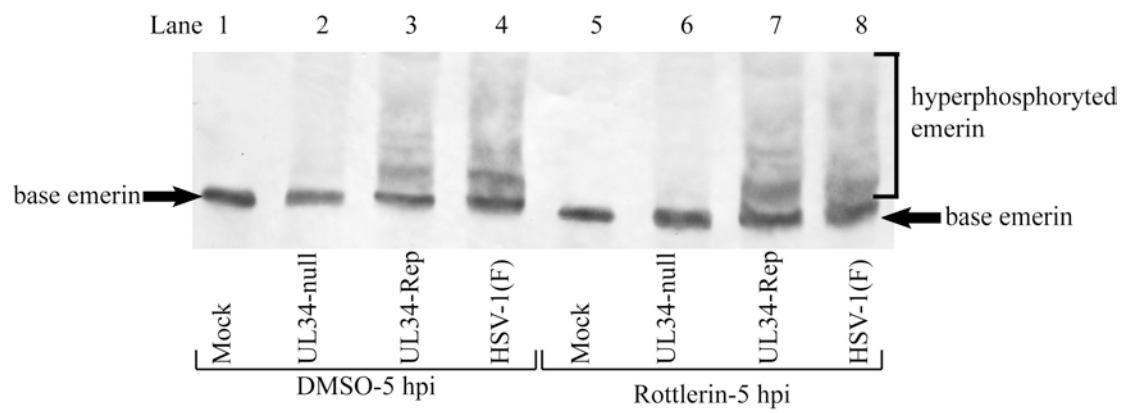
Figure 2.5. Emerin is not required for HSV-1(F) replication. Replicate cultures Wild-type Mouse Embryonic Fibroblasts (MEF+/y) and emerin-null MEFs (EM-/y) were infected at an MOI of five with WT HSV-1(F), US3-null, or US3-null-Repair. Residual virus was removed or inactivated with a low-pH wash, and at the indicated times total culture virus was titrated on Vero cells. Virus yields are expressed as plaque forming units (PFU) per milliliter. Each data point represents the mean of three independent experiments. Error bars indicate standard deviation from the mean.

MEF SSGC
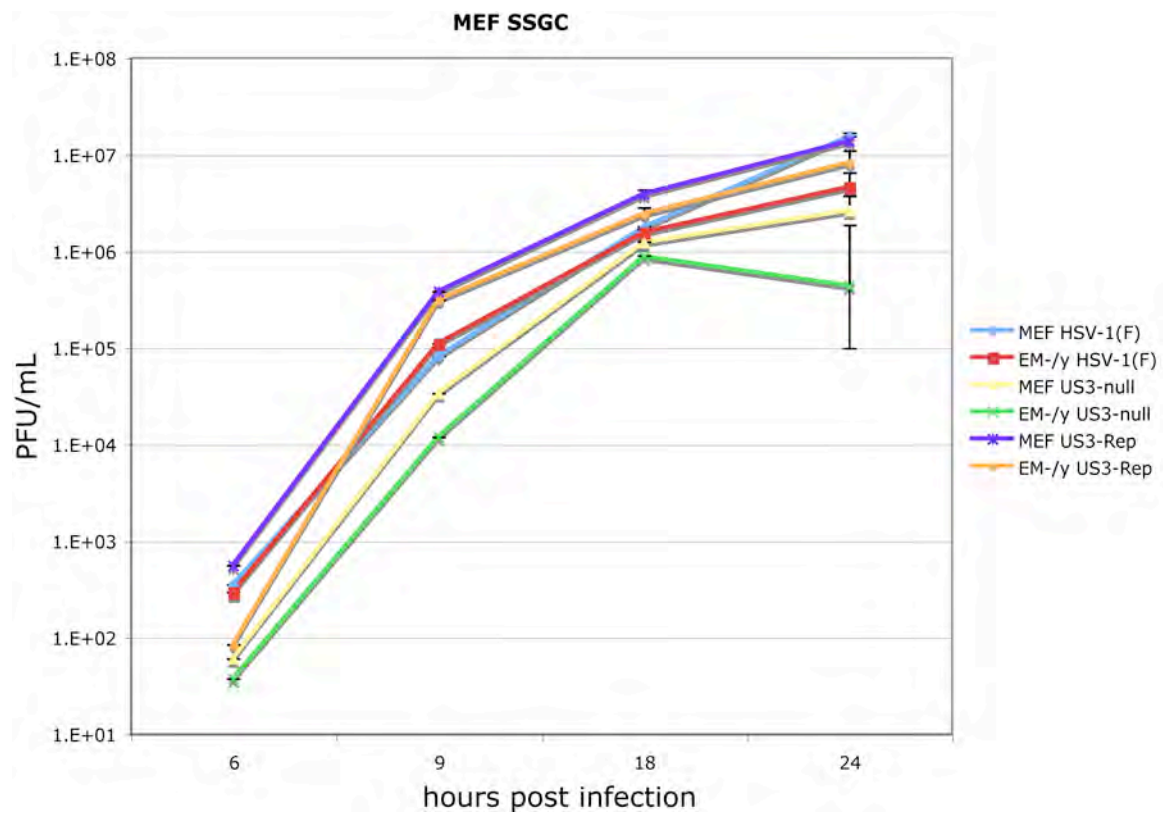
Figure 2.6. SHis-EGFP-Emerin localization in HSV-1(F) infection. A)
The SHis-EGFP-emerin plasmid. **B)** Schematic of the SHis-EGFP-emerin fusion
protein. **C)** HEp-2 clonal cell line B2 stably expressing SHis-EGFP-emerin fusion
protein was mock infected or infected with five PFU/cell of HSV-1(F). At 16 hpi
cells were fixed with four percent formaldehyde, and GFP fluorescence was
observed.

A

CMV promotor 1...588
Streptactin-Binding 598...627
His 631...654
EGFP 655...1370

ColE1 origin 4797...5479

SHispEGFPemerinC1.ape
5575 bp

Emerin 1387...2183

Kan/neoR 3474...4265

SV40 early promoter 3240...3436

F1 ori 2501...2941

B

N-terminus   C-terminus

| Strep | 8xHis | EGFP | Emerin (human) |

C

S-His-EGFP-Emerin

i

ii

Mock   HSV-1(F)

HEp-2 B2 cell line
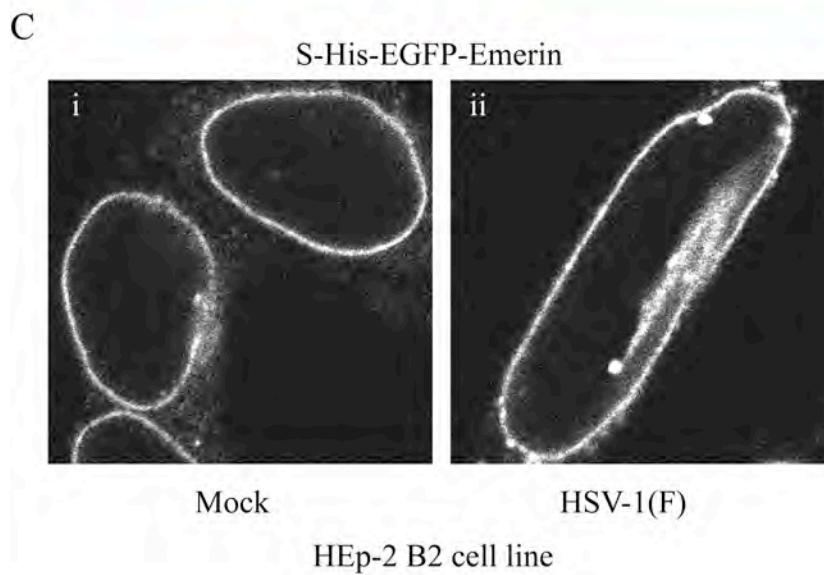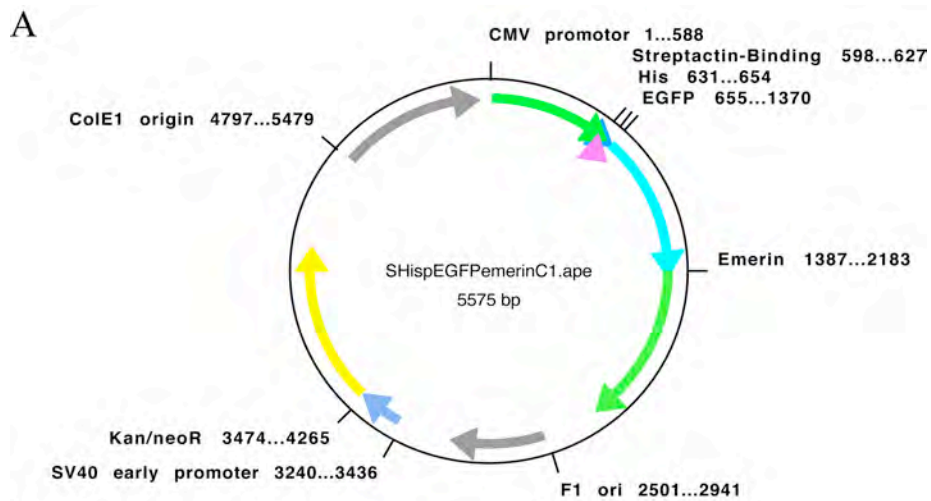
CHAPTER III

INHIBITION OF PKC ISOFORMS BLOCKS

EFFICIENT GROWTH AND NUCLEAR EGRESS

OF HERPES SIMPLEX VIRUS TYPE 1

Abstract

The nuclear lamina is thought to be a steric barrier to the herpesvirus capsid. Disruption of the lamina accompanied by phosphorylation of lamina proteins is a conserved feature of herpesvirus infection. In HSV-1-infected cells, protein kinase C (PKC) alpha and delta isoforms are recruited to the nuclear membrane and PKC delta has been implicated in phosphorylation of emerin and lamin B. We tested two critical hypotheses about the mechanism and significance of lamina disruption. First, we show that chemical inhibition of all PKC isoforms reduced viral growth five-fold (0.5 log) and inhibited capsid egress from the nucleus. However, specific inhibition of either conventional PKCs or PKC delta does not inhibit viral growth. Second, we show hyperphosphorylation of emerin by viral and cellular kinases is required for its disassociation from the lamina. These data support hypothesis that phosphorylation of lamina components mediates lamina disruption during HSV nuclear egress.

Introduction

      All herpesviruses assemble newly formed capsids in the nucleus of the host cell. To escape the nucleus, the capsids must traverse the inner and outer nuclear membranes via an envelopment/de-envelopment process at the nuclear envelope (NE) to release un-enveloped capsids into the cytoplasm (179). The inner nuclear membrane (INM) is not, however, freely accessible to large macromolecular complexes like a herpesvirus capsid. The INM is supported by a complex meshwork of proteins called the nuclear lamina (1, 92, 291).

      The lamina mesh is primarily composed of intermediate filament-related proteins called lamins of which there are three types: A, B, and C. The lamin A gene is alternatively spliced to generate A and C types while the two B types are encoded by separate genes (91). The lamin meshwork is anchored to the INM via interactions with lamin associated proteins (LAPs) (291). Many LAPs such as emerin, the lamin B receptor (LBR), MAN1, and LAP2β are inner nuclear membrane-bound proteins that bind both lamins and chromatin (111). The organization of the nuclear periphery suggests that it may present multiple barriers to herpesvirus envelopment. The presence of chromatin attached to the lamina and the organization of the lamin proteins themselves may each present a steric barrier. The spacing of structural elements of the lattice (about 50 nm) is too small to allow passage of a herpesvirus capsid, and physical measurements suggest that the lamin network is quite stiff and resistant to deformation and therefore unlikely to bend around a capsid during envelopment (1, 203). It is likely that the lamina must be disrupted in order for capsids to gain access to the INM.

      Infection with wild-type herpesviruses results in changes in nuclear architecture consistent with disruption of the nuclear lamina, including: (i) enlargement of the nucleus demonstrated for HSV-1 and HCMV (19, 217, 249); (ii)

change in the shape of the nucleus from a smooth ovoid to something that more closely resembles a raisin in contour, demonstrated for HSV and HCMV (19, 98, 217, 248, 249); (iii) changes in the localization of both A and B type lamin proteins from a smooth, even lining of the INM to an uneven distribution showing gross thickening of the lamin layer at some sites and small perforations in the layer at other sites, demonstrated for HSV-1, HSV-2, HCMV, MCMV, and EBV (19, 35, 36, 98, 145, 217, 223, 248, 249); (iv) masking and unmasking of monoclonal antibody epitopes on the lamin proteins that indicate a change in the conformation or associations of the lamin proteins, seen with HSV-1 (223); (v) redistribution of LAPs including LBR, LAP2β, and emerin in herpes simplex infections (20, 142, 186, 242, 248).

Mitosis requires disruption of the nuclear lamina (reviewed in (169) which is mediated by phosphorylation of lamins and LAPs by cellular kinases including cyclin dependent kinase 1 (Cdk1), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), protein kinase A (PKA), casein kinase II, and AKT (91, 92, 169). Phosphorylation disrupts the lamina protein-protein and protein-DNA interactions. A growing body of evidence suggests that herpesviruses adapt this mechanism and induce phosphorylation of nuclear lamina components to gain access to the INM. HSV-1, HSV-2 and HCMV infections induce phosphorylation of all three types of lamins (36, 170, 188, 190, 207). EBV and MCMV infections induce phosphorylation of at least lamin A/C (145, 191). LAPs are also phosphorylated during herpesvirus infection. Emerin is hyperphosphorylated and disconnected from the lamina during HSV -1 and -2 infections however the mechanism and significance of this modification remains untested (142, 186). It is unknown which emerin residues are modified during HSV infection and if these modifications are required for its disassociation from the NE.

Phosphorylation of lamina components during infection is mediated by both viral and cellular kinases. Herpesviruses encode a conserved protein kinase called

CHPK (Conserved Herpesvirus Protein Kinase), which shows similarities to Cdk1 in function and substrate specificity (115, 121). Cdk1, also known as cdc2, has also been implicated in HSV-2 lamina disruption via its putative role in emerin phosphorylation (186). Involvement of the beta- and gamma-herpesvirus CHPKs in nuclear lamina disruption and nuclear egress has been established (135, 145), and the HCMV CHPK phosphorylates lamin proteins on previously defined CDK-dependent sites (98). The HSV-1 CHPK, pUL13, apparently does not directly phosphorylate nuclear lamina proteins, but the HSV-2 pUL13 homolog has the ability to disrupt lamin localization in transiently transfected cells and to directly phosphorylate lamins *in vitro* (36). Alpha-herpesviruses encode a second serine/threonine protein kinase designated pUS3 in HSV. pUS3 mediates phosphorylation of lamina components, including lamin A/C, and emerin, and regulates the degree of lamina disruption (20, 142, 190).

During HSV-1, MCMV, and HCMV infections, PKC isoforms are recruited to the NE by viral proteins that are required for lamina disruption, suggesting that PKC activity may contribute to lamina-disrupting phosphorylation events (191, 207). There are ten PKC isoforms divided into three groups that differ in their activation mechanisms and all isoforms may be involved in herpesvirus-mediated lamina disruption. Conventional PKC isoforms (cPKCs), such as protein kinase c alpha (PKC alpha), require an efflux of calcium and diacylglycerol (DAG) for activation. Novel PKC (nPKC) family members, such as PKC delta (encoded by the *PRKCD* gene), are activated by DAG in a calcium-independent manner. Atypical PKCs (aPKC) such as PKC zeta do not require either for activation (221).

Recruitment of PKCs to the NE appears to be isoform specific. Although not all ten isoforms were tested, both PKC alpha and PKC delta, but not PKC zeta, were recruited to the NE upon HSV-1 infection (207). Treatment of HSV infected cultures with Rottlerin, a widely used putative PKC delta inhibitor, blocked lamin B

phosphorylation (207). These data suggested a role for PKC alpha and delta but not zeta in nuclear egress. Although PKC recruitment and concurrent phosphorylation of lamina proteins has been repeatedly demonstrated, it has yet to be shown if PKC function is required for nuclear egress. It is also unknown if PKC mediated phosphorylation of LAPs, such as emerin, regulates the LAP-lamin association in the infected cell.

Recruitment of PKCs to the NE in herpesvirus infections requires expression of the conserved proteins of the virus nuclear egress complex. In HSV, these proteins are called pUL31 and pUL34, and they form a complex that is required for events in lamina disruption including redistribution of lamin proteins, masking and unmasking of lamin epitopes during infection and full hyperphosphorylation and redistribution of emerin (142, 223). In HSV-1 infection, recruitment of both PKC alpha and PKC delta depends on pUL34 expression (207). In MCMV, M50/p38, the pUL34 homolog, is required to recruit cPKCs to the NE (191). HSV-1 induced emerin phosphorylation is dependent upon both pUS3 kinase activity and pUL34 expression. The pUL34 dependent component of emerin hyperphosphorylation is sensitive to inhibition by Rottlerin suggesting that PKC delta mediates emerin hyperphosphorylation (142).

Despite the evidence for herpesvirus-dependent lamina disruption, it should be emphasized that the hypothesis that lamina disruption is necessary for herpesvirus egress has not yet been rigorously tested largely because all of the viral and cellular activities that mediate lamina disruption may also have other functions in infection and in nuclear egress. Experiments that specifically isolate effects on lamina disruption have not been performed. Also, no study has yet demonstrated that the observed phosphorylation of lamina components causes their disconnection from other components of the lamina. The data presented in this article support both of these hypotheses by showing that (i) PKC family function is required both for

efficient replication of HSV-1 and for nuclear egress, and (ii) emerin localization in the infected cell is determined by phosphorylation state. Emerin hyperphosphorylation is not sensitive to pan-PKC or DN-PKC delta inhibition suggesting a role for a non-PKC isoform in emerin phosphorylation. This non-PKC isoform or Rottlerin sensitive kinase (RttSK) is sensitive to Rottlerin but not BIM I treatment.

Materials and Methods

Cells, viruses, and chemicals. HEp-2 and Vero cells were maintained as previously described (232). MCF-7 and BT-549 cells were maintained in RPMI containing 10% fetal calf serum. BT-549 cells were derived from the NCI-60 cell collection and were kindly provided by Jack Stapleton. The properties of HSV-1(F), vRR1072(tk+) (UL34-null mutant virus), vRR1202 (US3-null virus), vRR1204 (US3 kinase-dead virus K220A), and repair viruses for vRR1072(tk+) (vRR1072Rep) and vRR1202 (vRR1202Rep) were previously described and characterized (224, 232, 236). Rottlerin (Santa Cruz Biotechnology) was diluted in DMSO to a stock concentration of 10 mM and used to treat cells at 10 uM. Ro-31-7539 (Calbiochem) was diluted in DMSO to a stock concentration of 1mM and used to treat cells at 1 uM while bisindolylmaleimide I (BIM I- LC Laboratories) was diluted in DMSO to a stock concentration of 500 uM and used at 10 uM. For IFNβ (Interferon β) treatment, HEp-2 cells were serum starved in DMEM without serum overnight, and then exposed to 1000U of Human recombinant IFNβ (IBL) for 30 min. Phorbol 12-myristate 13-acetate (PMA) was kindly provided by Jeffery Meier (Sigma). PMA was used at 20 nM to activate PKCs for 30 min.

Plasmids and cell lines. pRR1072Rep, which expresses wild-type HSV-1 UL34 from its own promoter, has been previously described (232). The FLAG epitope-tagged rat wild-type and K376M (dominant negative) PKC delta expression

vectors were kindly provided by U. Kikkawa (129). pRR1342 (PRKCDwt-UL34 Duo) and pRR1343 (PRKCDdn-UL34 Duo), which express either wild-type or K376M FLAG epitope tagged rat PKC delta (respectively) and wild-type HSV-1 UL34 were constructed by ligation of the 3.05 kb XbaI-NgoMIV fragment of pRR1072Rep and the 5.44 kb fragment of the corresponding PKC delta expression vectors. In pRR1342 and pRR1343, PKC delta is constitutively expressed from the human cytomegalovirus major immediate early promoter (HCMV MIEP), and pUL34 is expressed in a HSV infection-dependent manner from its own promoter. The HuSH shRNA constructs against PKC delta (product number TR320468) were purchased from OriGene. In order to construct stable clonal cells lines expressing HuSH shRNAs against PKC delta, HEp-2 cells were transfected with individual HuSH shRNA constructs and then selected with 0.5 ug/mL puromycin for one week. Colonies were then scraped from the plate, transferred to individual cultures, and amplified. The HuSH cell lines shRNA-control, shRNA-PRKCD-1, shRNA-PRKCD-2, and shPRKCD-PRKCD-3 constitutively express the following HuSH plasmids respectively: TR30003, TI379089, TI379090, and TI379090. To create the Strep-His-EGFP-emerin plasmid, PCR amplification of EGFP from the EGFP-emerin-C1 plasmid from Y. Hiraoka (142) was performed with the following primers: 5'

CACAGCTAGCCATGGCGTGGAGCCACCCCCAGTTCGAAAAGGCGCACCATCACCATCACCATCACCATATGGTGAGCAAGGGCGAGGA 3' and 5' AGTTGTCCATGGATCTGAGTCCGGA 3'. The 0.8kb PCR product and EGPF-emerin-C1 were digested with Nhe1 and BspE1 and the PCR product was ligated into the 4.6 kb product of e EGFP-emerin-C1 digestion to create pRR1315. To create the retroviral expression plasmid for Strep-His-EGFP-emerin, Strep-His-EGFP-emerin was PCR amplified from pRR1315 using the following primers: 5'-CACAGTTAACGCTAGCCATGGCGTGGAGC-3' and 5'-

CACAGCTCGAGATCCTAGAAGGGGTTGC-3'. This 1.6kb PCR product and 5.9kb pLXSN vector were digested with Xho1 and Hpa1 and ligated to create pRR1319. pLXSN was kindly provided by A. Klingelhutz. The University of Iowa Vector Core used pRR1319 to generate a MoMLV pseudotyped retroviral transduction particle. These particles were used to transduce HEp-2 cells. 48 hr post transduction, cells were placed under G-418 selection for two weeks. Colonies were then scraped from the plate, transferred to individual cultures, and amplified to create the HEp-2 Strep-His-EFGP-emerin B2 cell line.

Indirect Immunofluorescence. Indirect immunofluorescence (IF) was performed for pUL34, emerin, lamin A/C and FLAG-epitope-tagged PKC delta as previously described (20, 224). Briefly, cells were fixed with 4% formaldehyde for 10 min, washed with phosphate-buffered saline (PBS), and then permeabilized and blocked in the same step with IF buffer (0.5% Triton X-100, 0.5% Sodium Deoxycholate, 1% BSA, 0.05% Sodium Azide diluted in PBS) for 1 hr. Primary antibodies were diluted in IF buffer as follows: chicken anti-UL34, 1:1000 (224); mouse monoclonal anti-lamin A/C, 1:1000 (Santa Cruz Biotechnology, sc-7292); mouse monoclonal anti-emerin, 1:500 (Santa Cruz Biotechnology, sc-25284); mouse monoclonal anti-FLAG M2, 1:1000 (Sigma, F1804). The procedure for STAT3-pTyr705 localization differed in that cells were fixed in cold methanol for 30 min, dried, and rehydrated in IF buffer. Rabbit polyclonal anti-STAT3-pTyr705 (Cell Signaling, #1938), was diluted 1:400.

Secondary antibodies were diluted 1:1000 in IF buffer as follows: Alexa Fluor goat anti-chicken IgG (Invitrogen, A11001), Alexa Fluor goat anti-mouse IgG (Invitrogen, A1104), Alexa Flour donkey anti-rabbit IgG (Invitrogen, A21206). Slo-fade II (Invitrogen, Molecular Probes) was used to mount cover slips on glass slides. All confocal microscopy work was done with a Zeiss 510 microscope except Figure X and Figure X, which were performed on a BioRad Multiphoton microscope.

SDS-PAGE sample preparations. For analysis of viral protein expression and PKC delta protein expression, infected or uninfected cultures containing 2×10^6 cells were washed twice with PBS, scraped into PBS, pelleted at low speed, total soluble protein was extracted with acetic acid, and precipitated with acetone as previously described (229). Total protein concentration was determined using BioRad Dc Protein Assay (BioRad Laboratories). MEK 1/2 lysates were prepared differently in that after collecting the treated cultures containing 2×10^6 cells after with a low speed spin, total soluble protein was extracted with SDS-PAGE sample buffer and PBS. This total cell lysate was denatured at 70C, sonicated twice for 30 seconds each, and then total protein concentration was determined with BioRad Dc Protein Assay (BioRad Laboratories).

Preparation of nuclear fractions. Nuclear-lamina fractions were prepared as described in (142).

Western blot. The antibodies and dilutions used were: mouse monoclonal anti-emerin, 1:500 (Santa Cruz, sc-25284); rabbit polyclonal anti-PKC delta, 1:400 (Santa Cruz, sc-937); mouse monoclonal anti-US11, 1:1000 (231); chicken monoclonal anti-UL34, 1:1000 (224); mouse monoclonal anti-VP5, 1:1000 (Meridian Life Science, #C05014M); mouse anti-actin, 1:1000 (Sigma, A3853); mouse monoclonal anti-gC, 1:1000 (Virusys, H1A022M); rabbit monoclonal anti-phos-MEK 1/2 Ser 217/221, 1:1000 (Cell Signaling, 9154); rabbit monoclonal anti-MEK 1/2 (Cell Signaling, 9122). Alkaline phosphatase conjugated anti-mouse, anti-rabbit, and anti-chicken secondary antibodies were incubated with the appropriate blot for 30 min (Sigma, mouse: A3562, rabbit: A3687 Aves Lab, chicken: AP1001).

Virus Growth. Single-step growth analysis was performed as previously described (225, 232). Briefly, replicate cultures of cells were infected at an MOI of five, and residual virus was removed or inactivated with a low-pH-buffer wash. Cells were untreated or treated with DMSO, Ro-31-7549 (1 uM), or BIM I (10 uM) at five

hpi. At 24 hpi, virus was harvested and total culture PFU were calculated by titration on Vero cells.

Transmission Electron Microscopy. Vero cells were infected with HSV-1(F) at an MOI of five and at five hpi cells were treated with DMSO or BIM I. At 16 hpi, cells were fixed with glutaraldehyde, processed, and analyzed by electron microscopy as described previously (232).

^{35}-S-methionine Labeling. 2×10^6 HEp-2 cells were uninfected or infected with five PFU/cell of HSV-1(F) and beginning at five hpi, the cells were treated with DMSO (D) or BIM I (B). At 9 hpi, the cells were starved for methionine by replacing the media with methionine minus media (Gibco, 21013) with DMSO or BIM I. Beginning at 14 hpi, each sample was labeled for two hr with 20 uCi of ^{35}S-methionine. Total cell lysates were prepared at 16 hpi by the acid preparation as described above (229). Total protein from equal numbers of cells was separated on an SDS-PAGE gel and visualized by autoradiography.

Complementation assays. Twelve-well cultures of Vero cells containing 4×10^5 cells were transfected with a total of 650 ng of plasmid DNA using 5 μl of LipofectAmine Reagent according to the manufacturer's instructions. Complementation assays were performed on the transfected cultures as previously described (18).

Inhibitor toxicity assays. 2×10^5 HEp-2 and Vero cells were plated in a 48 well dish in triplicate. 12 hr later, cells were treated with vehicle (DMSO), 10 uM Rottlerin, 10 uM BIM I, and 1 uM Ro-31-3549. 24 hr post-treatment, the ATPLite assay (Perkin-Elmer, #6016739) was followed per manufacture's directions. This was repeated three independent times to determine that at the indicated dosage, the kinase inhibitors did not induce significant toxicity compared to vehicle (DMSO). Since this assay measures the amount of ATP in the cell, this assay also controls for any reduction in the amount of PO_4 for phosphorylation of proteins. This could be an

issue in Rottlerin treated cells where mitochondrial uncoupling had been previously observed (253). ATPLite data was not included since at the concentrations used in these experiments, no toxicity was observed (data not shown).

Inhibitor signaling assays. 2×10^6 HEp-2 cells were placed into the presence of 10 uM BIM I, 1 uM Ro-31-7549 or vehicle for two hr, followed by a 30 min activation of PKCs with 20 nM PMA. The cells were washed twice with PBS, then pelleted at low speed, resuspened in PBS and SDS-PAGE buffer. Protein concentration was determined using the BioRad Dc Assay described above. These lysates were subjected to western blot analysis for MEK 1/2 and phos-MEK 1/2.

<div align="center">Results</div>

Inhibition of cellular kinases involved in lamina disruption inhibits viral replication. To test the hypothesis that PKC activity is required for late events of HSV-1 replication, specifically nuclear egress, activity of all PKC isoforms was blocked with the pan-PKC small molecule inhibitor bisindolylmaleimide I (BIM I) (271). HEp-2 cells were infected with five PFU/cell of WT HSV-1(F), treated with 10 uM BIM I or vehicle (DMSO) beginning at five hr post infection (hpi), and production of infectivity was measured at 24 hpi (Figure 3.1). This time of drug addition was chosen to allow at least the entry and IE (immediate early) gene expression phases to occur uninhibited. Ten uM BIM I has been previously shown to inhibit all PKC isoform activity (271). BIM I treatment reduced the infectivity compared to DMSO by nearly five-fold (0.5 log). Similar results were obtained in Vero cells and at 16 hpi (not shown).

Toxicity, determined using the ATPLite system, was not observed at the concentrations used for any of the inhibitors used in this thesis (except for MEFs, data not shown). BIM I inhibits PKC activity as previously reported, since BIM I treatment blocked PKC activation of the MAPK pathway (Figure 3.2) (220, 283).

Similar results were obtained in Vero cells (data not shown). Collectively, Figures 3.1 and Figure 3.2 suggest a significant role for PKC function in the late phase of HSV-1 replication.

PKC activity is required for extra-nuclear capsid accumulation. To determine if the decrease in viral infectivity produced by PKC inhibition is due to a block to primary envelopment, Vero cells were infected with five PFU/cell of WT HSV-1(F), treated with vehicle (Figure 3.3 A) or 10 uM BIM I (Figure 3.3 B) beginning at five hpi, fixed at 16 hpi, and processed for TEM analysis. DMSO-treated, WT virus-infected cells contained empty (A and B capsids (without DNA)) and full capsids (C capsids (with DNA)) in four distinct sub-cellular compartments: nucleus, perinuclear space\ER, cytoplasm (either as naked or membrane-enclosed capsids), and cell surface (membrane-enclosed capsids) (Figure 3.3). Capsids were counted in the four distinct compartments in 20 cells for each treatment condition and represented as a percentage of total capsids (Table 3.1). In DMSO-treated cells, 33% of virions were found in the nucleus. BIM I treatment increased the number of nuclear virions to 77% of total. While 46% of virions in DMSO-treated infected cells were found at the cell surface, only 9% of BIM I-treated virions were at the cell surface. BIM I-treated cells either had no or few capsids in the entire cell or the majority of the capsids were located inside the nucleus (Table 3.1).

Many of the WT virus-infected, BIM I-treated cells, displayed distinct characteristics of infection such as marginated chromatin (Figure 3.3 B), and yet had few or no capsids in the cell. Furthermore, the total number of capsids found in or on BIM I-treated cells was decreased when compared to vehicle-treated cells (Table 3.1, total DMSO-631 vs BIM I-223). To determine if there was a statistical difference in capsid accumulation, we compared the number of capsids per cell in DMSO- and BIM I-treated cells using an unpaired Student T-Test (Table 3.1, mean DMSO-31.6 vs BIM I-11.2) (http://www.physics.csbsju.edu/stats/t-test.html). Inhibition of PKC

with BIM I changed the mean number of capsids per cell significantly as evaluated by the T-Test (p=0.001). Collectively, these data suggest BIM I inhibition of PKC inhibits (i) accumulation of capsids and (ii) the ability of the capsids that are formed to egress from the nucleus.

PKC inhibition does not inhibit viral protein translation. The significant reduction in the fraction of extra-nuclear capsids produced in BIM I- treated, HSV-1(F)-infected cells compared to vehicle control cells, indicated that BIM I inhibits nuclear egress (Table 3.1). However, the reduction in the total number of capsids observed in the presence of the pan-PKC inhibitor suggested its effect on nuclear egress might result from inhibition of events preceding capsid assembly, including expression of genes required for capsid assembly and nuclear envelopment (Table 3.1).

To test the hypothesis that BIM I treatment inhibits late gene expression necessary for efficient capsid assembly and nuclear egress, accumulation of viral proteins was tested in cells infected in the presence of vehicle and BIM I. HEp-2 cells were infected with five PFU/cell of HSV-1(F) and at five hpi, the cells began treatment with DMSO (D) or BIM I (B). Total cell lysates were prepared at 16 hpi and protein from equal numbers of cells was separated on an SDS-PAGE gel and transferred to nitrocellulose (Figure 3.4 A). These samples were subjected to western blot analysis for the following proteins: VP5 (leaky-late gene product and capsid protein), pUS11 and gC (archetype true-late gene products), and pUL34 (leaky-late and required for nuclear egress). BIM I treatment in infected cells had little to no reproducible effect on the accumulation of VP5, gC, pUS11, or pUL34 compared to DMSO treatment (compare lanes three to two).

To test the hypothesis that BIM I treatment inhibits global late gene expression, synthesis of late viral proteins was tested in cells infected in the presence of vehicle or BIM I. HEp-2 cells were infected with five PFU/cell of HSV-1(F) and

beginning at five hpi, the cells were treated with DMSO (D) or BIM I (B). Beginning at 14 hpi, cells were labeled for two hr with ^{35}S-methionine. Total cell lysates were prepared at 16 hpi and protein from equal numbers of cells was separated on an SDS-PAGE gel and visualized by autoradiography (Figure 3.4 B). Upon infection with HSV-1(F), host protein synthesis was turned off and viral protein synthesis was evident in DMSO-treated samples (compare lanes two to four). When infected cells were treated with BIM I little or no effect on viral protein synthesis was observed (compare lanes one to two). BIM I treatment reduced mock-infected HEp-2 protein synthesis (compare lanes three to four) as previously observed (253, 283). These data suggest that BIM I mediated inhibition of nuclear egress is not due to inhibition of late gene expression, and are consistent with the hypothesis that inhibition of PKCs has specific inhibitory effects on both (i) accumulation of capsids and (ii) egress of accumulated capsids from the nucleus.

Neither conventional PKCs nor PKC delta kinase activity are uniquely required for HSV-1 replication. PKC alpha, a conventional (cPKC) isoform, is recruited to the NE during HSV-1 infection and the entire class of cPKCs is recruited to the rim during MCMV infection suggesting a conserved and specific role for cPKCs in herpesvirus nuclear egress (191, 207). To test the hypothesis that cPKC isoforms are required for optimal HSV-1 growth, cPKCs were inhibited with the small molecule inhibitor Ro-31-7549 (300). HEp-2 cells were infected with five PFU/cell of WT HSV-1, treated with 1 uM Ro-31-7549 or vehicle (DMSO) beginning at five hpi, and production of infectivity was measured at 24 hpi (Figure 3.5 A). Treatment with Ro-31-7549 had no significant effect on replication compared to vehicle. Ro-31-7549 inhibits cPKC (PKC alpha) signaling, as previously reported, since it partially blocked PKC signaling and activation of the MAPK pathway (Figure 3.6 A). Collectively, Figures 3.5 A and Figure 3.6 A do not suggest a unique, significant role for cPKC function in the late phase of HSV-1 replication. HSV-1

also recruits PKC delta to the NE and treatment with Rottlerin, a reported PKC delta inhibitor, reduces infection-induced phosphorylation of lamin B and emerin (142, 207). These data suggested that PKC delta contributed to lamina disruption and might be required for nuclear egress. It has become apparent with additional research, however, that there are many PKC delta-independent Rottlerin-dependent phenotypes (157, 253, 270, 283), necessitating the use of more specific methods to probe PKC delta function. We used three approaches to reduce PKC delta activity to test the hypothesis that PKC delta activity is specifically required for viral replication.

First, we inhibited PKC delta activity by over-expression of dominant-negative (DN) PKC delta (K376M) (129). Our strategy was to place a DN-PRKCD gene on the same plasmid as an essential viral gene and then perform a complementation assay with the virus lacking that essential gene. Two plasmids were created expressing both FLAG-PKC delta (WT or DN) and the viral gene UL34. These constructs are termed PRKCDwt-UL34 Duo or PRKCDdn-UL34 Duo. These plasmids allow for co-expression of both genes in the transfected cell, however UL34 will only be expressed upon infection with the UL34-null virus (vRR1072(tk+)) (or HSV-1) since the UL34 gene is under the control of its viral promoter. If PKC delta activity is required for HSV-1 replication, then the PRKCDdn-UL34 Duo plasmid should inhibit complementation of the UL34-null virus. We tested for efficient gene expression from the Duo plasmids (Figure 3.6 B). We also confirmed that DN-PKC delta (K376M-PRKCD) expressed from the Duo system retains the dominant negative function (Figure 3.6 C).

To test the hypothesis that PKC delta activity is required for viral growth, PKC delta activity was blocked with PRKCDdn-UL34 Duo in a complementation assay. Vero cells were co-transfected for 24 hr with a transfection efficiency control plasmid, pCMVβ, and no UL34 (pRR1072), WT UL34 (pRR1072Rep), PRKCDwt-UL34 Duo, or PRKCDdn-UL34 Duo. These Vero cells were infected with five

PFU/cell of UL34-null virus (vRR1072(tk+)) and at 18 hpi the amount of infectivity produced was measured by plaque assay on the UL34 complementing cell line RepAC (Figure 3.5 B). The complementation index for WT UL34 was set to 1.000 and the other conditions were calculated relative to that. No pUL34 resulted in little virus production, while pUL34 provided *in trans* supported replication as previously shown. Co-expression of either WT- or DN-PKC delta appeared to slightly reduce complementation (Figure 3.5 B). However, there was not significant difference between WT- and DN-PKC delta suggesting that DN-PKC delta has no specific inhibitory effect on complementation. The data in Figure 3.5 suggest that neither cPKCs nor PKC delta kinase function is required for efficient HSV replication.

Our second approach was to determine if reduction of PKC delta protein levels would depress viral replication. HEp-2 clonal cell lines constitutively expressing shRNA against human PKC delta were generated (Figure 3.7 A). There was not a significant difference between replication of HSV-1(F) in any of the shRNA-PKC delta cell lines and replication in the shRNA-control or untransfected control (3.7 B).

Our third approach was to evaluate HSV-1 replication in a cell line with little or no endogenous PKC delta protein. It is possible that 70% knockdown in HEp-2 cells was not sufficient to reduce the level of PKC delta total kinase activity to inhibit viral replication (Figure 3.7 A and B) or that the requirement for PKC delta during HSV-1 replication may be a cell type specific event. Using the NCI-60 microarray database, we identified a breast cancer epithelial cell line, BT-549, with reduced mRNA expression levels of PRKCD. The NCI-60 data indicated that BT-549 cells express low levels of PRKCD mRNA as compared to another breast cancer epithelial line, MCF-7, which have a median expression profile for PRKCD (http://dtp.nci.nih.gov/mtweb/search.jsp). MCF-7 cells express readily detectable amounts of PKC delta while BT-549 cells have almost undetectable levels of PKC

delta in mock or infected lysates (Figure 3.5 C, only mock shown) (117). The breast cancer cells were infected with five PFU/cell of WT HSV-1(F) and infectivity was determined at 24 hpi (Figure 3.5 D). There was not a significant difference in viral infectivity between MCF-7 and BT-549 cells (Figure 3.5 D). Collectively, results from shRNA-expressing and breast cancer cell comparisons suggest that protein level of PKC delta does not significantly alter WT HSV-1 replication (Figure 3.7 and Figure 3.5 D).

Emerin hyperphosphorylation is necessary for disassociation from the lamina during HSV-1 infection. Another goal of this study was to determine if phosphorylation of lamina components regulates their association with the nuclear lamina during herpesvirus infection. It has been previously hypothesized that recruitment of kinases to the NE and concurrent phosphorylation of nuclear lamina proteins is one mechanism of generating the necessary flexibility in the lamina for primary envelopment. However, it remains unknown if phosphorylation of nuclear lamina proteins, such as emerin, is required for their re-localization during HSV-1 infection. It also remains unknown if phosphorylation of lamina components, LAPs and lamins, is required for nuclear egress. Since HSV-1 induced emerin hyperphosphorylation is blocked in the absence of pUS3 kinase activity and in the presence of Rottlerin (142), these conditions were used as a tool to induce known phosphorylation states in emerin and to determine whether hyperphosphorylation of emerin is required for disassociation from the lamina.

HEp-2 cells were mock infected or infected with five PFU/cell of WT or pUS3 kinase-dead virus (vRR1204) and treatment with vehicle (DMSO) or Rottlerin began at five hpi. At 16 hpi, cells were fixed and stained for emerin and pUL34 and visualized by confocal microscopy (Figure 3.8 A). Mock-infected HEp-2 cells exhibited smooth emerin localization around the NE (Figure 3.8 Aa). WT-infected, vehicle-treated cells demonstrated two changes in the appearance of emerin reactivity

(142). (i) Most emerin is still smoothly distributed in the nuclear membrane and reveals the infection-dependent alteration of the contour of the NE from a smooth ovoid to a wrinkled ovoid. (ii) Some emerin is localized to blebs that appear both inside and outside the NE. In both places it co-localizes with pUL34 (Figure 3.8 Ad,e,f). One of the two changes in emerin appearance is lost in WT virus-infected, Rottlerin-treated cells. All emerin is smoothly distributed in the nuclear membrane and the interior and exterior membrane but the blebs are absent (Figure 3.8 A compare g to d). The contour of the NE is still altered to an irregular ovoid in the presence of Rottlerin (Figure 3.8 Ah,i), suggesting that the NE contour change is not dependent on Rottlerin-sensitive phosphorylation events.

It has been previously demonstrated that in the absence of pUS3 activity and in the presence of Rottlerin treatment, emerin modification is indistinguishable from that in uninfected cells (142). Based on the results from Figure 3.8 Aa-i, our hypothesis would predict that under these same conditions (pUS3-kinase dead and presence of Rottlerin), emerin localization would resemble that seen in mock-infected cells. pUS3 kinase-dead (vRR1204)-infected, vehicle-treated cells showed pUL34 and emerin co-localization in puncta around the NE indicating dissociation from the nuclear lamina (142) (Figure 3.8 Aj,k,l). As predicted, pUS3 kinase-dead (vRR1204)-infected, Rottlerin-treated cells showed emerin localization indistinguishable from mock (Figure 3.8 A compare m to a). Interestingly, although pUL34 and emerin are binding partners, pUL34 still localized in puncta (Figure 3.8 An) and no longer co-localized with emerin (Figure 3.8 Ao) (142). These results suggest that emerin hyperphosphorylation is necessary for its disassociation from the lamina and for its association with pUL34.

We previously reported pUL34-dependent, pUS3-independent emerin phosphorylation events are sensitive to Rottlerin treatment. These data suggested that pUL34-dependent, phosphorylation is mediated by the cellular kinase PKC delta

(142). However, the usefulness of Rottlerin as a PKC delta inhibitor has since been brought into question. Additionally, data presented in Figures 3.1, 3.3, and 3.5 suggest that while inhibition of the entire PKC family leads to a nuclear egress block, PKC delta nor cPKCs are not uniquely required for HSV replication. To test if the pUL34-dependent emerin phosphorylation event relies on the activity of a PKC isoform, HEp2 cells were mock infected or infected with five PFU/cell of WT or pUS3 kinase-dead (vRR1204) viruses. At five hpi, treatment began with vehicle (DMSO) (D), Rottlerin (R), or BIM I (B). At 16 hpi, lamina fractions were prepared; proteins were separated by SDS-PAGE, transferred to nitrocellulose, and then immunoblotted for emerin (Figure 3.8 B). As shown previously, infection with WT HSV-1 induces hyperphosphorylation of emerin, resulting in the appearance of multiple bands that migrate more slowly than the single major band seen in uninfected cells (compare lanes one and two) (142). Emerin hyperphosphorylation is reduced in the absence of pUS3 kinase activity (lane five) or in the presence of Rottlerin (lane four, middle band is most prominent), and is completely eliminated in cells infected with vRR1204 and treated with Rottlerin (compare lanes seven (one species) and eight). Treatment with BIM I, however, does not result in any change in hyper-phosphorylated species present in cells infected with either WT (compare lanes two and three) or pUS3 kinase-dead virus (compare lanes five and six, two species in each lane). Similar results were observed in Vero cells (data not shown). The lack of mobility shift upon BIM I treatment suggests that BIM I inhibition of PKC isoforms late in HSV-1 infection does not affect HSV-1 induced emerin hyperphosphorylation.

Emerin localization in the infected cell nuclear membrane depends upon its phosphorylation state yet BIM I treatment does not alter emerin's phosphorylation state (Figure 3.8 A and B). Therefore, we would predict BIM I treatment would not alter emerin localization. To test this prediction, the same experimental conditions were used as in Figure 3.8 A except PKCs were inhibited by treatment with vehicle

(DMSO) or BIM I beginning at five hpi. At 20 hpi, the cells were fixed in formaldehyde and prepared for confocal microscopy (Figure 3.8 C). Consistent with our hypothesis, emerin mislocalization and co-localization with pUL34 were not appreciably changed by treatment with BIM I in cells infected with either WT virus (Figure 3.8 C, compare panels d-f and g-i) or pUS3 kinase-dead virus (Figure 3.8 C compare panels j-l and m-o).

PKC delta is recruited to the nuclear envelope during HSV-1 infection (Figure 3.8 De) (207). Emerin appears to be re-localized in a phosphorylation dependent manner (Figure 3.8 A), however, inhibition of the entire PKC family did not block emerin hyperphosphorylation (Figure 3.8 B). To test for a specific role for PKC delta activity in HSV-1-induced emerin re-localization, cells from a stable EGFP-emerin-expressing HEp-2 cell line (described in Chapter II) were transfected with PRKCDwt-UL34 Duo or PRKCDdn-UL34 Duo. One day later, the cells were infected with 20 PFU/cell pUS3 kinase-dead virus. At 20 hpi, cells were fixed and processed for detection of FLAG and pUL34 by immunofluorescence (Figure 3.8 D). If PKC delta activity is required for emerin re-localization in the absence of pUS3 activity, then PRKCDdn-UL34 Duo transfected cells should be unable to localize emerin to puncta. Contrary to our hypothesis, emerin re-localization to puncta was observed in cells that express both PRKCDwt and PRKCDdn (Figure 3.8 D, compare panels d-f and g-i).

Discussion

The involvement of PKCs in nuclear egress of herpesviruses appears to be a conserved property of herpesviruses. Both PKC alpha and delta, but not zeta, are recruited to the NE in HSV-1 infection while cPKCs are recruited upon HCMV infection (191, 207). For HSV, HCMV and MCMV, PKC recruitment depends on expression of pUL34 (or its HCMV and MCMV homologs pUL50 and M50), further

suggesting a role in nuclear egress of capsids (191, 207). In the case of HCMV, recruitment of cPKCs to the NE also requires cPKC activity, since recruitment can be inhibited by the specific cPKC inhibitor Ro-31-7549 (191). Data presented here support the hypothesis that the PKC activity is required for replication of HSV and specifically, for nuclear egress of the virus capsid. Inhibition of all PKC isoforms by the pan-PKC inhibitor BIM I beginning at five hr post-infection, results in a five-fold reduction (0.5 log) in virus infectivity (Figure 3.1). The reduction in new virus production is accompanied by retention of capsids in the cell nucleus (Table 3.1) suggesting that it is at least partially attributable to inhibition of nuclear egress. This is the first evidence that PKC function is required for efficient nuclear egress and supports the hypothesis that phosphorylation-mediated nuclear lamina breakdown is important for herpesvirus nuclear egress.

Interestingly, our data also suggest a role for PKC function in formation or stability of capsids (Table 3.1). BIM I inhibition of PKC activity reduced the overall number of capsids in infected cells (DMSO-631 vs BIM I-223). The reduction in capsid number is not apparently due to overall diminished viral protein synthesis late in infection since we observed no inhibition of overall protein synthesis (Figure 3.4). The decrease in capsid number could be attributable an important function of PKCs in capsid assembly or stability, directly or indirectly through signaling events. These data suggest that of PKC activity is involved in (i) accumulation of capsids and (ii) egress of capsids that do accumulate from the nucleus.

The recruitment of conventional PKCs to the NE during HSV and CMV infection suggests specific roles for cPKCs in herpesvirus nuclear egress (191, 207). We tested for a unique role for cPKCs in HSV infection by use of a specific chemical inhibitor, and were surprised to observe that inhibition of cPKCs with Ro-31-7549 had no effect on replication (Figure 3.5 A). This suggests that cPKCs do not have a unique, specific role in HSV replication. Recruitment of PKC delta to the NE in a

pUL34-dependent manner in HSV-infected cells suggested that PKC delta might be required for HSV replication due to its proposed function in lamina disruption prior to nuclear egress (207). Similar to cPKCs, inhibition of PKC delta had no effect on viral replication (Figures 3.5 and Figure 3.7). These results for cPKCs and PKC delta, along with our findings that inhibition of the entire family (Figure 3.1) does inhibit HSV-1 replication are consistent with two hypotheses. First, it is possible that the functions of cPKCs and PKC delta are redundant. Second, it is also possible that these kinases are in fact irrelevant and that some other PKC isoform (perhaps another of the nPKCs or aPKC) plays the critical role in viral replication and nuclear egress.

Emerin and lamin B have both been reported to be phosphorylated by PKC delta in HSV-1-infected cells (142, 207). However, this conclusion was based on sensitivity of phosphorylation to Rottlerin. Since it has become clear that Rottlerin is not a specific PKC delta inhibitor in intact cells, we re-examined the dependence of emerin hyperphosphorylation on PKC activity. HSV-1-induced emerin hyperphosphorylation does not appear to be dependent on activity of a PKC isoform since its mobility in SDS-PAGE was not altered by the inhibition of all PKC isoforms with BIM I (Figure 3.8 B), and its infected cell localization was not altered by expression of DN-PKC delta (Figure 3.8 D). This creates a class of enzymes we termed Rottlerin sensitive kinases (RttSK) since they are sensitive to Rottlerin but not specific PKC delta inhibition. The RttSK responsible for pUL34-dependent emerin phosphorylation remains unknown at this time, but possible candidates include: AKT, PDK1, JNK1alpha, GSK3beta, PKA, and/or MSK1 (253).

Our data also show that protein synthesis in uninfected HEp-2 and Vero cells is dependent on PKC activity, but protein synthesis in wild-type HSV-infected cells is not (Figure 3.4). Therefore, HSV infection apparently protects the infected cell protein synthesis machinery from the effect of PKC inhibition. PKC isoforms have been identified mostly as positive regulators of translation in different cell types and

in response to different stimuli. Positive effects on translation have mostly been attributed to indirect signaling effects through Erk, MAPK or mTOR signaling pathways (2, 3, 104, 136, 233, 283), but cPKCs have also been reported to directly phosphorylate eIF4e (285). The difference between infected and uninfected cells in PKC dependence of protein synthesis suggests that HSV infection provides a functional substitute for PKC in maintenance of protein synthesis. HSV-1 is known to protect host protein synthesis from protein kinase R (PKR)-induced shut-off through pUS11 and ICP34.5 (183). It may be that the virus encodes or induces other activities to maintain robust synthesis of viral proteins.

Many lines of evidence suggest that localization and function of emerin is regulated by its phosphorylation state. Studies in a *Xenopus* oocyte cell-free system, demonstrated that emerin binding to barrier to auto-integration factor (BAF) was un-coupled by a mitotic phosphorylation event on Ser 175 (105). Emerin is phosphorylated in a cell cycle-dependent manner, with maximal phosphorylation observed during mitosis (67). Some Emery-Dreifuss Muscular Dystrophy (EDMD) patients with mutant emerin have increased emerin phosphorylation that correlates with emerin mislocalization away from the NE suggesting that phosphorylation regulates emerin localization and function and aberrant phosphorylation may contribute to EDMD pathogenesis (67, 164, 255). Emerin is hyperphosphorylated in HSV-1 infected cells, and this is correlated with disconnection of emerin from lamin A/C (142). From these data we hypothesized that emerin disassociation from the lamina is caused by its phosphorylation state (Figure 3.8 A). Using conditions shown previously to completely ablate emerin hyperphosphorylation, we observed that, in fact, these same conditions result in maintenance of emerin connections to the lamina but not to pUL34 (Figure 3.8 A). Although PKC activity appears key for HSV-1 growth due to a block in (i) nuclear egress and (ii) capsid accumulation, it does not appear to be involved in the hyperphosphorylation of emerin (Figure 3.8 B). This

was surprising since emerin hyperphosphorylation is sensitive to Rottlerin treatment
(Figure 3.8 B) (142). Emerin localization also is insensitive to PKC delta activity
(Figure 3.8 D), although this enzyme is recruited to the NE (Figure 3.8 Dg) (207). It
remains unknown which cellular enzyme mediates emerin hyperphosphorylation, and
if phosphorylation of emerin is required for nuclear egress.

All of these data are consistent with the following model (Figure 3.9). (1)
Emerin is bound to lamin A/C in the uninfected cell. It is also anchored to the INM
via its transmembrane domain. (2) Upon HSV-1 infection, pUL34 and pUL31 are
expressed and assemble a complex. pUL34 anchors the complex at the INM via its
transmembrane domain. The pUL34/pUL31 complex recruits PKC isoforms, pUS3,
and non-PKC Rottlerin sensitive kinase(s) to the NE. Emerin, lamin A, lamin C, and
lamin B are phosphorylated. Independently of pUL34/pUL31, pUS3 can be recruited
to phosphorylate emerin (3). Phosphorylated emerin no longer interacts with
phosphorylated lamin A/C creating the flexibility necessary for the capsid to bypass
the lamina and access INM for envelopment. In this model, emerin is the prototype
LAP and it remains to be determined if the localization and interactions of other
LAPs, such as MAN1 or LBR, are regulated by phosphorylation in the infected cell as
emerin appears to be.

Figure 3.1. PKC activity required for HSV-1 growth. Replicate cultures of HEp-2 cells were infected at an MOI of five with HSV-1(F). Cells were treated with DMSO or 10 uM pan-PKC inhibitor bisindolylmaleimide (BIM I) beginning at five hr post infection (hpi). Titer was determined at 24 hpi. Virus yields are expressed as Plaque Forming Units (PFU) per milliliter. Each data point represents the mean of five independent experiments. Error bars indicate standard deviation from the mean. p-values were determined via a paired Student T-Test.
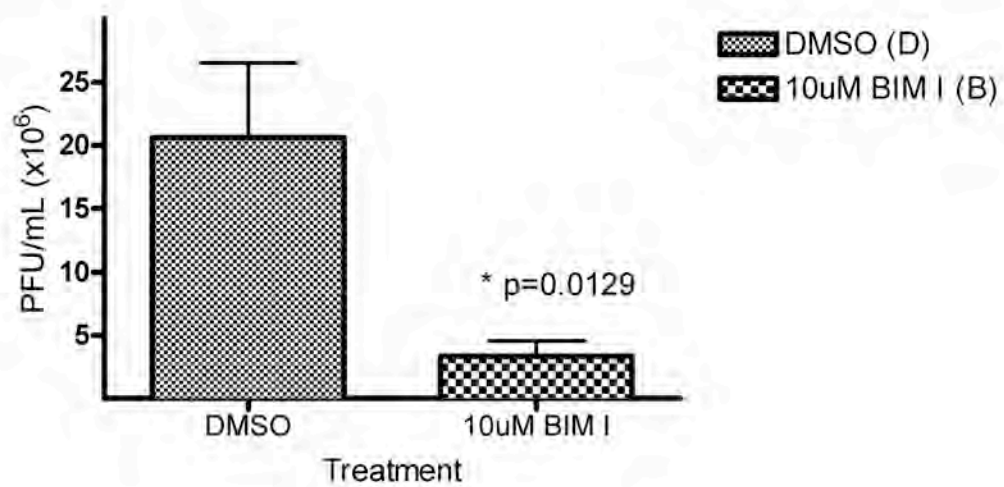
Figure 3.2. PKC inhibitor blocks PKC signaling. Digital images of total MEK 1/2 (bottom) and phos-MEK 1/2 (top) western blots. The MAPK pathway is activated following phorbol 12-myristate 13-acetate (PMA) activation of PKCs (220, 283). HEp-2 and Vero cells (not shown) were placed in the presence of 10 uM BIM I, or vehicle, for two hr and then activation of PKCs was induced by adding 20 nM PMA, or vehicle (DMSO), to the cells for 30 min. Total cell lysates were harvested and 40 ug of total protein were separated by SDS-PAGE, transferred to nitrocellulose, and then immunblotted for total or activated (phos-Ser 217/221) MEK 1/2. Vehicle (DMSO) treated HEp-2 cells have little detectable phos-MEK 1/2 (lane one). Stimulation with PMA induced a dramatic increase in the level of phos-MEK 1/2 but no change in the level of total MEK 1/2 (compare lane two to one). Pre-treatment of HEp-2 with BIM I, prior to PMA stimulation, returned the phos-MEK 1/2 level nearly to vehicle stimulation levels (compare lane three with one).

	1	2	3
DMSO	+	+	
PMA		+	+
BIM I			+

p-MEK 1/2

Total MEK 1/2
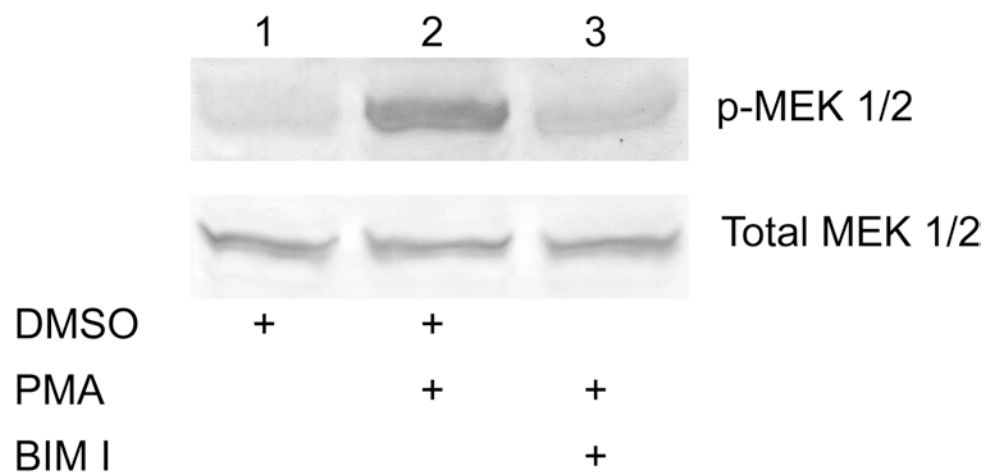
Figure 3.3. PKC inhibition blocks extra-nuclear capsid accumulation.

Shown are digital images of transmission electron micrographs (TEM) of Vero cells infected with five PFU/cell HSV-1(F). Cells were treated with vehicle beginning at five hpi with vehicle **(A)** or BIM I **(B)** fixed with glutaraldehyde at 16 hpi, and thin sectioned for TEM. The inset picture is approximately twice the magnification and shows an example of an empty capsid (A and B capsids) (arrow) and a full capsid (C capsid) (arrow head).
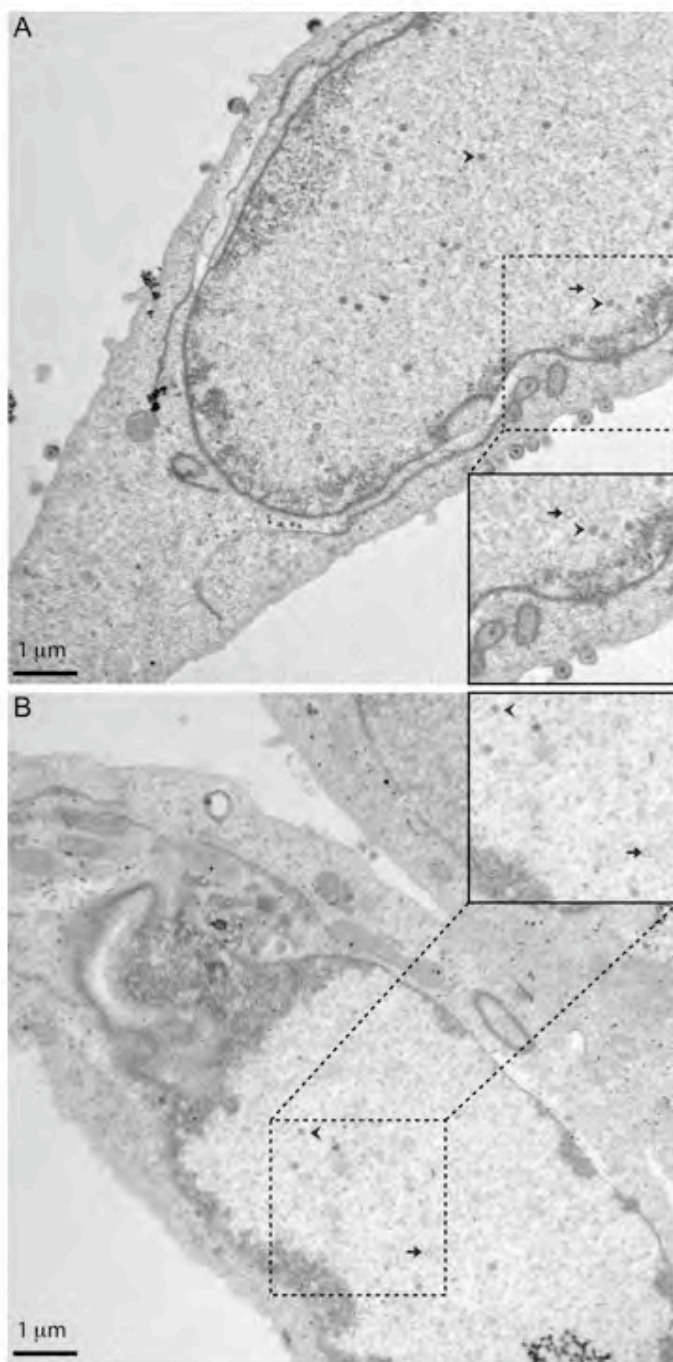
Table 3.1. BIM I treatment assembly and capsid intermediates. This table represents capsids from 20 cells for each treatment condition. The representative TEM images are shown in Figure 3.3. Vero cells were infected with five PFU/cell of HSV-1(F). Starting at five hpi, the cells began treatment with either vehicle (DMSO) or BIM I. At 16 hpi, the cells were fixed with gultaraldehyde, thin sectioned, and TEM analysis of egress intermediates was performed.

Cellular compartment	Capsid number (% of total capsids)	
	DMSO-treated	BIM I-treated
Nucleus	221 (33%)	159 (71%)
Perinuclear space/ER	1 (0%)	7 (3%)
Cytoplasm	129 (20%)	38 (17%)
Cell surface	290 (46%)	19 (9%)
Total	**631** (100%)	**223** (100%)
Mean Capsid/Cell	31.6	11.2
Median Capsid/Cell	29.0 ± 13.3^{a}	1.5 ± 11.1

[a]Average absolute deviation from median

**Figure 3.4. PKC activity required for cellular but not viral protein
synthesis.** **A)** Digital images of western blots of HEp-2 unlabeled total cell lysates
prepared from mock-infected cells or cells infected with five PFU/cell of HSV-1(F).
At 16 hpi, total cell lysates were prepared from equal numbers of cells. Cells were
DMSO (D) or BIM I (B) treated beginning at five hpi. Blots were immunoblotted
with monoclonal antibodies to VP5, pUL34, gC, and US11. **B)** Autoradiogram of ^{35}S-
methionine labeled HEp-2 cell lysates from A.

A

HSV-1(F)

Mock   D   B

VP5
gC
US11
UL34

1   2   3

B

HSV-1(F)   Mock
B   D   B   D
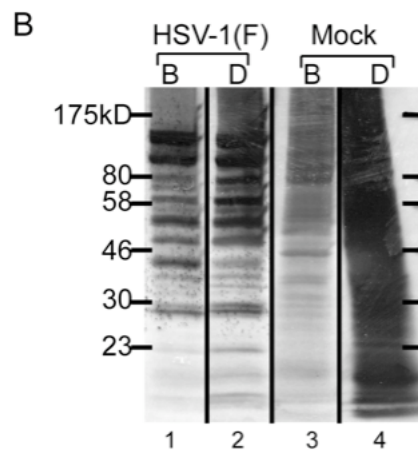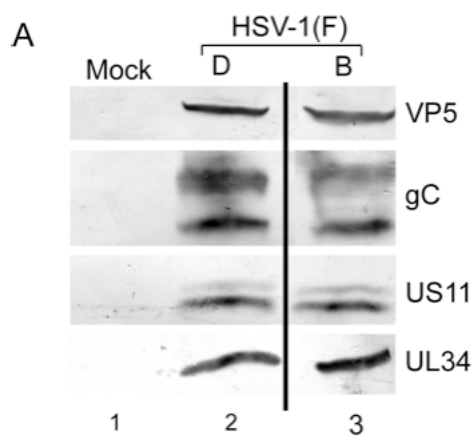
175kD
80
58
46
30
23

1   2   3   4

Figure 3.5. cPKCs nor PKC delta are uniquely required. **A)** Cells were treated vehicle (DMSO) or 1 uM Ro-31-7549 beginning at five hpi. Titer was determined at 24 hpi. Virus yields are expressed as PFU (PFU) per milliliter. Each data point represents the mean of six independent experiments. **B)** Vero cells were co-transfected for 24 hr with pCMVβ and either: pRR1072, pRR1072Rep, PRKCDwt-UL34 Duo or PRKCDdn-UL34 Duo. Complementation of the vRR1072(TK+) was performed five independent times as previously described (18). Error bars indicate standard deviation from the mean. p-values were determined via a paired Student T-Test. **C)** Digital images of western blots immunoblotted with polyclonal antibody to PKC delta (top) and monoclonal antibody to actin (bottom). Equal amounts of total protein from total cell lysates from MCF-7 or BT-549 cells were immunoblotted. **D)** Replicate cultures of MCF-7 and BT-549 cells were infected at an MOI of five with HSV-1(F). At 24 hpi total culture virus was titrated on Vero cells. Virus yields are expressed as PFU per milliliter. Each data point represents the mean of three independent experiments. Error bars indicate standard deviation from the mean.
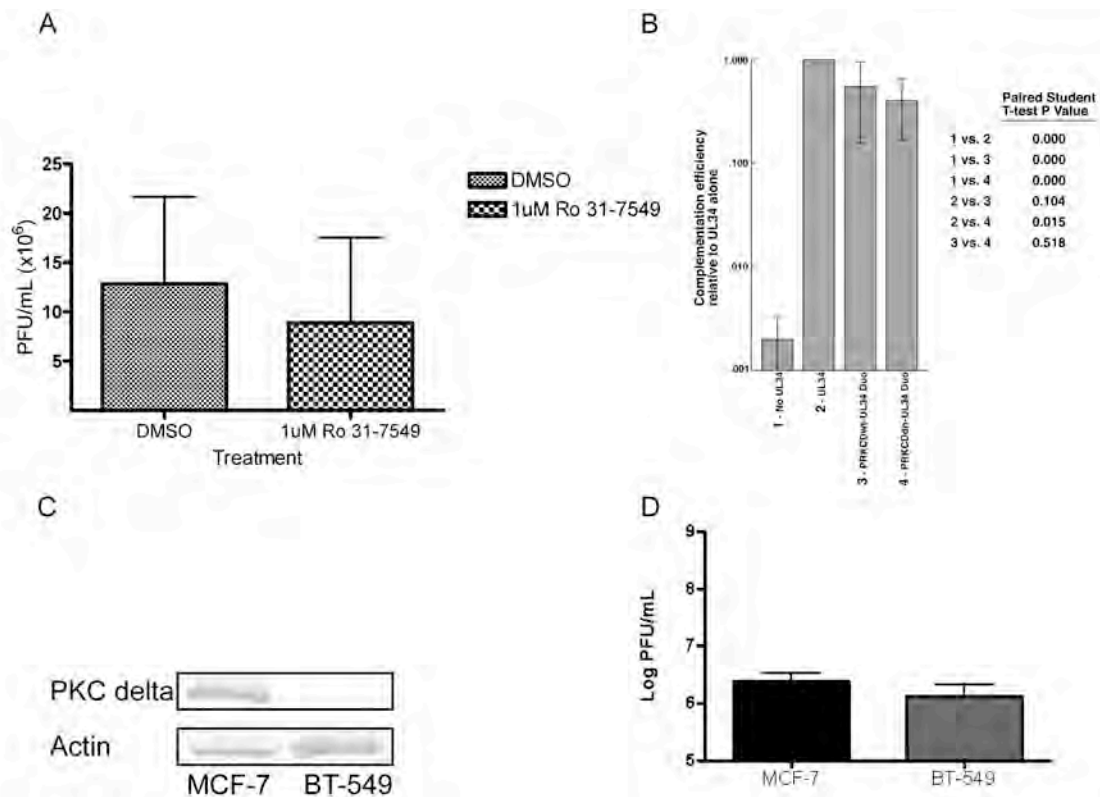
A

B

	Paired Student T-test P Value
1 vs. 2	0.000
1 vs. 3	0.000
1 vs. 4	0.000
2 vs. 3	0.104
2 vs. 4	0.015
3 vs. 4	0.518

C

PKC delta

Actin

MCF-7   BT-549

D

Figure 3.6. Confirmation of inhibition of cPKC and PKC delta activity.

A) Digital images of total MEK 1/2 (bottom) and phos-MEK 1/2 (top) western blots. A similar approach as described for BIM I, was used to test the ability of Ro-31-7549 to block cPKC signaling. HEp-2 cells were treated with 1 uM Ro-31-7549 or vehicle for two hr followed by a 30 minute incubation with 20 nM PMA. Total cell lysates were harvested and 40 ug of total protein were separated by SDS-PAGE. As expected, pre-treatment of HEp-2 with Ro-31-7549 for two hr, prior to PMA stimulation, reduced but did not eliminate the phos-MEK 1/2 level (compare lane four with one). Some induction of MEK1/2 phosphorylation in the presence of the cPKC inhibitor (lane 4) is expected due to nPKC and aPKC activities. **B)** Shown are digital confocal images. To test for efficient gene expression from the Duo plasmids, Vero cells transfected with PRKCDwt-UL34 Duo (a-f) or PRKCDdn-UL34 Duo (g-l) for 24 hr. Cells were then uninfected (a, b, c, g, h, i) or infected with UL34-null virus (vRR1072(tk+)) at an MOI of 20 (d, e f, j, k l). At 20 hpi, cells were fixed and stained with antibodies directed against Flag-PKC delta (left column) or pUL34 (center column). PKC delta is represented in red and pUL34 in blue. Similar results were observed in HEp-2 cells (data not shown). **C)** Shown are digital confocal images. To confirm that DN-PKC delta (K376M-PRKCD) functions as a dominant negative and that expression from the Duo plasmid retains this function, downstream signaling of PKC delta was tested. It was previously demonstrated that DN-PKC delta inhibited STAT3 Tyr 705 phosphorylation and consequent nuclear re-localization (86). HEp-2 cells transfected with PRKCDwt-UL34 Duo (a-f) or PRKCDdn-UL34 Duo (g-l) for 24 hr. Cells were serum starved overnight and un-treated (a, b, c, g, h, i) or treated with 1000U of IFNβ for 30min (d, e f, j, k l). Cells were fixed and stained with antibodies directed against Flag-PKC delta (left column) or p-STAT3 (center column). Transfected (white arrow) and untransfected (white arrowhead) cells.

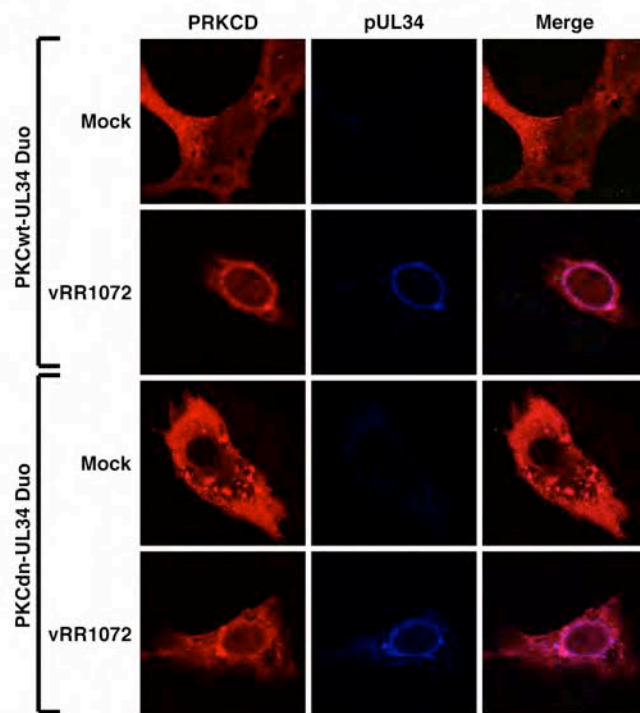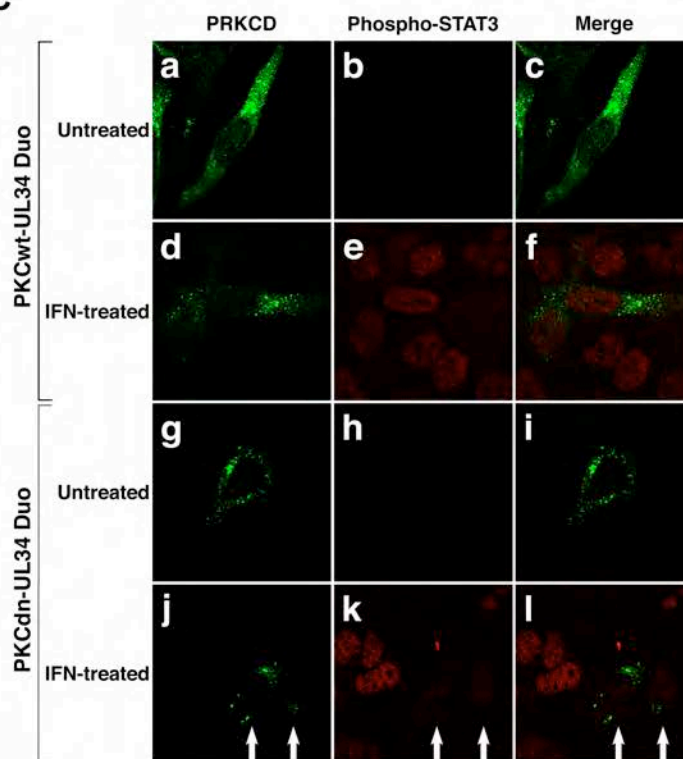**A**

Lane	1	2	3	4	
					p-MEK 1/2
Treatment					Total MEK 1/2
DMSO	+				
Ro-31-7549		+		+	
PMA			+	+	
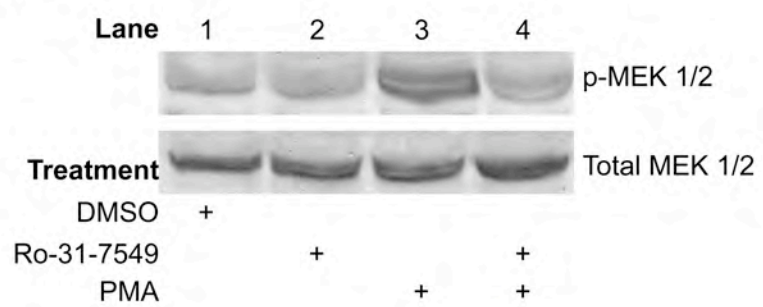
B

C

Figure 3.7. Reduced PKC delta protein levels does not significantly alter HSV-1 replication. A) The shRNA-control cell line stably expresses a non-effective shRNA, while shRNA-PRKCD-1, shRNA-PRKCD-2, and shRNA-PRKCD-3 are independently isolated clones expressing shRNAs directed towards human PRKCD mRNA. Shown are digital images of western blots immunoblotted with polyclonal antibody to PKC delta (top) and monoclonal antibody to actin (bottom) of total cell lysates of shRNA HEp-2 cell lines. Lanes 1, 4, 5, and 6 contain 40 ug of total protein, while lane 2 and 3 contain 20 ug and 10 ug respectively. This is our standard curve to compare PKC delta protein levels in the negative control cell line and knockdown lines. Using the intensity of PKC delta expression from the shRNA-PRKCD lysates lanes (lanes 4, 5, 6), we estimated the level of PKC delta knockdown by comparing the intensity to the appropriate level in the three shRNA-control lanes. shRNA-PRKCD-1 and shRNA-PRKCD-3 express approximately 30% of the control level of PKC delta while expression in shRNA-PRKCD-2 cells is reduced by 50% (compare lanes 4 and 6 to 3 and 5 to 2). **B)** Replicate cultures of HEp-2 and shRNA cells were infected at an MOI of five with HSV-1(F) virus. At 24 hpi total culture virus was titrated on Vero cells. Virus yields are expressed as PFU (PFU) per milliliter. Each data point represents the mean of three independent experiments. Error bars indicate standard deviation from the mean.

A

	shRNA-control	shRNA-control	shRNA-control	shRNA-PRKCD-1	shRNA-PRKCD-2	shRNA-PRKCD-3
PKC delta						
Actin						
ug total protein	40	20	10	40	40	40
Lane #	1	2	3	4	5	6

B

Log PFU/mL

- HEp-2
- shRNA-control
- shRNA-PRKCD-1
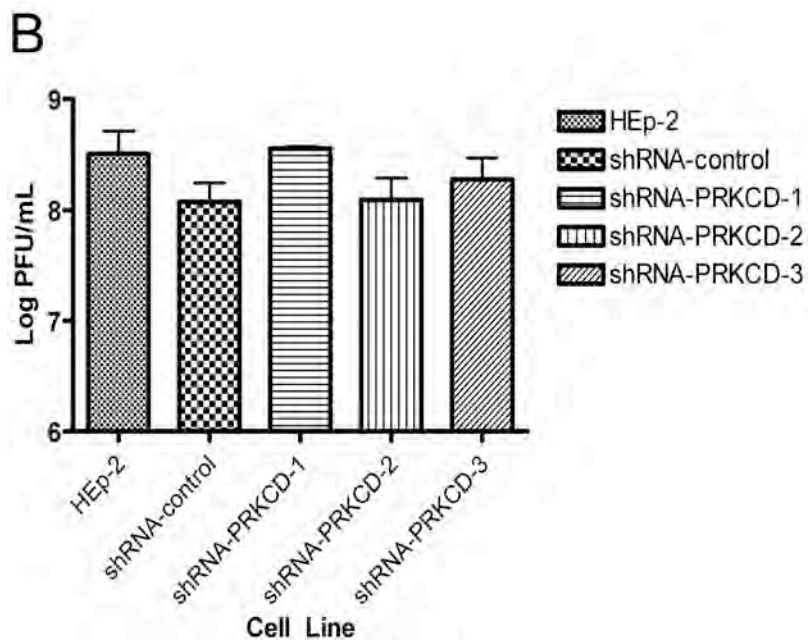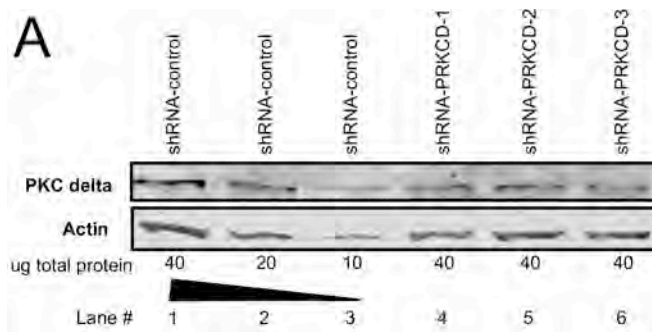- shRNA-PRKCD-2
- shRNA-PRKCD-3

Cell Line

Figure 3.8. Disruption of emerin localization in HSV-1-infected HEp-2 cells is sensitive to pUS3-mediated and Rottlerin sensitive kinase phosphorylation events but not BIM I treatment or PKC delta activity. A) Shown are digital confocal images of optical sections taken near the middle of the nuclei of HEp-2 cells that were uninfected (a-c) or infected with WT HSV-1(F) (d-i) or pUS3 kinase-dead (vRR1204) (j-o) with five PFU/cell for 20 hr and treated with vehicle (a-f and j-l) or Rottlerin (g-i and m-o) beginning at five hpi. Cells were stained with antibodies directed against emerin (left column) or pUL34 (center column). Emerin is represented in red and pUL34 in green. **B)** Digital image of western blot for emerin in HEp-2 cell nuclear lamina preparations. Cells were mock infected or infected with five PFU/cell of either WT or pUS3 kinase-dead (vRR1204) HSV-1(F). At five hpi, cells began treatment with vehicle (D), BIM I (B), or Rottlerin (R) and were harvested for lamina preparations at 16 hpi. The arrow in lane four indicates the predominant middle species, while the dots in lanes five, six, and seven indicate double or single emerin species. **C)** Shown are digital confocal images of optical sections taken near the middle of the nuclei of HEp-2 cells that were uninfected (a-c), HSV-1(F) (d-i), or pUS3 kinase-dead (vRR1204) (j-o) infected with five PFU/cell for 20 hr and treated with vehicle (a-f and j-k) or BIM I (g-i, and m-o) beginning at five hpi. Cells were stained with antibodies directed against emerin (left column) or pUL34 (center column). Emerin is represented in green and pUL34 in red. **D)** Digital confocal images taken near the center of nuclei of EGFP-Emerin HEp-2 B2 cells. Cells were un-transfected (a, b, c) or transfected with PRKCDwt-UL34 Duo (d, e, f) or PRKCDdn-UL34 Duo (g, h, i) for 24 hr. Cells were then infected with pUS3 kinase-dead (vRR1204) at an MOI of 20 for 20 hr. Cells were fixed with formaldehyde and stained with antibodies directed against FLAG-PKC delta (middle column) or pUL34 (right column). Emerin is represented in green, PKC delta in blue, and pUL34 in red.

A

	Emerin	pUL34	Merge
Mock DMSO	a	b	c
HSV-1(F) DMSO	d	e	f
HSV-1(F) Rottlerin	g	h	i
vRR1204 (K220A) DMSO	j	k	l
vRR1204 (K220A) Rottlerin	m	n	o

C

	Emerin	pUL34	Merge
Mock DMSO	a	b	c
HSV-1(F) DMSO	d	e	f
HSV-1(F) BIM I	g	h	i
vRR1204 (K220A) DMSO	j	k	l
vRR1204 (K220A) BIM I	m	n	o

B

Lane	1	2	3	4	5	6	7	8
emerin								
Treatment	Mock	D	B	R	D	B	R	Mock
		HSV-1(F)			vRR1204 K220A			

D

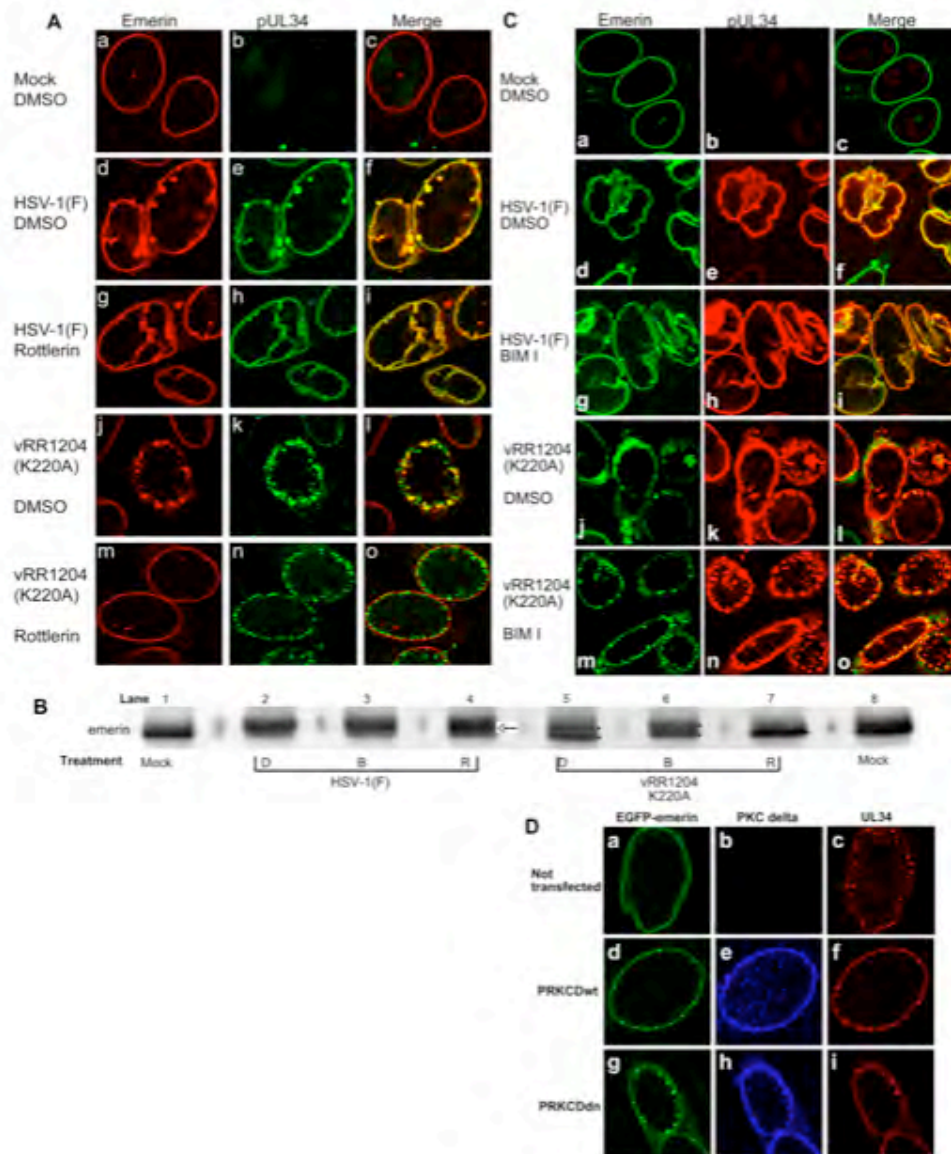|	EGFP-emerin	PKC delta	UL34
Not transfected	a	b	c
PRKCDwt	d	e	f
PRKCDdn	g	h	i

Figure 3.9. Model of HSV-1 induced nuclear lamina disruption. (1) In the un-infected cell, emerin is bound to the lamins via interaction with lamin A/C. Emerin is intended to be a prototype LAP protein and may behave in the cell similarly to MAN1 or other LAPs. (2) Upon infection with HSV-1, pUL34 and pUL31 are expressed and accumulated as a complex at the inner nuclear membrane (INM). pUL34/pUL31 recruits pUS3, the alpha-herpesvirus viral kinase, PKCs, and Rottlerin Sensitive Kinases (RttSK) to the NE to phosphorylate emerin, lamins and pUL34/pUL31. Arrows indicate that pUS3 and RttSK are known to be necessary for phosphorylation of emerin, pUL34/pUL31, and lamin B. (3) Phosphorylation of lamins and emerin, prevents there binding and creates flexibility within the lamina necessary for nuclear egress. PKCs, RttSK, and pUS3 are depicted near the INM due to their localization at the NE during infection.
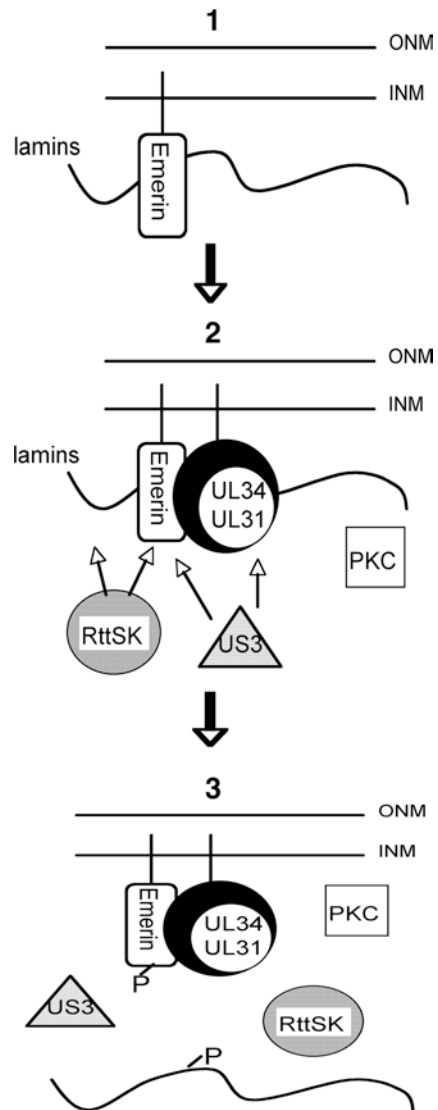
CHAPTER IV

IMPORTANCE OF ROTTLERIN SENSITVE KINASES

TO HSV-1(F) GROWTH

Abstract

Rottlerin treatment of HSV-1 infected cells has been previously associated with a decrease in HSV-1 induced phosphorylation of lamin B and emerin (142, 206). This study attempted to test the novel hypothesis that inhibition of lamina disruption via inhibition of Rottlerin Sensitive kinase(s) (RttSKs) would (i) inhibit HSV induced lamin A/C disruption and (ii) HSV-1 nuclear egress and therefore virus growth. Treatment of infected cells with the kinase inhibitor Rottlerin resulted in a growth inhibition 20-fold greater than treatment with a pan-PKC inhibitor. Rottlerin treatment was also associated with severe depression of viral late-gene expression, suggesting that non-PKC, RttSK activity was required for efficient viral late protein translation. TEM analysis suggested that RttSK(s) are required for both nuclear egress and capsid accumulation or formation. This second requirement was mostly likely due to the novel finding that RttSKs are required for infected cell protein synthesis. Another novel finding of this study, was pUS3 and PKC isoforms have redundant, cell-type-dependent activities required for efficient late-gene viral protein translation. Contrary to our hypothesis, lamin A/C, an emerin binding partner and phosphorylated during infection, virus induced-disruption was not altered by RttSK inhibition. This suggests that (i) HSV-1 encodes different mechanisms for lamin A/C, emerin, and lamin B disruption and (ii) RttSK(s) are not involved in lamin A/C disruption.

<center>Introduction</center>

Rottlerin has been marketed as a specific PKC delta inhibitor because at ten uM (IC_{50}=3-6) it does not inhibit other PKC isoforms (94). To inhibit other PKC isoforms, five to 17 times more of the substance must be used (94). It does, however, at ten-20 uM, inhibit other cellular kinases such as calmodulin kinase II, AKT, PKA, JNK1, PDK1, MSK-1 and GSK3 beta (50, 253). Rottlerin may also have many PKC-delta independent protein and kinase activity effects. These Rottlerin-dependent, PKC delta-independent effects include: induction of autophagy, inhibition of neuronal transport, inhibition of PKC delta tyrosine signaling in bone marrow-derived mast cells (BMMC), up-regulation of heme oxygenase-1 (HO-1) expression, apoptosis induction due to ER stress via CHOP, and mitochondrial uncoupling (149, 157, 158, 205, 252, 256, 262). The human emerin protein contains kinase recognition sequences for the Rottlerin sensitive kinases: AKT, PDK1, JNK1 alpha, GSK3beta, PKA and MSK1 (21). Human emerin hyperphosphorylation induced by HSV-1 infection was sensitive to Rottlerin treatment (Figure 2.3 and 2.4) (142).

Despite the evidence for PKC delta recruitment to the nuclear envelope, this particular PKC isoform does not appear uniquely required for HSV growth (Chapter III) (143). It also is clear that the chemical Rottlerin inhibits cellular enzymes outside of the PKC family that phosphorylate emerin (Chapter 3.8 C). These data prompted the naming of this class of kinases as "Rottlerin sensitive Kinases" (RttSK). These kinase(s) are sensitive to Rottlerin but are not limited to PKC delta (253). The RttSK responsible for pUL34-dependent emerin phosphorylation remains unknown but possible candidates include: AKT, PDK1, JNK1 alpha, GSK3beta, PKA and/or MSK1 (253) (Figure 3.8).

Although Rottlerin inhibition may not be specific to PKC delta, we can use it as a tool to understand HSV lamina disruption. It appears that the class of RttSKs is important in phosphorylating emerin and lamin B (142, 206). While PKC delta may not

the critical kinase, we sought to determine if RttSK activity was required for (i) lamina disruption (ii) and if RttSK dependent-phosphorylation induced disruption is necessary for herpesvirus nuclear egress.

This was the first study to look at how RttSK(s) are involved in disruption of the nuclear lamina. The data presented in this chapter demonstrate novel findings that (i) RttSKs are required for efficient single-step growth of HSV-1 due to a block in both nuclear egress and viral protein synthesis; (ii) lamin A/C (an emerin binding partner) localization in the infected cell is not regulated by RttSK(s) unlike emerin suggesting that the virus has multiple mechanisms for disruption of lamina components; (iii) In a cell-type dependent manner, pUS3 and PKC isoforms have a redundant function maintaining viral protein synthesis.

<u>Materials and Methods</u>

Cells, viruses, and chemicals. HEp-2 and Vero cells were maintained as previously described (232). The properties of HSV-1(F), vRR1072(tk+) (UL34-null mutant virus), vRR1202 (US3-nullvirus), vRR1204 (pUS3 kinase-dead virus K220A), and repair viruses for vRR1072(tk+) (vRR1072Rep) and vRR1202 (vRR1202Rep) were previously described and characterized (224, 232, 236). Rottlerin (Santa Cruz Biotechnology) was diluted in DMSO to a stock concentration of 10 mM and used to treat cells at 10 uM. Bisindolylmaleimide I (BIM I- LC Laboratories) was diluted in DMSO to a stock concentration of 500 uM and used at 10 uM used to treat cells.

Indirect Immunofluorescence. Indirect immunofluorescence (IF) was performed for pUL34, and lamin A/C as previously described (20, 224). Briefly, cells were fixed with 4% formaldehyde for 10 min, washed with phosphate buffered saline (PBS), and then permeabilized and blocked in the same step with IF buffer (0.5% Triton X-100, 0.5% Sodium Deoxycholate, 1% BSA, 0.05% Sodium Azide diluted in PBS) for 1

hour. Primary antibodies were diluted in IF buffer as follows: chicken anti-UL34, 1:1000 (224); mouse monoclonal anti-lamin A/C, 1:1000 (Santa Cruz Biotechnology, sc-7292).

Secondary antibodies were diluted 1:1000 in IF buffer as follows: Alexa Fluor goat anti-chicken IgG (Invitrogen, A11001), Alexa Fluor goat anti-mouse IgG (Invitrogen, A1104). Slo-fade II (Invitrogen, Molecular Probes) was used to mount cover slips on glass slides. All confocal microscopy work was done with a Zeiss 510 microscope.

SDS-PAGE sample preparations. For analysis of viral protein expression, infected or uninfected cultures containing 2×10^6 cells were washed twice with PBS, scraped into PBS, pelleted at low speed, total soluble protein was extracted with acetic acid, and precipitated with acetone as previously described (229). Total protein concentration was determined using BioRad Dc Protein Assay (BioRad Laboratories).

Western blot. The antibodies and dilutions used were: mouse monoclonal anti-US11, 1:1000 (231); chicken monoclonal anti-UL34, 1:1000 (224); mouse monoclonal anti-VP5, 1:1000 (Meridian Life Science, #C05014M); mouse anti-actin, 1:1000 (Sigma, A3853); mouse monoclonal anti-gC, 1:1000 (Virusys, H1A022M). All antibodies were diluted in three percent (w/v) GT-TBS) as described in Chapter II. Alkaline phosphatase conjugated anti-mouse, anti-rabbit, and anti-chicken secondary antibodies were incubated with the appropriate blot for 30 min (Sigma, mouse: A3562, rabbit: A3687 Aves Lab, chicken: AP1001).

Virus Growth. Single-step growth analysis was performed as previously described (225, 232). Briefly, replicate cultures of cells were infected at an MOI of five, and residual virus was removed or inactivated with a low-pH-buffer wash. Cells were untreated or treated with DMSO or Rottlerin, at five, seven, nine, and twelve hpi. At 20 hpi (for Figure 4.9 B), virus was harvested and total culture PFU were calculated by titration on Vero cells. For Figure 4.1, virus was harvested at the indicated times prior to total culture PFU calculation by titration on Vero cells.

Transmission Electron Microscopy.   Vero cells were infected with HSV-1(F) at an MOI of five and at five hpi cells were treated with DMSO or Rottlerin. At 16 hpi, cells were fixed with glutaraldehyde, processed, and analyzed by electron microscopy as described previously (232).

^{35}-S-methionine Labeling. 2×10^6 HEp-2 cells were uninfected or infected with five PFU/cell of HSV-1(F) and beginning at five hpi, the cells were treated with DMSO (D), Rottlerin (R), or BIM I (B). At nine hpi, the cells were starved for methionine by replacing the media with methionine minus media (Gibco, 21013) with DMSO, Rottlerin, or BIM I. Beginning at 14 hpi, each sample was labeled for two hr with five uCu of ^{35}S-methionine. Total cell lysates were prepared at 16 hpi by the acid preparation as described above (229). Total protein from equal numbers of cells was separated on an SDS-PAGE gel and visualized by autoradiography.

Inhibitor toxicity assays. 2×10^5 HEp-2 and Vero cells were plated in a 48 well dish in triplicate. 12 hr later, cells were treated with vehicle (DMSO) or Rottlerin. 24 hr post-treatment, the ATPLite assay (Perkin-Elmer, #6016739) was followed per manufacture's directions. This was repeated three independent times to determine that at the indicated dosage, these kinase inhibitors did not induce significant toxicity compared to vehicle (DMSO). ATPLite data was not included since HEp-2 and Vero cells both tolerated treatment.

Results

Rottlerin inhibits viral growth. PKC delta is recruited to the nuclear rim in HSV-1-infected cells and Rottlerin treatment blocks emerin and lamin B phosphorylation (142, 207). These data suggest that RttSK(s) are important in lamina remolding prior to herpes nuclear egress, and suggest the hypothesis that RttSK activities may be necessary for nuclear egress of HSV capsids. To test the hypothesis that RttSKs are required for late events of HSV-1 growth, HEp-2 cells were infected with five PFU/cell of WT HSV-

1(F), treated with Rottlerin or vehicle (DMSO) beginning at five hpi, and the production of virus infectivity was measured at various times post infection (Figure 4.1). Rottlerin treatment of WT virus-infected cells reduced peak infectivity production by 100-fold (2 log) compared to vehicle treatment in HEp-2, Vero, and SNB-19 cells (Vero and SNB-19 data not shown). These data suggest that inhibition of RttSK(s) blocks efficient single-step growth of HSV-1. Notably, the Rottlerin treatment-dependent drop in infectivity was markedly enhanced (Rottlerin 100-fold decrease Figure 4.1) above inhibition of the PKC family alone (BIM I 5-fold decrease (Figure 3.1)). This 20-fold increase in inhibition suggests that Rottlerin may also inhibit kinases outside of the PKC family that are important for growth of HSV. Toxicity, determined using the ATPLite system, was not observed at the concentrations used for any of the inhibitors used in this study (data not shown).

HSV-1-induced emerin hyperphosphorylation is dependent on pUS3 and kinases sensitive to Rottlerin (142). These RttSKs may be acting directly or indirectly to induce emerin phosphorylation. This dual requirement also suggests that pUS3 and RttSKs might have overlapping or redundant functions in HSV-1 infection. Deletion of pUS3 leads to at most a 20-fold (1.3 log) reduction (236) while inhibition of RttSK(s) individually lead to a modest growth inhibition of 100-fold (2 logs) (Figure 4.1 WT virus). However, we hypothesized that loss of both activities would result in the dramatic inhibition of late events in HSV-1 replication since they both are known to phosphorylate components of the nuclear lamina. To test this hypothesis, HEp-2 cells were infected with five PFU/cell of US3-null (vRR1202) or pUS3 homologous-repair virus (vRR1202Rep), treated with Rottlerin or vehicle (DMSO) beginning at five hpi, and production of infectivity was measured at the indicated times post infection (Figure 4.1). As previously observed, the US3-null (vRR1202) virus had a 10-fold (one-log) decrease in peak infectivity compared to WT HSV-1(F) in HEp-2 cells (236). Rottlerin treatment of US3-null (vRR1202)-infected cells reduced viral growth to background levels. A

similar degree of virus growth inhibition was observed when RttSK were inhibited with Rottlerin in HSV-1 infected Vero and SNB-19 cells (not shown). The pUS3 homologous-repair virus (vRR1202Rep) responded to Rottlerin treatment similarly to WT virus with a 100-fold (two-log) decrease in peak infectivity (Figure 4.1).

Rottlerin treatment blocks extra-nuclear capsid accumulation and capsid assembly. To determine if the reduced infectivity or new virus production in the absence of RttSK activity is due to a block in nuclear egress (Figure 4.1), Vero cells were infected with five PFU/cell of WT HSV-1(F), treated with Rottlerin, or vehicle beginning at five hpi, fixed at 16 hpi, and processed for TEM analysis (Figure 4.2). DMSO-treated, WT virus-infected cells contained empty (non-DNA capsids) and full (DNA-containing) capsids in the same four distinct sub-cellular compartments as observed in Figure 3.3 (Figure 4.2 A). Most capsids found in Rottlerin-treated cells were localized in the nucleus and no mature virus particles were found at the cell surface (Figure 4.2 B). Capsids were counted in the four distinct compartments in ten cells for each treatment condition and represented as a percentage of total capsids (Table 4.1). While DMSO-treated cells demonstrated 35% of virions in the nucleus at 16 hpi, Rottlerin-treated cells retained a higher proportion of nuclear virions (81%). Additionally, while 54% of virions in DMSO-treated infected cells were found at the cell surface, no cell surface-associated virions were detected in Rottlerin-treated infected cells. Treatment with Rottlerin also decreased the total number of capsids by roughly three fold (Table 4.1, DMSO-353 vs Rtt-110).

RttSKs act prior to nine hpi for maximal inhibitory effect. The significant reduction in the fraction of extra-nuclear capsids produced in Rottlerin-treated, HSV-1(F)-infected cells compared to vehicle control cells, indicated this inhibitor blocked nuclear egress (Table 4.1). However, the reduction in the total number of capsids observed in the presence of this inhibitor suggested its effect on nuclear egress might result from inhibition of viral life cycle events preceding capsid assembly, including

expression of genes required for capsid assembly and nuclear envelopment. Results from TEM and the SSGC (Figure 4.1) suggested that Rottlerin inhibits viral growth in at least two ways: (i) blockage of viral events prior to egress and (ii) blockage of nuclear egress of capsids that were properly assembled. The five hpi time of addition for Rottlerin was selected previously for two reasons: (i) this time of addition showed the maximal inhibition of emerin hyperphosphorylation and (ii) to allow early viral life cycle events to proceed in the absence of the drug (142). The second issue may be important if RttSKs are essential for entry, IE, or E gene expression. These viral lifecycle steps are essential for replication of viral genomes and assembly of new capsids prior to nuclear egress. However, since TEM data (Figure 4.2 and Table 4.1) suggested that this drug was having effects on multiple viral lifecycle events, we wanted to test the hypothesis that the egress defect could be un-coupled from the assembly defect(s). If RttSK activities on egress and capsid assembly could be un-coupled, then at some time point infection inhibition of RttSK would inhibit egress but not assembly or vice versa. If the activities could not be uncoupled, then at every point post-infection the kinases were inhibited both activities would be affected in the same manner.

To test the uncoupling hypothesis, HEp-2 cells were infected with five PFU/cell of HSV-1(F) or US3-null (vRR1202). The cells began treatment with DMSO (-) or Rottlerin (+) at five, seven, nine or 12 hpi and total cell lysates were harvested at 20 hpi. These samples were subjected to western blot analysis for the following proteins: VP5 (leaky-late and major capsid protein), pUS11 (archetype true-late gene product), and pUL34 (leaky-late and required for nuclear egress) (Figure 4.3 A). Rottlerin treatment at five hpi, significantly reduced pUS11 and VP5 protein levels in infected cells compared to DMSO treatment in WT and US3-null infections (compare lanes 2 and 3, 4 and 5). Interestingly, pUL34 expression was not altered by significantly altered by Rottlerin treatment (compare lanes two and three, four and five). As Rottlerin treatment was delayed, accumulation of VP5 and pUS11 increased (compare lanes 14 and 15 to two and

three, 16 and 17 to four and five). In the absence of US3, the effect of Rottlerin treatment on VP5 and UL34 expression was not as severe as in the WT infected samples at five, seven, or nine hpi (compare lane three and five, nine and seven, 13 and 11). Similar results were obtained in Vero cells (data not shown).

We also wanted to test if Rottlerin effect on viral growth (Figure 4.1) was dependent on time of addition and could be correlated to the effect on late-gene expression by performing a time-course of additions of Rottlerin in a viral growth assay (Figure 4.3 B). HEp-2 cells were infected with five plaque forming units (PFU)/cell of WT, US3-null (vRR1202), or US3-null-homologous Repair (vRR1202Rep) virus, treated with Rottlerin or vehicle (DMSO) beginning at five, seven, nine, or 12 hpi, and production of infectivity was measured at 20 hpi (Figure 4.3 B). As observed in Figure 4.1, addition of Rottlerin at five hpi, leads to a dramatic reduction in infectivity of WT, US3-null, and US3-null-Rep viruses (Figure 4.3 B). The longer infection is allowed to precede without Rottlerin, the smaller the effect of the drug (compare 12 hpi to five hpi, Figure 4.3 B). This proceeded in a linear fashion in theWT and US3-null-Repair infections demonstrating that the effects of Rottlerin on (i) late-gene expression and (ii) new virus production cannot be uncoupled in this assay (compare to Figure 4.3 A). In the US3-null infected cells, Rottlerin treatment at five, seven, and nine hpi lead to a two-log decrease in PFU. At 12 hpi treatment, the addition of Rottlerin did not appear to make a significant difference to US3-null replication. This altered the response kinetics of the WT virus (linear) and US3-null (non-linear) to Rottlerin. Similar results were observed in Vero cells (data not shown).

RttSKs are required for efficient viral and cellular translation. Data from Figure 4.3 A suggested that RttSK(s) were important early (five to nine hpi) for either (i) protein synthesis or (ii) protein stability. To test the hypothesis that Rottlerin inhibits global protein synthesis, synthesis of viral late-gene proteins were tested in cells infected in the presence of DMSO, Rottlerin, and BIM I. HEp-2 cells were infected with five

PFU/cell of HSV-1(F) and beginning at five hpi, the cells were treated with DMSO (D), Rottlerin (R), or BIM I (B). Beginning at 14 hpi, cells were labeled for two hr with ^{35}S-methionine. Total cell lysates were prepared at 16 hpi and protein from equal numbers of cells was separated by SDS-PAGE and visualized by autoradiography (Figure 4.4 A). Both Rottlerin and BIM I treatment reduced mock-infected HEp-2 and Vero protein synthesis (compare lanes two and three to one, Figure 4.4 A and B) as previously observed (253, 283) (Figure 3.3). Rottlerin treatment inhibited uninfected protein synthesis to a larger degree than BIM I (compare lanes two and three, Figure 4.4 A and B). Upon infection with HSV-1(F), host protein synthesis was turned off and viral protein synthesis was evident in DMSO-treated samples (compare lane one and four). When infected cells were treated with Rottlerin, viral protein synthesis was significantly decreased (compare lane five to four) but BIM I treatment had little or no effect on viral protein synthesis (compare lane six to four). In contrast to BIM I treatment, Rottlerin significantly inhibits synthesis and accumulation of viral late proteins and its effects of nuclear egress cannot be uncoupled form its other effects on protein translation (4.4 A, compare lane five and six). Similar results were obtained in Vero cells (Figure 4.4 B).

pUS3 and PKC have redundant functions for viral protein synthesis in HEp-2 but not Vero cells. It has been recently demonstrated that pUS3 is involved in regulating viral protein synthesis (40). To determine if PKC or RttSKs are functioning redundantly with pUS3 for efficient viral late-gene protein synthesis, HEp-2 cells were infected with five PFU/cell of US3-null (vRR1202) or US3-null-homologous Repair (vRR1202Rep). The samples were treated and prepared as described above for WT virus (Figure 4.4 A). US3-null infected-Rottlerin treated cells were not additionally inhibited for protein translation compared to WT (compare lane eight to five) but US3-null infected-BIM I treated cells were (compare lane nine to six). Results with the US3-null-Repair were comparable to WT infection (compare lanes ten-12 to four-six). Results differed in Vero cells (Figure 4.5 B) in that addition of BIM I to US3-null infected cell

did not produce a block to translation as it did in HEp-2 cells (compare lanes nine from Figure 4.5 A and B).

Lamin A/C disruption is not affected by Rottlerin treatment. Lamin A/C, an emerin binding partner, is also redistributed in a pUS3- and pUL34- dependent manner during HSV infection (20). In US3-null or pUS3-kinase dead infections, lamin A/C is disruption is more extreme than WT infection and large holes in the lamin A/C are created (20). Lamin A/C is a substrate of pUS3 while lamin B is phosphorylated upon HSV-1 infection by a RttSK (188, 207). It is unknown if RttSK(s) regulate lamin A/C phosphorylation and disruption. Additionally, emerin is a lamin A/C binding-partner that is regulated by RttSK-dependent phosphorylation in the infected cell (43) (Figure 3.8 A).

To test the hypothesis that lamin A/C localization would be sensitive to pUS3-dependent and Rottlerin-sensitive phosphorylation, HEp-2 cells were infected with US3 kinase-dead (vRR1204) virus at five PFU/cell and treatment began at five hpi with either vehicle (DMSO) or Rottlerin. At 20 hpi, the infected, treated cells were fixed and stained for lamin A/C and pUL34, and visualized by confocal microscopy (Figure 4.6). The holes created in the lamin A/C network are most easily viewed in confocal microscopy with an optical plane near the top of the infected nuclei (20). DMSO-treated, US3 kinase-dead (vRR1204)-infected cells demonstrated the previously observed extreme lamin A/C disruption resembling Swiss cheese (Figure 4.6 A) with pUL34 staining in puncta (Figure 4.6 B) that do not co-localize with lamin A/C (Figure 4.6 C). No appreciable change was observed upon treatment with Rottlerin, indicating that neither lamin A/C nor pUL34 localization is sensitive to Rottlerin treatment (Figure 4.6 D,E,F).

Discussion

Caution: Rottlerin ahead. Recruitment of PKC delta to the nuclear rim in a pUL34-dependent manner in HSV-infected cells suggested that PKC delta might be required for HSV replication due to its function in lamina disruption prior to nuclear

egress (207). We, and others, have previously used Rottlerin as a specific PKC delta inhibitor and found that Rottlerin treatment inhibits HSV-induced lamin B and emerin phosphorylation (142, 207). It has since become clear that the effectiveness and specificity of Rottlerin (Rtt) as a PKC delta inhibitor in intact cells are highly questionable, and a functional role for PKC delta in lamin B and emerin phosphorylation in HSV infected cells needs to be re-examined (253).

Rottlerin treatment significantly inhibits virus growth. Treatment of infected cells with the putative PKC delta inhibitor Rottlerin (Figure 4.1) resulted in 20-fold greater fold (~1.3 log) decrease in inhibition of virus infectivity than treatment with a pan-PKC inhibitor (BIM I) (Figure 3.1). This 20-fold decrease suggests that non-PKC, Rottlerin-Sensitive Kinase (RttSK) activity also contributes to HSV-1 infectivity. TEM analysis suggested that RttSK are required for: (i) nuclear egress of capsids that are formed and (ii) capsid accumulation or formation (Figure 4.2 and Table 4.1). Rottlerin also appears to significantly inhibit late gene protein synthesis and accumulation (Figure 4.3 and 4.4), an event critical to virion assembly. Thus it appears that Rottlerin has at least two effects on the HSV-1 life cycle that cannot be uncoupled in a WT infection: (i) inhibition of viral protein synthesis and (ii) nuclear egress. At this time, all that can be concluded is that the capsids that are assembled in the presence of Rottlerin cannot egress from the nucleus efficiently.

Rottlerin's effects on protein translation created the possibility that nuclear egress was blocked due to failure to express or accumulate the essential nuclear egress protein: pUL34. However, it appears this does not happen in Rottlerin-treated cells (Figure 4.3 A). It seems reasonable to assume that the few capsids that are made in the presence of Rottlerin, do have pUL34 for nuclear egress. It is interesting that pUL34, a leaky-late gene, is not inhibited as severely as VP5 or pUS11 (Figure 4.3A). It is also interesting that in the absence of pUS3, pUL34 seems further stabilized in the presence of Rottlerin

(lanes three to five). Further analysis of pUL34 protein stability or translation regulation could determine the mechanism of this protection.

Protein translation inhibition. Data from Chapter III demonstrated that protein synthesis in uninfected HEp-2 and Vero cells is dependent on PKC activity, but protein synthesis in wild-type HSV-infected cells is not (Figure 3.5 and Figure 4.4). Therefore, HSV infection apparently protects the infected cell protein synthesis machinery from the effect of PKC inhibition. There are observable changes at 80K, 56K, and 40K but they were not reproducible. These data suggest that BIM I mediated inhibition of nuclear egress is not due to inhibition of late gene expression, and are consistent with the hypothesis that inhibition of PKCs has a specific inhibitory effect on nuclear egress (Chapter III). PKC isoforms have been identified mostly as positive regulators of translation in different cell types and in response to different stimuli. Positive effects on translation have mostly been attributed to indirect signaling effects through Erk, MAPK or mTOR signaling pathways (2, 3, 104, 136, 233, 283), but cPKCs have also been reported to directly phosphorylate eIF4e (285). The difference between infected and uninfected cells in PKC dependence of protein synthesis suggests that HSV infection provides a functional substitute for PKC in maintenance of protein synthesis. HSV-1 is known to protect host protein synthesis from protein kinase R (PKR)-induced shut-off through pUS11 and ICP34.5 (183). It may be that the virus encodes or induces other activities to maintain robust synthesis of viral proteins.

RttSKs, in contrast to PKCs, are critical to maintenance of protein translation in HSV-1 infected cells (Figure 4.4). Rottlerin is thought to inhibit protein translation in uninfected cells via the inactivation of the mTOR pathway (253, 283). It has been previously documented that herpesviruses activate the mTOR pathway (4, 28, 183, 280, 281). It therefore seems likely that viral protein translation is blocked in the presence of Rottlerin due to the inability to activate the mTOR pathway. This hypothesis however will have to be directly tested. This could be accomplished by testing activation of

different mTOR components in Rottlerin-treated HSV-1 infected cells or testing HSV-1 infectivity (and translation) in MEFs that lack components of the mTOR pathway.

Rottlerin inhibits most (pUS3- and non-pUS3 dependent) late-viral protein translation in both Vero and HEp-2 cells. Unlike BIM I, Rottlerin severely inhibits protein translation in both infected and uninfected cells (Figure 4.4). Both pUS3-dependent and –independent translation is sensitive to RttSK inhibition. This makes interpretation of any viral growth data impossible due to the multiple points of action of Rottlerin (Figure 4.1). The analysis of protein synthesis, however, helps illuminate the results from the time-course data (Figure 4.3 A and B). When infection is allowed to proceed longer, more viral proteins are made at WT levels prior to the shut off of protein synthesis by Rottlerin treatment. Rottlerin treatment at 12 hpi has little effect on viral growth because most of the late proteins have been made, the effects are not being amplified by decreasing amounts of genome replication, and most of the capsids that were made previous to 12 hpi, egressed normally (Figure 4.3 A and B). The Rottlerin effects on viral growth cannot be delineated and these results should be a caution to scientists about the use of small molecule inhibitors in live cells.

pUS3 and PKC isoforms act redundantly for viral late-protein translation in HEp-2 cells. PKC inhibition and pUS3 deletion leads to a dramatic decrease in viral late-protein translation in HEp-2 cells (Figure 4.4 A lane nine). Interestingly, this did not occur with BIM I treatment in Vero cells (Figure 4.4 B, lane nine). This suggests that pUS3-dependent translation is regulated in different cell types by different schemes. One possible explanation of the mechanism of the cell type specificity, is expression of cellular kinases that have redundant functions with pUS3 that are only expressed in Vero cells but not HEp-2. The identity of the cellular kinase or regulation pathway remains unknown but additionally study would add to our current understanding of (i) pUS3 function in viral translation, (ii) PKC regulation of viral translation, and (iii) regulation of redundant pUS3 functions with cellular kinases.

Lamin A/C virus induced-disruption was not altered by RttSK inhibition.
Localization of lamin A/C and pUL34 appear to be regulated by non-RttSK(s) (Figure
4.5). Emerin binds to both lamin A/C and pUL34 (43, 142). Lamin A/C is also
phosphorylated by pUS3 (188). Using conditions shown previously to completely ablate
emerin hyperphosphorylation, it appeared that, in fact, emerin connections to the lamina
were maintained (Figure 3.8 A). Using these same conditions, it appears that lamin A/C
and pUL34 do not follow emerin's relocalization in the absence of pUS3 and presence of
Rottlerin (Figure 4.6). It appears that HSV-1 has developed multiple mechanisms for
dealing with the different lamina components. These data would also suggest that the
mechanism of lamina disruption required for nuclear egress is not mediated by a "one
size fits all kinase". It would be of interest to determine what molecular signal
determines pUL34-emerin and emerin-lamin A/C interactions during HSV infection. It
would also be interesting to determine if the RttSK that is required for HSV-1-induced
emerin hyperphosphorylation, is the same as the lamin B. It is also imperative to the
herpes egress and EDMD fields that the identity of the infection–induced phosphorylated
residue on emerin is identified and if phosphorylation of that residue on emerin does
indeed detach emerin from binding to lamin A/C.

Figure 4.1. Rottlerin Sensitive kinases (RttSK) are important for HSV-1(F)

SSSG. Replicate cultures of HEp-2 cells were infected at an MOI of five with HSV-1(F),

US3-null (vRR1202), or US3 null-homologous Repair (vRR1202-Rep) virus. Cells were

DMSO or 10 uM Rottlerin treated beginning at five hpi. Virus yields are expressed as

plaque forming units (PFU) per milliliter. Each data point represents the mean of at least

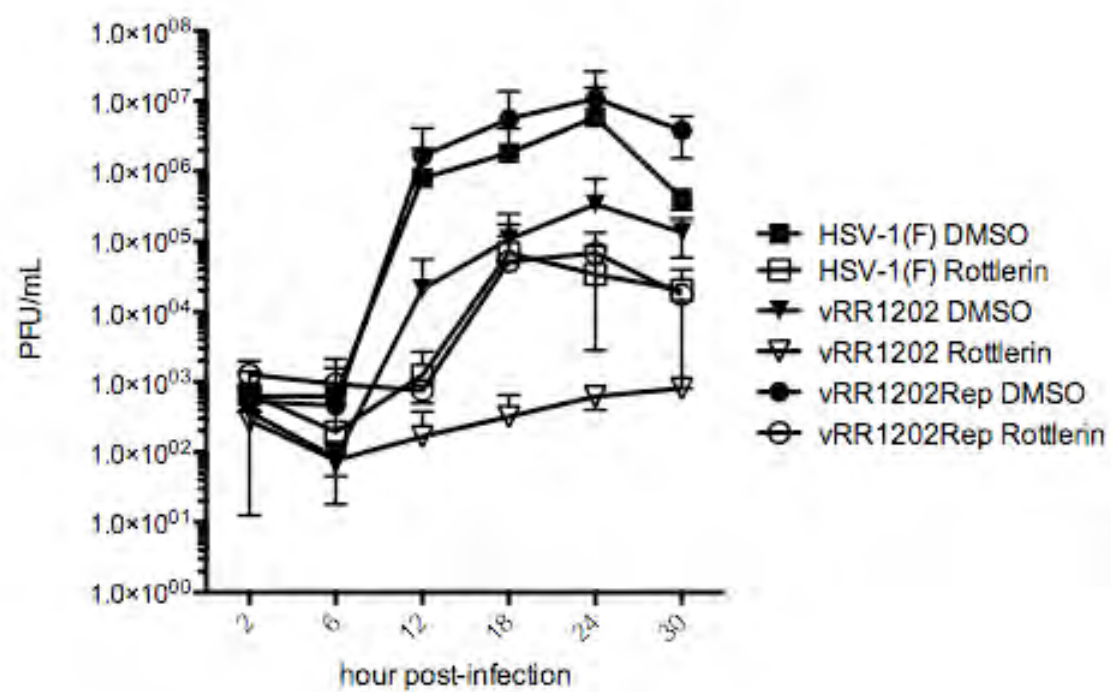three independent experiments. Error bars indicate standard deviation from the mean.

Figure 4.2. Rottlerin inhibits capsid accumulation and nuclear egress.

Shown are digital images of transmission electron micrographs (TEM) of Vero cells infected with five PFU/cell HSV-1(F). Cells were treated with vehicle beginning at five hpi with vehicle **(A)** or Rottlerin **(B)** fixed with glutaraldehyde at 16 hpi, and thin sectioned for TEM. Arrowheads indicate capsid location in the following cellular compartments: 1, nuclear; 2, perinuclear/ER; 3,cytoplasm; 4, cell surface. The inset pictures are twice the magnification and show an example of an empty capsid (*) and a full capsid (#).
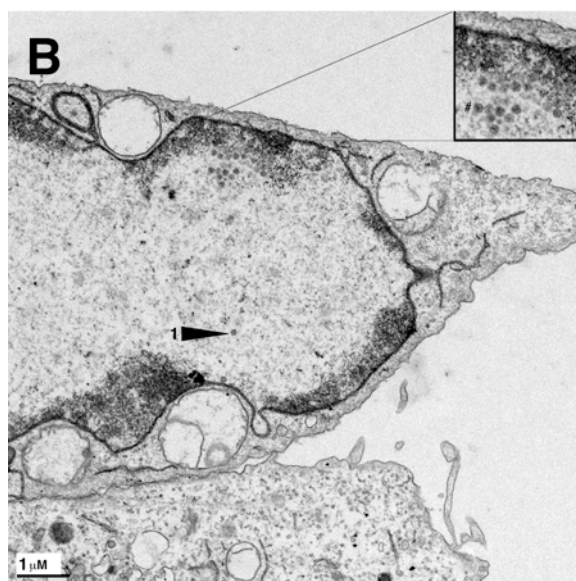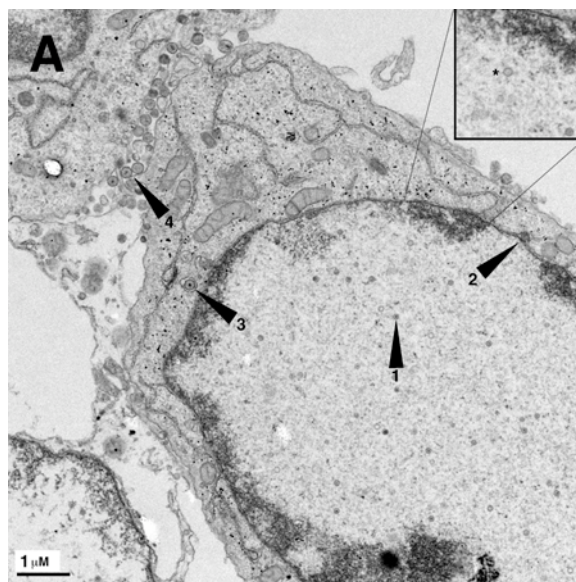
Table 4.1. Rottlerin treatment assembly and capsid intermediates. This table represents capsids from ten cells for each condition. Representative TEM images are shown in Figure 4.2. Vero cells were infected with five PFU/cell HSV-1(F). Cells were treated with vehicle beginning at five hpi with vehicle (DMSO) or Rottlerin, fixed with glutaraldehyde at 16 hpi, and thin sectioned for TEM.

Cellular compartment	Capsid number (% of total capsids)	
	DMSO-treated	Rottlerin-treated
Nucleus	124 (35%)	89 (81%)
Perinuclear space/ER	4 (1%)	8 (7%)
Cytoplasm	34 (10%)	13 (12%)
Cell surface	191 (54%)	0 (0%)
Total	**353** (100%)	**110** (100%)

Figure 4.3. RttSKs act early (five-nine hpi) in infection and effect accumulation of some viral genes. A) Digital images of western blots of total cell lysates of HEp-2 cells mock infected or infected with WT or US3-null (vRR1202) HSV-1(F) at an MOI of five for 20 hr. Cells were vehicle (-) or Rottlerin (+) treated at five, seven, nine, and 12 hpi. Blots with equal amounts of total protein were immunoblotted with monoclonal antibodies to VP5, US11, and UL34**. B)** Replicate cultures of HEp-2 cells were infected with WT, US3-null (vRR1202), or US3-null-homologous-Repair (vRR1202Rep) HSV-1(F) at an MOI of five for 20 hr. Cells were treated as described in **A**. At 20 hpi, total virus was prepared and titered on Vero cells. Each bar indicates the average of at least three independent experiments. Error bars indicate standard deviation.
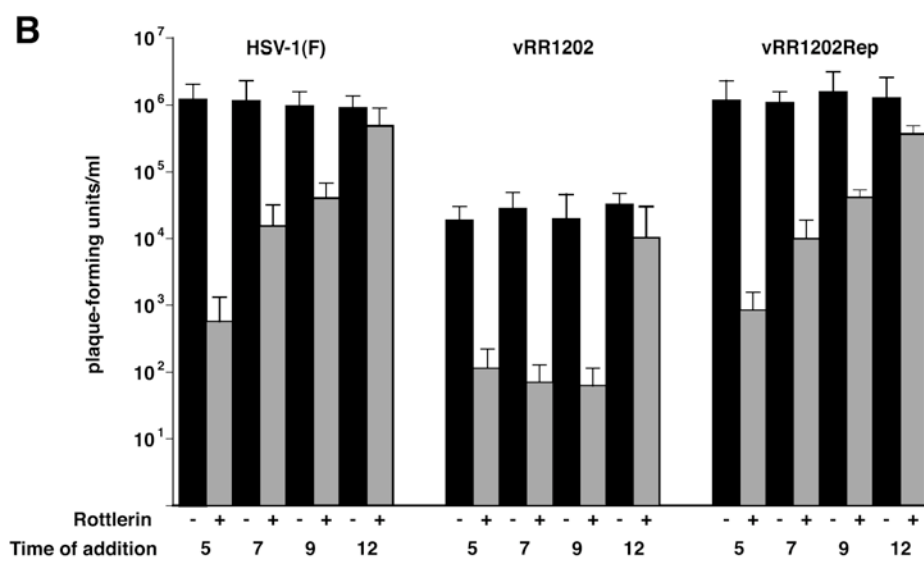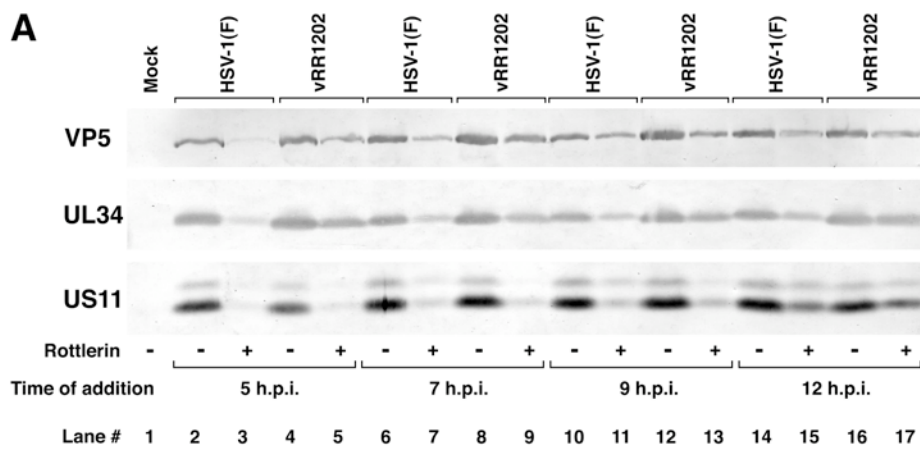.

Figure 4.4. RttSKs are required for cellular and viral protein synthesis and PKCs act redundantly with pUS3 in HEp but not Veros cells for efficient protein synthesis. Autoradiogram of ^{35}S-methionine labeled HEp-2 **(A)** or Vero **(B)** cell lysate from mock infected or infected with five PFU/mL of WT, US3-null (vRR1202), or US3-null-homologous-Repair (vRR1202Rep) HSV-1(F). Cultures were treated with DMSO (D), Rottlerin (R), or BIM I (B) at five hpi and at 16 hpi, total cell lysates were prepared from equal numbers of cells.

A. HEp-2
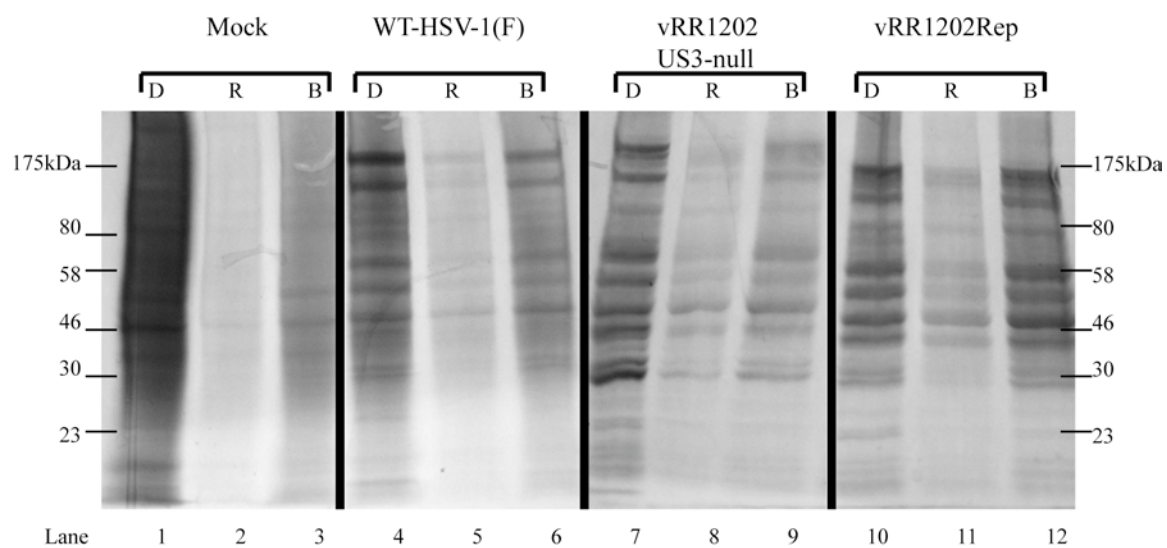
B. Vero
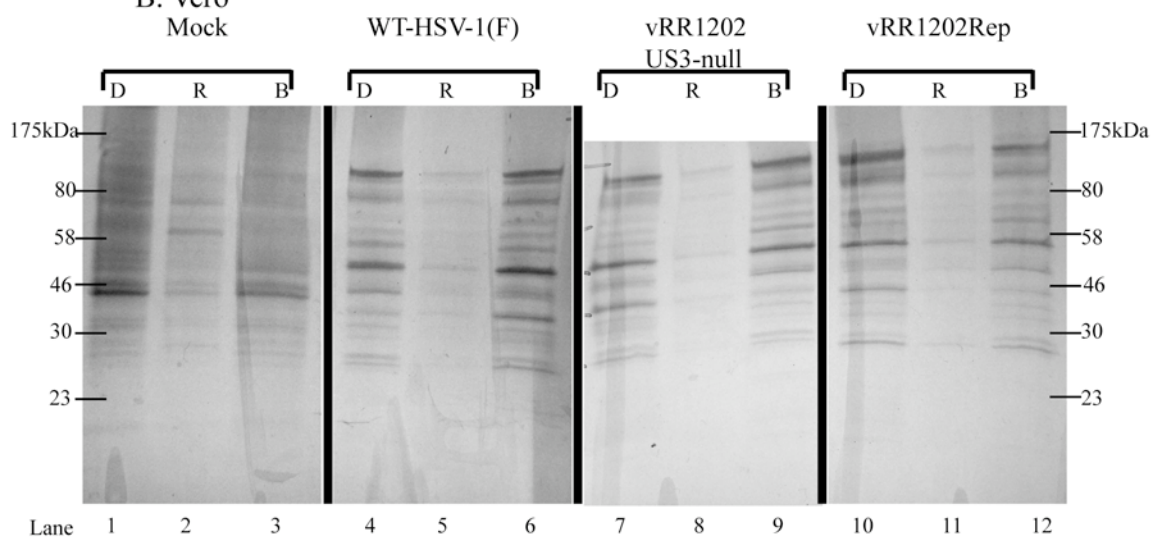
Figure 4.5. Rottlerin treatment of pUS3-kinase-dead-infected HEp-2 cells does not alter lamin A/C disruption. Shown are digital confocal images of optical sections taken near the top of the nuclei of HEp-2 cells infected with US3-kinase-dead recombinant vRR1204 virus at a MOI of five for 20 hr and treated with vehicle (Panels A-C) or Rottlerin (Panels D-F) beginning at five hpi. Cells were fixed with formaldehyde and stained with antibodies directed against lamin A/C (left column) or UL34 (center column). Lamin A/C is represented in red and pUL34 in green.

| Lamin A/C | UL34 | Merge |
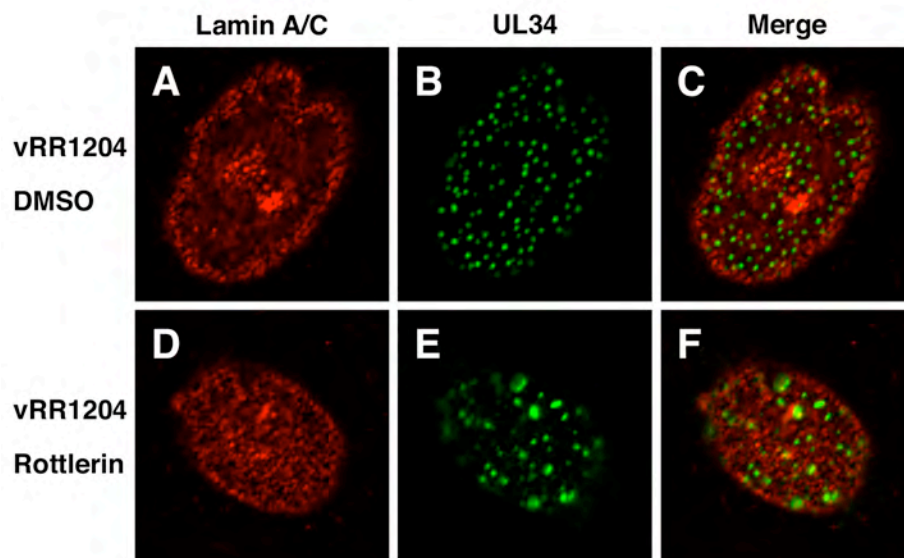
CHAPTER V

THE US3-NULL GROWTH DEFECT IN BT-549 CELLS

Abstract

The HSV-1 US3 protein has many reported functions in nuclear lamina disruption, de-envelopment of the perinuclear viron, protection from apoptosis, maintenance of infected cell protein synthesis, and cytoskeletal rearrangement. In most cell lines previously tested, however deletion of US3 resulted in only a minor decrease in PFU production (10-20 fold 1-1.3 log decrease compared to WT viruses). BT-549 cells are unusual in that they show an additional 10-fold decrease in US3-null (vRR1202) virus infectivity defect to produce an approximant 100-fold (2-log) decrease in infectious PFU. Late-viral translation and late-protein accumulation of gC, pUS11, pUL34, and VP5 proteins appeared normal. The pUS3-de-envelopment defect was not detectably enhanced in BT-549 cells. Cells did not demonstrate an enhanced sensitivity to apoptosis. Lamin A/C disruption occurred via similar mechanism in both MCF-7 and BT-549 cells. Interestingly, emerin was extensively hyperphosphorylated in a US3-null infection, yet was not redistributed along the NE. These data support a model that critical residues must be phosphorylated for emerin disconnection from lamina binding partners. This model also suggests that BT-549 cells, may not express the pUL34-dependent cellular kinase that is responsible for the critical phosphorylation event that disconnects emerin from the NE.

Introduction

The data presented thus far suggests that the nuclear lamina is a structural barrier for a herpes capsid. Specific phosphorylation events that disrupt specific interactions within the nuclear lamina are likely required for capsid primary envelopment (Chapter III). Alpha-herpesvirus encode two serine/threonine protein kinases (PK): pUL13 (CHPK) and pUS3 (213, 214). pUS3 directly phosphorylates lamina components (lamin A, C, and B1) and regulates the amount of disruption (20, 142, 189). BT-549 cells are a breast cancer cell line that express low levels of PKC delta protein and perhaps other cellular kinases (Figure 3.5) (117). If BT-549 cells lack the cellular kinase(s) that disrupts the specific interactions required for primary envelopment within the lamina, this cell may be especially prone to defects in viral growth in an US3-null state. This chapter focuses on the function of pUS3 in nuclear egress and nuclear lamina disruption.

pUS3: the alpha-herpesvirus kinase. pUS3 is the alpha-herpesvirus viral serine/threonine PK with a highly conserved kinase domain (175). Although there is some sequence variation in the N-termini, the US3 gene of alpha-herpesviruses HSV-1 and Pseudorabies virus (PRV) both function in primary envelopment (126, 224). pUS3 phosphorylates the consensus sequence RRR X(S/T) but is a known promiscuous kinase *in vitro* suggesting that regulatory co-factors may be key in determining pUS3 substrates in the infected cell (49, 188). Emerin contains this consensus sequence where serine 49 would be modified but direct phosphorylation has yet to be demonstrated (226). Some of the viral and cellular pUS3 substrates include: pUL34, pUL31, pUS9, emerin, lamin A/C, and lamin B1 (49, 142, 188, 189, 236). HSV-1 pUS3 is thought to function in: (i) blocking apoptosis, (ii) de-envelopment at the ONM, (iii) redistribution and phosphorylation of nuclear lamina components including the envelopment complex (pUL34 and pUL31), (iv) downregulation of gB expression, (v) re-organization of the actin cytoskeleton to promote cell-to-cell spread of virions (reviewed (184). When the

functions of HSV-1 and HSV-2 pUS3 were directly compared, it was determined that there are a few distinct differences between the two kinases (184). Unlike HSV-1 pUS3, HSV-2 pUS3 kinase function is not involved in nuclear egress, localization of the envelopment complex, or expression of gB (184).

Cellular kinases may complement the loss of US3. Deletion of the HSV-1, HSV-2, or PRV US3 gene leads to minor impairment of viral infectivity, 10-20-fold (1-1.3 log), in tissue culture and 10^4 fold-decrease in PFU in mice (176). It is still not understood why deletion of a multifunctional protein like pUS3 in HSV-1, HSV-2, or PRV demonstrates only modest growth defects in tissue culture (10-20 fold), while deletion leads to significant impairment of viral PFU production (10^4–fold) and virulence (ID_{50} down 10^5-fold) *in vivo* (8, 10, 124, 176, 213). This creates a disparity about pUS3 function between *in vivo* and tissue culture models of infection. These data suggested that pUS3-anti-apoptotic function is critical *in vivo*, but not in tissue culture PFU production. It also suggests that cellular kinases are able to compensate for the loss of US3 and even in the absence of US3 function, they are performing their pUS3-redundant function resulting in only small differences in PFU production in tissue culture models of infectivity. However, the minor differences in PFU production in the absence of US3 in tissue culture make it very difficult to separate and determine the function of exact gene sequences of the many biological functions attributed to pUS3.

Apoptosis, programmed cell death, is one mechanism of innate immunity. By triggering apoptosis, the infected cell can eliminate the pathogen, thus suppression of apoptosis is critical for the propagation of many viruses (131, 269). HSV-1 and -2 infections induce rapid and widespread changes in the host cell such as cell rounding and nuclear lamina disruption (20, 120, 186, 189, 190). However, classic hallmarks of apoptosis, genomic DNA fragmentation and condensed nuclei, are mostly absent from HSV-1 infected cultured cells such as Vero cells (192, 199). WT HSV-2, but not HSV-1, can induce apoptosis in a small percentage of HEp-2 cells (130). Infection with HSVs

actually can protect cells from apoptotic stimuli such as sorbital-mediated osmotic shock and thermal shock (11, 132). HSV mediated resistance to apoptosis was determined to occur early, three to six hpi (11, 199). The viral genes implicated in the anti-apopotic effects of HSV are: ICP4, ICP27, ICP22, US3, ICP34.5, gJ, gD, and LAT (199). Of all the viral genes mentioned for their possible anti-apoptotic roles, US3 is the only gene for which it has been demonstrated to protect cells from apoptosis *in vitro* and *in vivo*. HSV-1 US3 gene is required to block fragmentation of DNA in the infected cell (153). The authors had previously attributed the anti-apoptotic function to alpha 4 but subsequently determined the deletion mutant virus for alpha 4 also had a deletion for US3 and the US3 PK function actually conveyed the anti-apoptotic function lost in the mutant (153). It has been suggested that pUS3 anti-apoptotic function could be crucial *in vivo* but dispensable in cultured cells.

Lamina disruption and primary envelopment. Infection with wild-type herpesviruses results in changes in nuclear architecture. These changes are consistent with disruption of the nuclear lamina, including: (i) enlargement of the nucleus (19, 217, 249); (ii) change in the shape of the nucleus (19, 98, 217, 248, 249); (iii) changes in the localization of both A- and B- type lamin proteins (19, 35, 36, 98, 145, 217, 223, 248, 249); (iv) masking and unmasking of monoclonal antibody epitopes on the lamin proteins (223); (v) redistribution of LAPs including lamin B receptor (LBR), LAP2β, and emerin in herpes simplex infections (20, 142, 186, 242, 248).

pUS3 kinase activity is known to regulate HSV-induced nuclear lamina disruption (20, 142, 186, 188, 190). Phosphorylation disrupts the lamina protein-protein and protein-DNA interactions within the lamina and the role of pUS3 in lamina disruption is likely due to its kinase function in increasing lamina solubility and not to its role in suppressing apoptosis (20, 92, 169). As described in Chapters II and III, a growing body of evidence suggests that herpesviruses induce phosphorylation of nuclear lamina components to gain access to the INM for primary envelopment. In Chapter III, data was

presented data that PKC isoforms act redundantly for HSV-1 nuclear egress and capsid assembly (Figure 3.1, 3.2, and 3.4). pUS3 is also known to directly phosphorylate lamin A, C, and B1 and is required for WT levels of emerin hyperphosphorylation but it is unknown which residue is phosphorylated by pUS3 on emerin (although likely serine 49) or if phosphorylation of lamina components is required for primary envelopment (142, 188, 189, 226). HSV-2 pUL13 rather than pUS3 can directly phosphorylate lamins (36). It has been previously hypothesized that recruitment of kinases (PKC and pUS3) to the NE and concurrent phosphorylation of nuclear lamina proteins is one mechanism of generating the necessary flexibility in the lamina for primary envelopment (Chapter III). In Figure 3.8, we presented data to suggest that phosphorylation by pUS3 and Rottlerin sensitive Kinases (RttSK) is required for emerin re-localization during HSV-1 infection. It also remains unknown if phosphorylation of lamina components, LAPs and lamins, is required for nuclear egress. It also remains unknown if nuclear egress is blocked when emerin disruption is blocked.

De-envelopment. Another important function of pUS3 is facilitating de-envelopment of the primary virion (178). After envelopment of a herpes capsid at the inner nuclear membrane (INM), the enveloped particle is trapped between the INM and outer nuclear membrane (ONM) (Figure 5.8 A). The envelope of the perinuclear viron must then fuse with the ONM to release the capsid into the cytoplasm (179). Mutant viruses that lack pUS3 or catalytic pUS3 activity do not perform this step of virus maturation efficiently (Figure 5.8 C) (128, 236). Deletion of pUS3 in HSV-1 and PRV lead to build up of enveloped virus particles in "bags" between the INM and ONM (128, 224, 236). It is unknown why the deletion of US3 leads to the clustering or "bags" of enveloped particles in the peri-nuclear space, rather than even distribution unfused particles.

BT-549: A new model for pUS3 study. BT-549 express low levels of PKC delta protein (Figure 3.5) (117). We hypothesized that BT-549 cells may lack the cellular

kinase(s) required for disrupting interactions between nuclear lamina proteins prior to primary envelopment, in an US3-null infection. If BT-549 cells do not express cellular kinases that can complement for the pUS3 function, we predict a large reduction in PFU production due to a block in primary envelopment. BT-549 epithelial breast cancer cell line was isolated in 1978 by WG Coutinho and EY Lasfargues. The cells are from a female Caucasian woman with ductal carcinoma of the breast that had metastasized to three of seven regional lymph nodes (140). The established cell line is polymorphic with multinucleated cells exhibiting many long extensions (http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.as px?ATCCNum=HTB-122&Template=cellBiology). These cells have been used to determine the function of different cellular proteins in anoikis (anchorage induced cell death), p53 control, galectin-3 function, and tumor phenotype analysis (74, 123, 194, 257, 279). This cell line is part of the NCI-60 panel of cell lines whose mRNA profiles have been extensively analyzed (http://dtp.nci.nih.gov). MCF-7 cells are also human breast cancer epithelial cells part of the NCI-60 panel. BT-549 cells are from a highly invasive tumor while MCF-7cells are not (211). MCF-7 cells were first isolated in 1970 and have helped advance the understanding of cancer cell biology (258). MCF-7 cells form domes in tissue culture and retain the ability to respond to many in vivo signaing molecules such as estrogen (258). The morphology of BT-549 and MCF-7 cells is strinkingly different and suggests that it is possible that multiple proteins may be differentially expressed between the two lines.

This chapter contains data suggesting that BT-549 cells are an interesting new tissue culture model system for studying pUS3 function. In the absence of pUS3, in BT-549 cells US3-null infectivity is reduced by 73-fold and in MCF-7 cells infectivity is reduced 8.7-fold compared to WT virus. The reduction in infectivity does not appear to be due to a pUS3-dependent block in late-gene protein synthesis or accumulation. Nor, does it appear to be due to a dramatic block in de-envelopment of perinuclear virions.

Emerin is reorganized in MCF-7 but not BT-549 cells in a pUS3 dependent manner.

Interestingly, emerin is hyperphosphorylated in BT-549 US3-null infected cells. Lamin

A/C disruption occurs via a similar manner in both cell lines. Increased apoptosis does

not appear to be responsible for the 73-fold drop. These data support the model the

critical emerin residue in BT-549 cells infected with the US3-null virus is not

phosphorylated (due, perhaps, to the absence of a US3-complementing cellular kinase).

This allows emerin to remain associated with binding partners in the nuclear lamina thus

blocking primary envelopment.

<div align="center">Materials and Methods</div>

Cells, viruses, and chemicals. Vero cells were maintained as previously

described (232). MCF-7 and BT-549 cells were maintained in RPMI containing 10%

fetal calf serum. BT-549 cells were derived from the NCI-60 cell collection and were

kindly provided by Jack Stapleton. The properties of HSV-1(F), vRR1202 (US3-null

virus), vRR1204 (US3 kinase-dead virus K220A), and vRR1202-null-homologous repair

(vRR1202Rep) was previously described and characterized (224, 232, 236).

SDS-PAGE sample preparations. For analysis of viral protein expression,

infected or uninfected cultures containing 2×10^6 cells were washed twice with PBS,

scraped into PBS, pelleted at low speed, total soluble protein was extracted with acetic

acid, and precipitated with acetone as previously described (229). Total protein

concentration was determined using BioRad Dc Protein Assay (BioRad Laboratories).

Equal number of cells and equal total protein concentration were compared.

Western blot. The antibodies and dilutions used were: mouse monoclonal anti-

US11, 1:1000 (231); chicken monoclonal anti-UL34, 1:1000 (224); mouse monoclonal

anti-VP5, 1:1000 (Meridian Life Science, #C05014M); mouse monoclonal anti-gC,

1:1000 (Virusys, H1A022M). Alkaline phosphatase conjugated anti-mouse, anti-rabbit,

and anti-chicken secondary antibodies were incubated with the appropriate blot for 30

minutes (Sigma, mouse: A3562, rabbit: A3687 Aves Lab, chicken: AP1001). Equal number of cells and equal total protein concentration were compared.

Virus Growth. Single-step growth analysis was performed as previously described (225, 232). Briefly, replicate cultures of cells (10^6) were infected at an MOI of five, and residual virus was removed or inactivated with a low-pH-buffer wash. At 16 hpi (and 24 hpi), virus was harvested and total culture PFU were calculated by titration on Vero cells.

Transmission Electron Microscopy. BT-549 cells were infected with HSV-1(F) or US3-null (vRR1202) at an MOI of five. At 16 hpi, cells were fixed with glutaraldehyde, processed, and analyzed by electron microscopy as described previously (232).

^{35}-S-methionine Labeling. 2×10^6 BT-549 cells were uninfected or infected with five PFU/cell of HSV-1(F), vRR1202 (US3-null virus), or vRR1202Rep (US3-null homologous repair). At 10 hpi, the cells were starved for methionine by replacing the media with methionine minus media (Gibco, 21013). Beginning at 14 hpi, each sample was labeled for two hours with five uCu of ^{35}S-methionine. Total cell lysates were prepared at 16 hpi by the acid preparation as described above (229). Total protein from equal numbers of cells was separated on an SDS-PAGE gel and visualized by autoradiography.

Indirect Immunofluorescence. Indirect immunofluorescence (IF) was performed for pUL34, emerin, and lamin A/C as previously described (20, 224). Briefly, cells were fixed with 4% formaldehyde for ten minutes, washed with phosphate-buffered saline (PBS), and then permeabilized and blocked in the same step with IF buffer (0.5% Triton X-100, 0.5% Sodium Deoxycholate, 1% BSA, 0.05% Sodium Azide diluted in PBS) for one hr. Primary antibodies were diluted in IF buffer as follows: chicken anti-UL34, 1:1000 (224); mouse monoclonal anti-lamin A/C, 1:1000 (Santa Cruz

Biotechnology, sc-7292); mouse monoclonal anti-emerin, 1:500 (Santa Cruz

Biotechnology, sc-25284).

Secondary antibodies were diluted 1:1000 in IF buffer as follows: Alexa Fluor goat anti-

chicken IgG (Invitrogen, A11001), Alexa Fluor goat anti-mouse IgG (Invitrogen,

A1104). Slo-fade II (Invitrogen, Molecular Probes) was used to mount cover slips on

glass slides. All confocal microscopy work was done with a Zeiss 510 microscope.

NCI-60 gene-expression of cellular kinases. The candidate kinases were

previously determined based on sensitivity to Rottlerin and the presence of a recognition

site in human emerin. mRNA levels were found for the following genes: AKT, AKT1, v-

AKT, GSK3B, PRKACG, PRKACA, PRKCAB, RPS6KA5 (MSK1), PDK1, and

MAPK8 (JNK1) (http://dtp.nci.nih.gov/mtweb/index.jsp). mRNA amounts were

calculated as fold change from NCI-60 calculated mean (MCF mRNA level experiment

#x/mean mRNA level of all cells for experiment #x). At least three independent

experiments were included into a calculation of the mean "fold change from mean."

Error bars indicate standard deviation from the mean from the "fold change from mean."

All mRNA levels can be found at http://dtp.nci.nih.gov/mtweb/index.jsp.

<div align="center">Results</div>

US3-null virus is down ~100-fold in BT-549 cells. BT-549 cells are a breast

cancer cell line that express undetectable levels of PKC delta protein (Figure 3.5 C)

(117). pUS3 directly phosphorylates lamina components (emerin and lamin A, C, and

B1) and regulates the amount of disruption (20, 142, 189). If BT-549 cells lack the

cellular kinase(s) that disrupts the specific interaction within the lamina required for

primary envelopment, this cell would be especially prone to defects in viral growth in an

US3-null state.

To test the hypothesis that US3-null (vRR1202) virus would replicate poorly in

BT-549 cells in the absence of pUS3 complementing cellular kinases such as PKC delta,

production of infectivity of WT, US3-null (vRR1202), and US3-null Repair (vRR1202Rep) in BT-549 and MCF-7 cells was measured at 16 hpi (Figure 5.1). As previously determined, replication of WT virus was not significantly different between the two breast cancer cell types, despite their different morphology and PKC delta expression levels (Figure 3.5 D and Figure 5.1). Deletion of pUS3 from the viral genome reduces growth in most tissue culture cells from 10-20 fold (1-1.3 log) (124, 213, 236, 278). Growth of US3-null in BT-549 cells however was repressed 73-fold compared to WT virus, while growth of US3-null in MCF-7 cells was reduced 8.7 fold (Figure 5.1). The homologous repair virus US3Rep restored virus growth to WT levels in both cell lines (Figure 5.1).

Late-gene expression was unimpaired in BT-549 cells. In addition to pUS3 function in de-envelopment, apoptosis, and lamina disruption, there are data to suggest that pUS3 functions to regulate viral protein synthesis (41). Data presented in Chapter IV, suggested that pUS3 and PKC function redundantly to maintain viral protein synthesis in HEp-2 cells (Figure 4.4 A). This suggested that the defect in US3-null growth in BT-549 cells could be due to the absence of a cellular kinase required for complementing the lack of pUS3 for viral protein synthesis.

To test the hypothesis that the US3-null replication defect in BT-549 cells is due to a pUS3-dependent block in late-protein synthesis, accumulation of late viral proteins were tested in MCF-7 and BT-549 cells infected with five PFU/cell of WT, US3-null (vRR1202), and US3-null homologous repair (vRR1202Rep) virus. Beginning at 14 hpi, cells were labeled for two hours with ^{35}S-methionine. Total cell lysates were prepared at 16 hpi and protein from equal numbers of cells were separated on an SDS-PAGE gel and visualized by autoradiography (Figure 5.2 A). Upon infection of MCF-7 or BT-549 cells with WT HSV-1(F), host protein synthesis was turned off and viral protein synthesis was evident (compare lanes one to two, and five to seven and eight). When MCF-7 cells were infected with US3-null or US3Rep, little or no effect on global viral protein synthesis was

observed (compare lanes three and four to two). BT-549 cells infected with US3-null virus had some changes in some specific late proteins, but the effect was subtle (compare lane seven to six, <30K, 40K). Repair of the US3 gene in the mutant virus genome returned the pattern to WT infection (compare lane eight with six).

To evaluate the effect of deletion of US3 on specific vial genes, the samples from Figure 5.2 A were subjected to western blot analysis for the following proteins: VP5 (major capsid protein), pUS11 and gC (archetype true-late gene products), and pUL34 (required for nuclear egress). Un-labeled samples prepared as described above were separated by SDS-PAGE and transferred to nitrocellulose (Figure 5.2 B). In MCF-7 cells, deletion of US3 resulted in the reduced expression of pUL34 (compare lanes three, five, and seven). In BT-549 cells, deletion of US3 did not appear to make a significant difference (compare lanes four, six, and eight) yet these cells appear to make less viral protein than the same number of MCF-7 cells (compare lanes three and four, five and six, and seven and eight). Equal total protein was also compared and demonstrated similar results (data not shown).

BT-549 infected cells do not have exaggerated US3-null de-envelopment defect. pUS3 is known to be involved in de-envelopment of the perinuclear viron (127, 236). Deletion or catalytic inactivation of pUS3 leads to build of enveloped virus particles between the INM and ONM (Figure 5.8 C). This build-up of virus particles is thought to lead to the concurrent decrease in virus titer since the infectious particles are trapped, however this has never been conclusively demonstrated (179, 184). To test the hypothesis that the increased growth defect of US3-null in BT-549 cells is due to an exacerbated defect in de-envelopment, BT-549 cells were infected with five PFU/cell of WT HSV-1(F) or US3-null (vRR1202) virus. At 16 hpi, the cells were fixed with gluteraldehyde and processed for electron microscopy (Figure 5.3). The WT infected BT-549 cells (Figure 5.3 A) demonstrated full (DNA containing capsids) and empty

(non-DNA) capsids in the nucleus. Cytoplasmic intermediates were also observed, however, enveloped cell surface particles were not (Figure 5.3 A and B).

As with the WT infected cells, US3-null (vRR1202) infected BT-549 cells demonstrated empty and full capsids in the nucleus (Figure 5.3 B). The previously documented de-envelopment defect of US3-null(vRR1202) can be readily observed in the inset picture (Figure 5.3 B). There are multiple enveloped particles in one "bag". These particles have failed to fuse their peri-nuclear envelope with the ONM and complete maturation in the cytoplasm (236) (Figure 5.8 C). However, the number of virus particle per bag or the number of bags per cell was not more extreme than previously documented defects in HEp-2 or Vero cells.

BT-549 cells hyperphosphorylate but do not disconnect emerin from the lamina in US3-null infection. pUS3 is a negative regulator of lamina disruption and a direct mediator of lamina phosphorylation (20, 142, 188). Inabilities to disrupt the nuclear lamina without pUS3 kinase activity or excessive disruption in the absence of pUS3 regulation are two possible hypotheses for the US3-null growth defect in BT-549 cells.

To determine if emerin HSV-induced re-localization (Figure 5.4 A) and hyperphosphorylation (Figure 5.4 B) occurs as previously mapped in other cell types (Chapter II), BT-549 cells were mock or WT, US3-null (vRR1202), US3-kinase dead (vRR1204), and US3-null homologous repair (vRR1202Rep) infected with ten PFU/cell. At 16 hpi, the cells were fixed and stained with monoclonal antibodies to emerin and pUL34 (Figure 5.4 A) or fractioned for a lamina prep. A western blot was performed for emerin from the lamina fraction (Figure 5.4 B). Mock-infected BT-549 cells demonstrated irregular nuclear size and shape, nuclear pleomorphism (Figure 5.4 Aa). Emerin was localized in a smooth pattern but the membrane appears wrinkled with many undulations and indentions and a bleb pinching off from the top (Figure 5.4 Aa). Mock-infected HEp-2 cells, which have a 20-fold (1.3 log) decrease in US3-null growth and are

also cancer cells, have a uniform in nuclear cell shape (Figure 3.8 Aa). No clumps or areas devoid of emerin staining were observed in mock-infected BT-549 cells, just highly irregular shaped nuclei (Figure 5.4 Aa). Interestingly, emerin localization and extreme nuclear pleomorphism can be used as a diagnostic tool for aggressive breast cancers and is associated with a poor prognosis (32).

Emerin was re-localized to clumps or thickenings around the NE in WT-infected BT-549 cells (Figure 5.4 Ad). Most of the emerin staining was observed within the nuclei while some pUL34 is outside the NE (Ad and Ae). Emerin and pUL34 colocalize at most instances at the NM, even in the blebs (Af). Infection with US3-null (vRR1202) resulted in characteristic pUL34 puncta (Ah) but smooth emerin localization along the NM (compare Ah to Ag). Mock-infected emerin localization was not the same as US3-null (vRR1202) infected but emerin was not re-localized to the pUL34-puncta (compare top cell to bottom cell in Ag, Ah, and Ai). The failure to re-localize emerin to the pUL34-puntua was also true in US3-kinase-dead infected BT-549 cells (Aj to Ak). Repair of the US3 gene returned the localization of emerin to the WT-infected phenotype (compare Am to Ad).

Emerin hyperphosphorylation in WT HSV-1 infected cells is dependent on pUS3 and pUL34 (Figure 2.1) (142). The pUL34-dependent component of emerin phosphorylation is mediated by cellular kinase(s) sensitive to Rottlerin (Figure 2.4) (142). Inhibition of both pUS3- and Rottlerin sensitive (pUL34-dependent) kinases prevented both emerin hyperphosphorylation and disconnection from the lamina (Figure 2.3 and 3.8 A). Thus, the failure to re-localize emerin in the US3-null infected BT-549 cells suggested that emerin may not be hyperphosphorylated by the pUL34-dependent kinase in this cell type. To test this hypothesis, we performed a lamina prep on BT-549 infected cells with the conditions described above (Figure 5.4 B). Uninfected BT-549 cells demonstrated two major species (lane one). Upon infection with WT virus, the two major emerin species become highly retarded in migration, and more

hyperphosphorylated species appear (lane two). Infection with US3-null virus (vRR1202) did not decrease the hyperphosphorylated "smear" of emerin also found in the WT-infected lane (compare three to two) but did return the major (base) emerin species to run at the same rate as mock infected (compare three to one). The same pattern was observed with US3-kinase dead-infected cells (lane four). Repair of the US3 gene returns the phosphorylation pattern to something indistinguishable from WT (compare five to two). UL34-null infected emerin phosphorylation is similar to US3-null in that the major species of emerin is migrating faster than that in WT-infected samples (compare lane six to two or seven). Hyperphosphorylation was reduced and the "super-shift" species was also gone in both US3-null and UL34-null (compare lane three and six). Repair of the UL34 gene returns the phosphorylation to WT-levels and pattern (compare seven to two). Collectively, these data suggest that emerin in US3-null infected BT-549 cells was extensively hyperphosphorylated, yet was not sufficient to disconnect emerin from the lamina. This also provides evidence for the hypothesis that phosphorylation of a specific residue(s) is critical for disrupting emerin localization and connection with lamina, not overall hyperphosphorylation levels (Figure 2.1) (142). Emerin HSV-1-induced disruption in MCF-7 cells was similar to previously observed phenotypes in HEp-2 or Vero cells (Figure 5.5) (142).

Mechanism of pUS3-dependent lamin A/C disruption is similar in two different nuclei shapes. Emerin localization in uninfected BT-549 cells resembled emerin and lamin localization in EDMD and progeria mutant cells (88). In many types of laminopathies, the similar lobules, undulations, or blebs arise as in cancerous cells (32). The E145K in the rod domain of lamin A (*LMNA*) results in progeria mutation presumably due to the inability of the E145K LMNA to associate with other lamins (264). The localization of LMNA dermal fibroblasts is these mutant cells is very similar to emerin in cancerous uninfected BT-549 cells (264) (Figure 5.4). If the BT-549 cells innately have a misregulated or deformed nuclei and then in the absence of pUS3, the

exaggerated disruption of the nuclear envelope could lead to decreased virus production. This synergistic effect could perhaps lead to an additive effect on apoptotic signaling.

To test the hypothesis that lamin A/C disruption would be exaggerated in US3-null infected BT-549 cells, lamin A/C localization was compared to MCF-7. Both cell lines were infected with ten PFU/cell WT, US3-null (vRR1202), US3-kinase dead K220A (vRR1204), and US3Rep virus or left uninfected for 16 hr. The cells were then fixed and stained with monoclonal antibodies against pUL34 and lamin A/C. Un-infected BT-549 cells had lamin A/C distribution in a wrinkled and blebby pattern. One of the large blebs is pointed out in Figure 5.5 Aa. Un-infected MCF-7 cells had a more uniform and round shape as assayed by the localization of lamin A/C (Figure 5.5 Ba). WT HSV-1(F) infection of BT-549 cells resulted in wrinkled lamin A/C and thickening in some areas, but the large blebs are absent (Ad). MCF-7 WT infection differed in that the nuclei did not demonstrate significant shape change or wrinkling (Bd). Additionally, some of the pUL34 appears outside of the NE (Be). BT-549 infection with the US3-null virus (Ag) or US3-kinase dead virus (Aj) did not significantly alter the WT infection induced changes in lamin A/C localization. pUL34 localization was in the characteristic puncta (Ah and Ak) (236). MCF-7 infection with the mutant US3 viruses did not alter lamin A/C localization but did have an effect on LMNA epitope masking (Bg and Bi, compare top and bottom cell) (223). Repair of the US3 gene returns both the BT-549 and MCF-7 lamin A/C and pUL34 localizations to a WT phenotype (Am-o and Bm-o compare to Ad-f and Bd-f).

Search for the RttSK. One aim of this thesis was to determine the mechanism of pUS3 redundancy with host cell kinases. pUS3 has sequence similarity with AKT (PKB) and PKA, but is also considered a promiscuous kinase (184, 188, 213). pUS3 is not thought to have functional redundancy with AKT (14). The nuclear lamina is thought to be structural barrier for a herpesvirus yet the specific phosphorylation events required for disruption of specific interactions within the lamin prior to capsid primary envelopment,

have not yet been demonstrated. This is in part due to the multiple and redundant functions of the viral and cellular kinases. However, if BT-549 cells lack the cellular kinase that disrupts the lamina, this cell would be especially prone to defects in viral growth in an US3-null state. To narrow the possible pUS3-redundant kinase candidates, it is likely (but not limited to) these kinases are also sensitive to Rottlerin (Chapter IV). We hypothesized that the defect in US3-null growth in BT-549 cells was due to the absence of or reduction in a Rottlerin sensitive kinase such as: AKT (PKB), PKA, JNK1, PDK1, GSK3beta, and MSK1.

To determine if BT-549 cells do not express a cellular kinase that could compensate for the absence of pUS3, we compared mRNA levels of AKT (PKB), PKA, JNK1, PDK1, GSK3beta, and MSK1 between MCF-7 and BT-549 cells using the NCI-60 compare website (http://dtp.nci.nih.gov/mtweb/index.jsp). This comparison found differences in mRNA expression in the 1-2 fold range between the two cell lines (Figure 5.7). The largest difference in mRNA expression between the two cell lines for the examined genes was for JNK1 (Figure 5.7).

Discussion

The HSV-1 US3 protein has many reported functions such as nuclear lamina disruption, de-envelopment of the perinuclear viron, protection from apoptosis, and maintenance of infected cell protein synthesis. It remains unknown why deletion of this multifunctional protein results in only a minor decrease in PFU production (10-20 fold decrease compared to WT viruses). BT-549 cells are unusual in that they show an additional 10-fold decrease in US3-null (vRR1202) virus infectivity defect to produce an approximant 100-fold decrease in infectious PFU. Late-viral translation and late-protein accumulation proteins appeared normal. The pUS3-de-envelopment defect was not detectably enhanced in BT-549 cells. Cells did not demonstrate an enhanced sensitivity to apoptosis. Lamin A/C disruption occurred via similar mechanism in both MCF-7 and

BT-549 cells. Interestingly, emerin was extensively hyperphosphorylated in an US3-null infection, yet was not redistributed along the NE.

Nuclear lamina disruption and pUS3: Similarities of Herpes, Cancer, and laminopathies. Nuclear lamina disruption is one pUS3-regulated process that could be responsible for the additional 10-fold drop in titer (Figure 5.1). To determine if BT-549 US3-null infected cells were competent for lamina disruption or excessively disrupted the lamina, we tested emerin re-localization and hyperphosphorylation (Figure 5.4 A and B). In BT-549-US3-null infected cells, emerin is highly hyperphosphorylated but is not re-localized to pUL34-containing puncta as previously observed (142) (Figure 5.4 Ag). While the failure to relocalize emerin does not explain what kinase or phosphorylation event was directly responsible for the drop in PFU production, it did pose more interesting questions about how emerin phosphorylation regulates its localization depending on the kinase environment. The HSV-1(F) infection-induced phosphorylated residues on emerin should be identified in BT-549 and other relevant cell types (Vero or HEp-2). It is possible that emerin localization in infected cells could be due to (i) phosphorylation site preference, (ii) emerin protein sequence, and/or (iii) cellular kinase expression. It would also be interesting to determine which residues are pUS3-dependent or pUL34-dependent since both viral proteins are required for WT levels of hyperphosphorylation (142) (Figure 2.1). Determining HSV-1-dependenet phosphorylated residues will allow evaluation of how emerin's associations with LMNA or LMNC is regulated during infection, mitosis, or aberrantly during laminopathies and cancer.

Emerin phosphorylation can be linked to its function. In the un-infected cell, emerin is maximally phosphorylated at mitosis, but phosphorylation can occur at any time (105). In nuclei from patients with EDMD, emerin is aberrantly hyperphosphorylated suggesting that this is one possible mechanism for emerin to contribute to EMDM pathology (67). Previous data suggested that emerin

phosphorylation on critical residues was responsible for its disconnection from the lamina during disassembly of the nuclear envelope prior to mitosis (105, 226). It appears that in BT-549 cells that emerin is highly hyperphosphorylated in the US3-null infection yet was still associated with the NE in spite of its pUL34-dependent phosphorylation (Figure 5.4 B, 2.1, and 5.9). This is interesting from several points of view. While in Vero cells emerin hyperphosphorylation is extensive, in HEp-2 cells phosphorylation is limited to two or three different species (Figure 2.1) (142). Regardless of the level of hyperphosphorylation, it appears that the few critical phosphorylation events in HEp-2 cells were sufficient to disconnect emerin from lamin A/C (142). In BT-549 US3-null infected cells, emerin was associated with the NE although it was hyperphosphorylated (Figure 5.4). These data from BT-549 supported the original hypothesis from HEp-2 cells suggesting limited phosphorylation was sufficient to completely disconnect emerin from lamin A/C (Figure 2.1) (142). Furthermore, phosphorylation of a critical or perhaps, multiple critical residues in emerin is required to mediate its disconnection from the NE (Figures 5.4 and 5.9). In the future, fine mapping of emerin phosphorylation sites and the relationship of phosphorylation to localization in different cell types would be highly informative.

The BT-549 and MCF-7 cells are part of a cell line panel whose genetic profile has been extensively evaluated (http://dtp.nci.nih.gov/mtweb/index.jsp). In Figure 5.7, we attempted to take advantage of the bio-informatic power of this system to identify the pUS3 complementing kinase, however it appears that the answer is more complicated than first anticipated. The mRNA fold change in kinases with known recognition sequences in emerin, were not larger than 2-fold (Figure 5.7) although the change in growth in US3-null virus was 10-fold (Figure 5.1). This is likely due to our small data size of only two cell lines. In the future using the entire NCI-60 panel and Compare algorithm, it is possible that a more extensive comparison of cell lines (or just all the

breast cancer lines) and US3-null growth could be performed to determine which cellular gene is necessary for complementing US3-null growth.

These data suggest a model in BT-549 un-infected cells emerin is attached to the INM via the TM domain and interactions with lamin A/C (Figure 5.9 A). This cell line does not express at all or high levels of cellular kinase that can be recruited by pUL34 for emerin phosphorylation or can function redundantly with pUS3 (Figure 5.9 B). Infection with WT HSV-1 leads to direct pUS3-dependent phosphorylation of emerin (B). Upon US3-null infection of BT-549 cells, emerin is not phosphorylated on the subset of residues that are *critical* to emerin disconnection because emerin is localized with its lamina-binding partners (C). The interactions between lamin A/C and emerin are maintained, and the capsid is retained within the nucleus (C). The few capsids that do escape, are trapped within the perinuclear space, and are viewed in TEM as 'bags' (Figure 5.2 and 5.8). The bags do not appear larger due to the double-block to primary envelopment and de-envelopment (Figure 5.8). One alternative hypothesis to this model is that BT-549 cells express a mutant emerin that cannot be relocalized because the mutant emerin cannot be phosphorylated by the pUL34-dependent cellular kinases (Figure 5.9 D).

De-envelopment. pUS3 mutants fail to efficiently fuse the perinuclear envelope with the outer nuclear membrane (ONM) (178) (Figure 5.8 C). A model of the normal egress intermediates observed via TEM in WT HSV-1-infected Vero cells is modeled in (Figure 5.8 A). Capsids are produced and observed in the greatest proportion the nucleus as naked capsids and as enveloped capsids on the cell surface (Figure 5.8 A). When a block to primary envelopment is placed on the system by removing an essential gene (such as pUL34 or pUL31) complete capsids are found inside the nucleus, but few enveloped capsids are found on cell surface and in the cytoplasm (Figure 5.8 B). A block to de-envelopment at the ONM leads to build up of enveloped particles in "bags" between the INM and ONM (Figure 5.8 C).

In Figure 5.2, the hypothesis was tested if in the BT-549 breast cancer cells, fusion the perinuclear particle with the ONM in an US3-null infection was excessively inhibited. We expected if the hypothesis was correct, then more enveloped capsids would be found per 'bag'. This hypothesis appears to be incorrect or unable to be determined via TEM. US3-null virus particles were formed but 'bags' of viruses were found between the two nuclear membranes (236) (Figure 5.2 C). However, the size of these bags (Figure 5.8 C) or the number of virus particles in the bags was not exceptionally large and it did not appear to be responsible for the additionally 10-fold decrease in virus production (Figure 5.1). The alternative hypothesis is that there is block to primary and de-envelopment (Figure 5.8 D). If both primary envelopment and de-envelopment were both inhibited, this would be difficult to detect. The block in primary envelopment would cause fewer capsids to enter the peri-nuclear space, but the block to de-envelopment would keep them in this space (Figure 5.8 D). Since BT-549 cells lack of cell surface particles, this double defect is not detectable. Thus, the role of pUS3 in de-envelopment cannot be completely ruled out at this time for the defect in BT-549 cells.

Global protein translation is not additionally impaired. As mentioned before pUS3 is thought to phosphorylate TSC2 in the mTOR pathway to regulating virus protein translation (40). This thesis has also presented data suggesting a redundant role for pUS3 and PKC in protein translation in a cell type dependent manner (Figure 4.4). These data suggested the possibility that the infectivity drop in BT-549 cells could be due to a defect in expression of an essential protein or global protein synthesis. Global late-protein translation does not appear to be 10-fold additionally inhibited in BT-549 cells (Figure 5.2 A) nor did accumulation of essential late-genes (Figure 5.2 B). While there are some bands that are altered in intensity in BT-549 US3-null infected cells, the change is not equivalent to an additional 10-fold change (Figure 5.2, compare lane seven to six, <30K, 40K). While the defect in growth is likely not due to translation, the effect of a minor change in a regulatory protein on PFU production should not be ignored.

Apoptosis and US3-null in BT-549. Apoptosis protection of the infected cell is
another function of pUS3, yet to achieve a 10-fold decrease in virus production, 90% cell
death would have to occur. Increased cell death or plasma membrane blebbing was not
observed (data not shown) suggesting that a lack of apoptosis protection was not the
defect responsible for the additional 10-20-drop in titer in BT-549 cells (Figure 5.1).
Future molecular analysis of apoptosis induction would be appropriate to accurately
determine that US3-null infection of BT-549 cells is not responsible for the 10-fold drop
in PFU. Caspases cleave lamins during HSV infection and apoptosis to disassemble the
NE and nuclear pores but aberrantly as well to cleave lamins during carcinogenesis (29,
141, 234). Future analysis of apoptosis should include examination of a known apoptotic
maker(s) such as: PARP, caspase-3, caspase-6, or cytochrome-c release in uninfected and
HSV-1(F) infected MCF-7 and BT-549. The nuclear lamina is a sensor of apoptosis but
it does not appear that in Vero or HEp-2 cells lamina disruption is mediated though
apoptotic signaling (20, 91).

Figure 5.1. US3-null virus has additional 10-fold growth defect in BT-549 cell line. Replicate cultures of BT-549 and MCF-7 cells were infected at an MOI of five with HSV-1(F), US3-null (vRR1202), or US3-null-homologous-Repair (vRR12020Rep) virus. Cultures were washed with low-pH buffer to remove unbound virus at two hpi and virus stocks were made at 16 hpi. Virus yields are expressed as plaque forming units (PFU) per milliliter as determined on Vero cells. Each data point represents the mean of three independent experiments. Error bars indicate standard deviation from the mean.
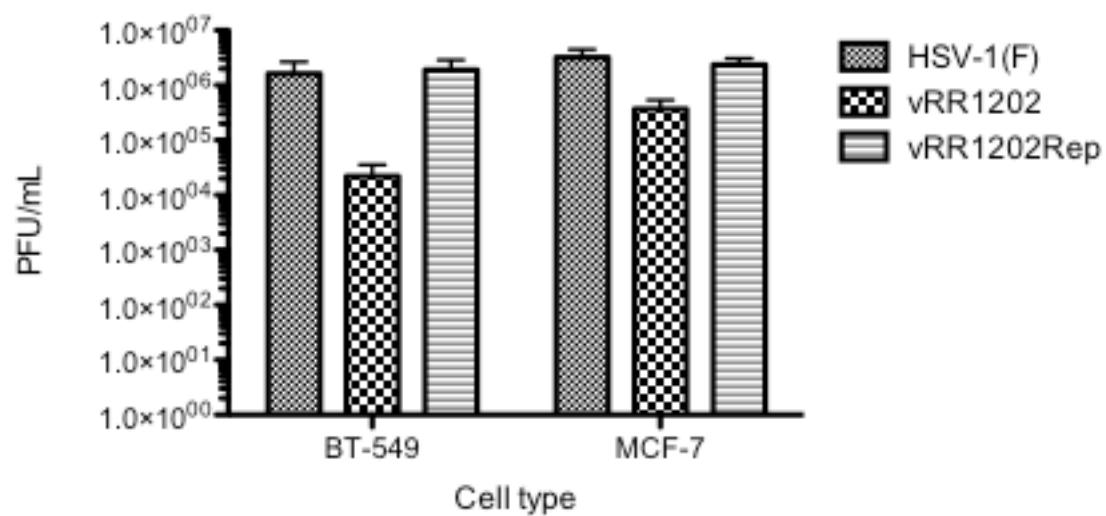
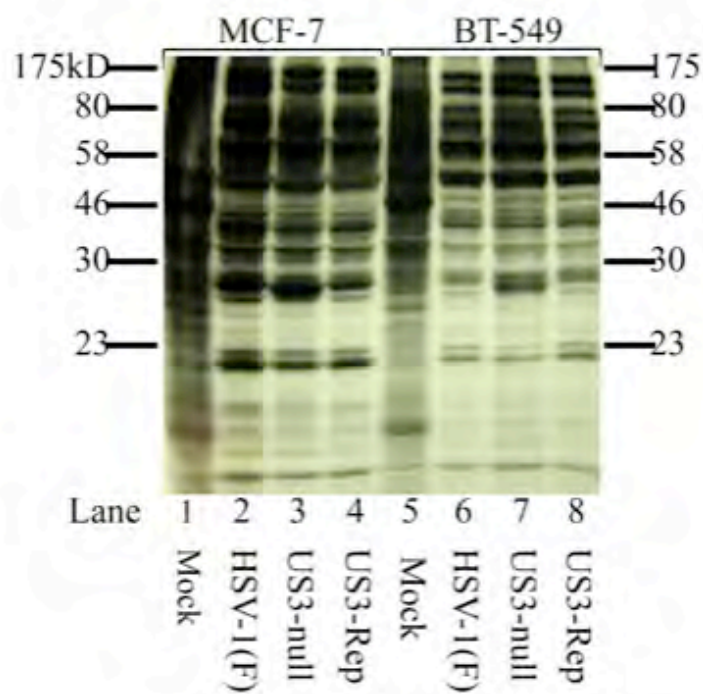HSV-1(F) Replication in Breast Cancer Cell Lines

Figure 5.2. Late-gene expression is not reduced in US3-null infected BT-549 cells. Digital images of **A)** Autoradiogram of ^{35}S-methionine labeled MCF-7 and BT-549 cell lysates from prepared from mock-infected cells or cells infected with five PFU/cell of HSV-1(F), US3-null (vRR1202), or US3 homologous repair (vRR12020Rep) virus. At 16 hpi, total cell lysates were prepared from equal numbers of cells. **B)** Western blots of unlabeled total cell lysates prepared as described in **A**. Blots were immunoblotted with monoclonal antibodies to VP5, gC, US11, and pUL34.

A

MCF-7   BT-549

175kD —   — 75
80 —   — 80
58 —   — 58
46 —   — 46
30 —   — 30
23 —   — 23

Lane   1   2   3   4   5   6   7   8

Mock | HSV-1(F) | US3-null | US3-Rep | Mock | HSV-1(F) | US3-null | US3-Rep

B

Lane   1   2   3   4   5   6   7   8

VP5

gC

US11

pUL34

| MCF-7 | BT-549 | | MCF-7 | BT-549 | | MCF-7 | BT-549 | | MCF-7 | BT-549 |

Mock   HSV-1(F)   1202   1202Rep
                        US3-null   US3-null Repair
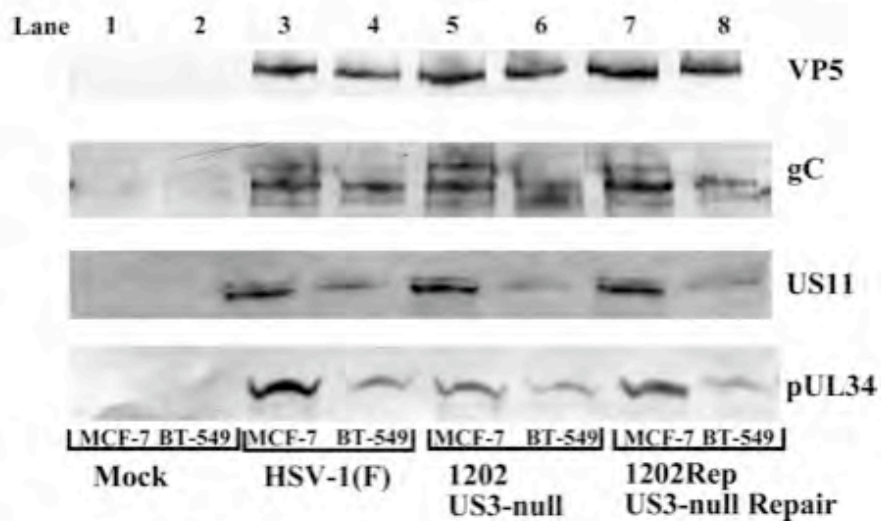
174

Figure 5.3. US3-null (vRR1202) egress intermediates do not show extreme defects in BT-549 cells. Shown are digital images of transmission electron micrographs (TEM) of BT-549 cells infected with five PFU/cell HSV-1(F) **(A)** or vRR1202 (US3-null) **(B)**. The cells were fixed with glutaraldehyde at 16 hpi, and thin sectioned for TEM. The inset picture is approximately twice the magnification.
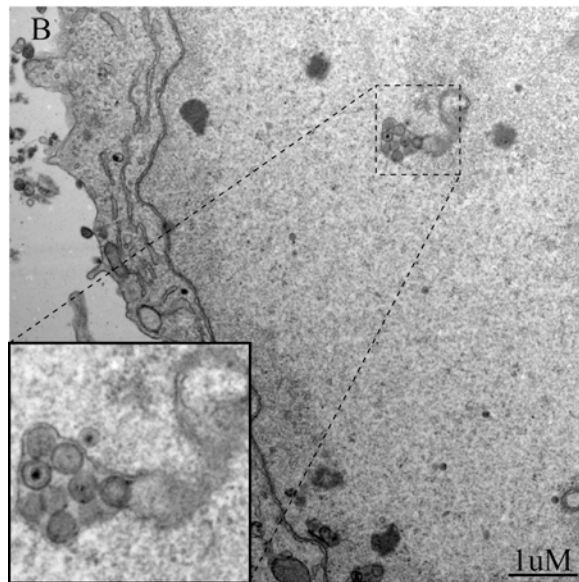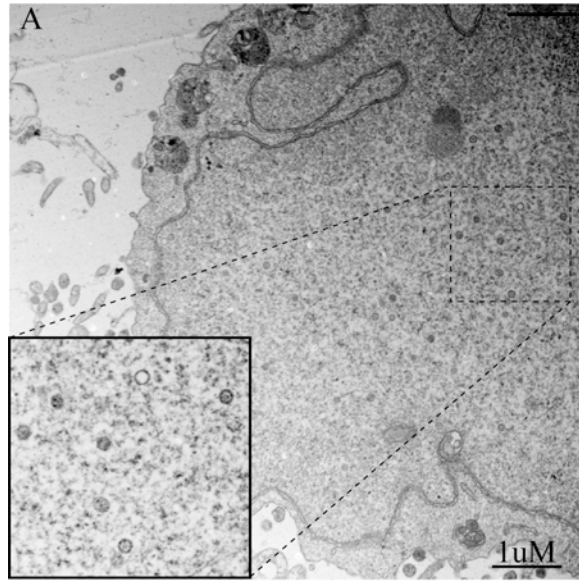
Figure 5.4. Emerin localization and modification HSV-1(F) infected BT-549 cells. A) Shown are digital confocal images of optical sections near the center of the nuclei of BT-549 cells. Zoom indicates zoom level for digital manipulation. Cells were uninfected (a-c) or infected with WT HSV-1(F) (d-f), US3-null (vRR1202) (g-i), US3 kinase dead-K220A (vRR1204) (j-l), or US3-null homologous Repair (vRR1202Rep) (m-o) with ten PFU/cell for 16 hr. The cells were then fixed and stained with monoclonal antibodies to emerin and pUL34. Emerin is represented in green and pUL34 in red. **B)** Digital image of a western blot for emerin from BT-549 nuclear lamina preparations. 1.2 x 10^7 cells were infected as described in **A.** At 16 hpi, the cells were subjected to fractionation as described in the methods. The lamina preparations were then separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for emerin. Red arrow indicates the high molecular weight emerin species.
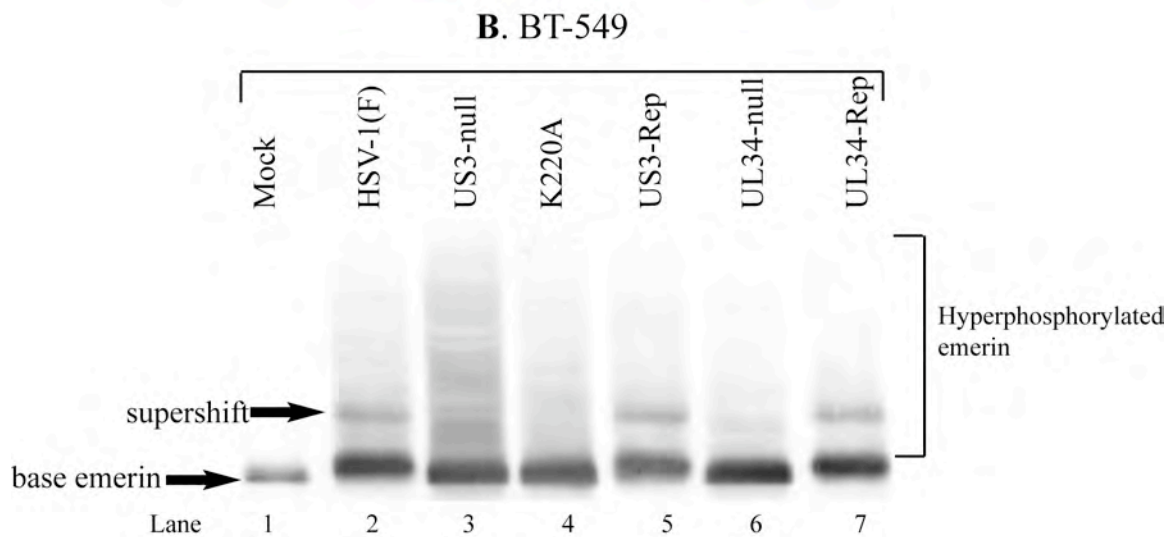
A. BT-549

	Emerin	pUL34	Merge	Zoom
Mock	a	b	c	
HSV-1(F)	d	e	f	
vRR1202 (US3-null)	g	h	i	
vRR1204 (K220A)	j	k	l	
vRR1202Rep US3-Repair	m	n	o	

B. BT-549

Lane 1 Mock, 2 HSV-1(F), 3 US3-null, 4 K220A, 5 US3-Rep, 6 UL34-null, 7 UL34-Rep

supershift

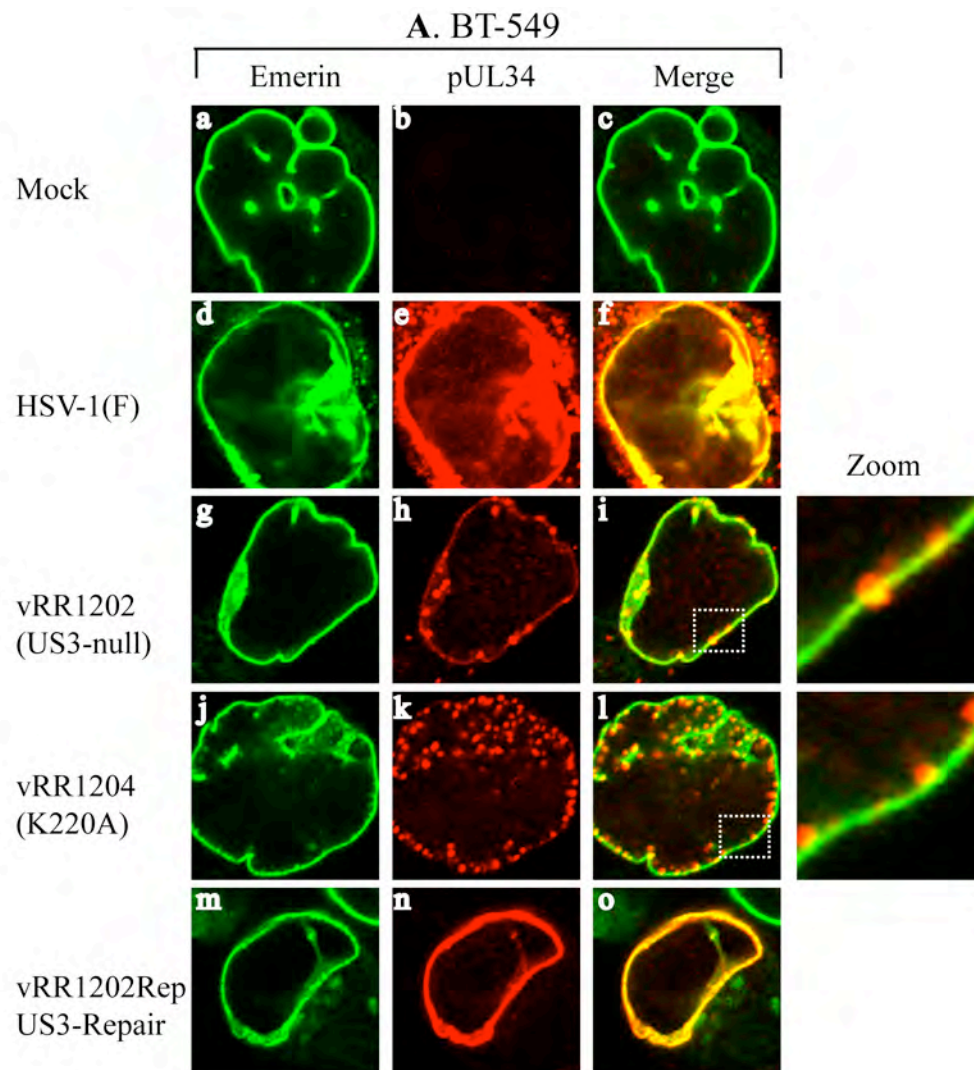base emerin

Hyperphosphorylated emerin

Figure 5.5. Emerin localization in HSV-1(F) infected MCF-7 cells. Shown are digital confocal images of optical sections near the center of the nuclei of MCF-7 cells. Cells were uninfected (a-c) or infected with WT HSV-1(F) (d-f), US3-null (vRR1202) (g-i), US3 kinase dead-K220A (vRR1204) (j-l), or US3-null-homologous-Repair (vRR1202Rep) (m-o) with ten PFU/cell for 16 hr. The cells were then fixed and stained with monoclonal antibodies to emerin and pUL34. Emerin is represented in green and pUL34 in red.
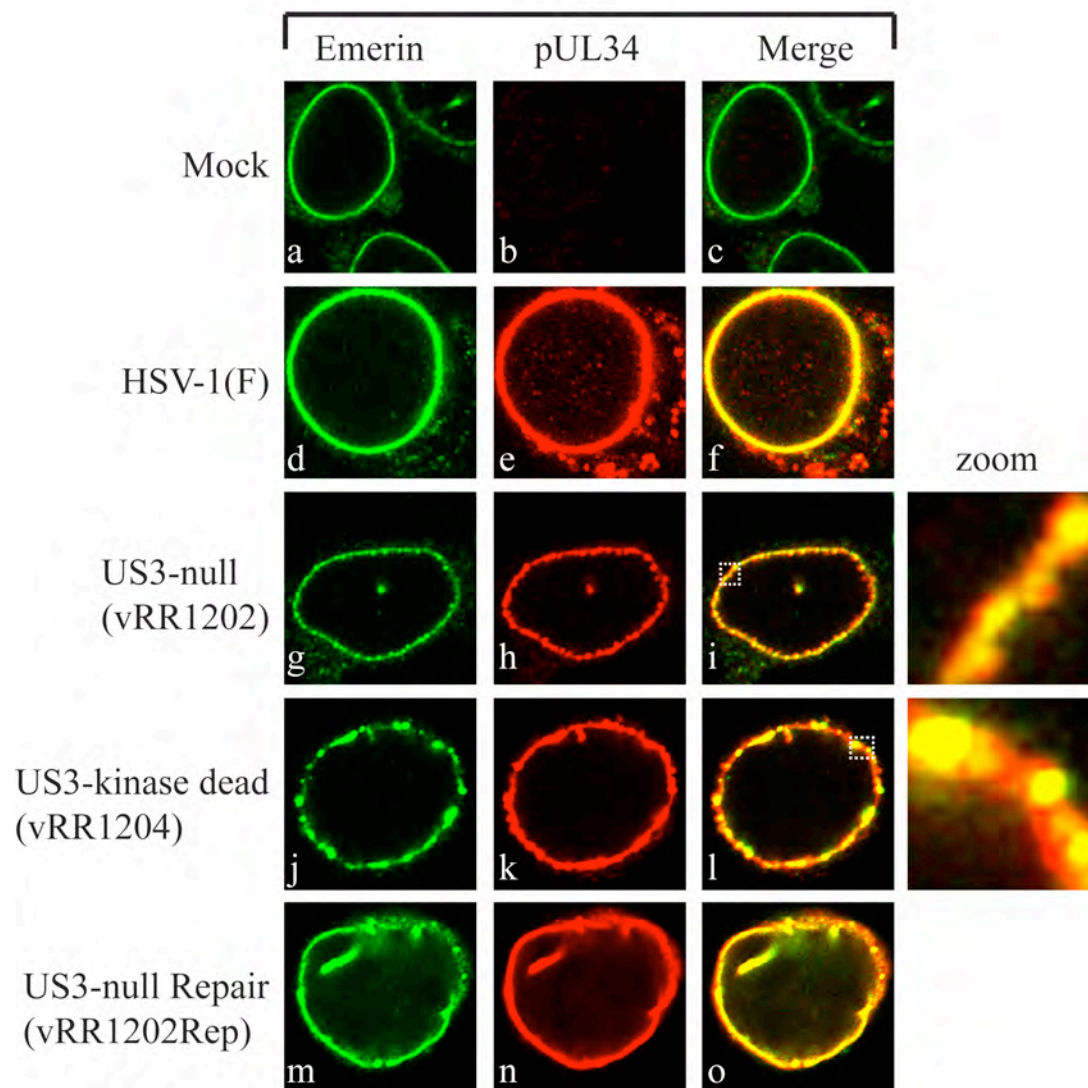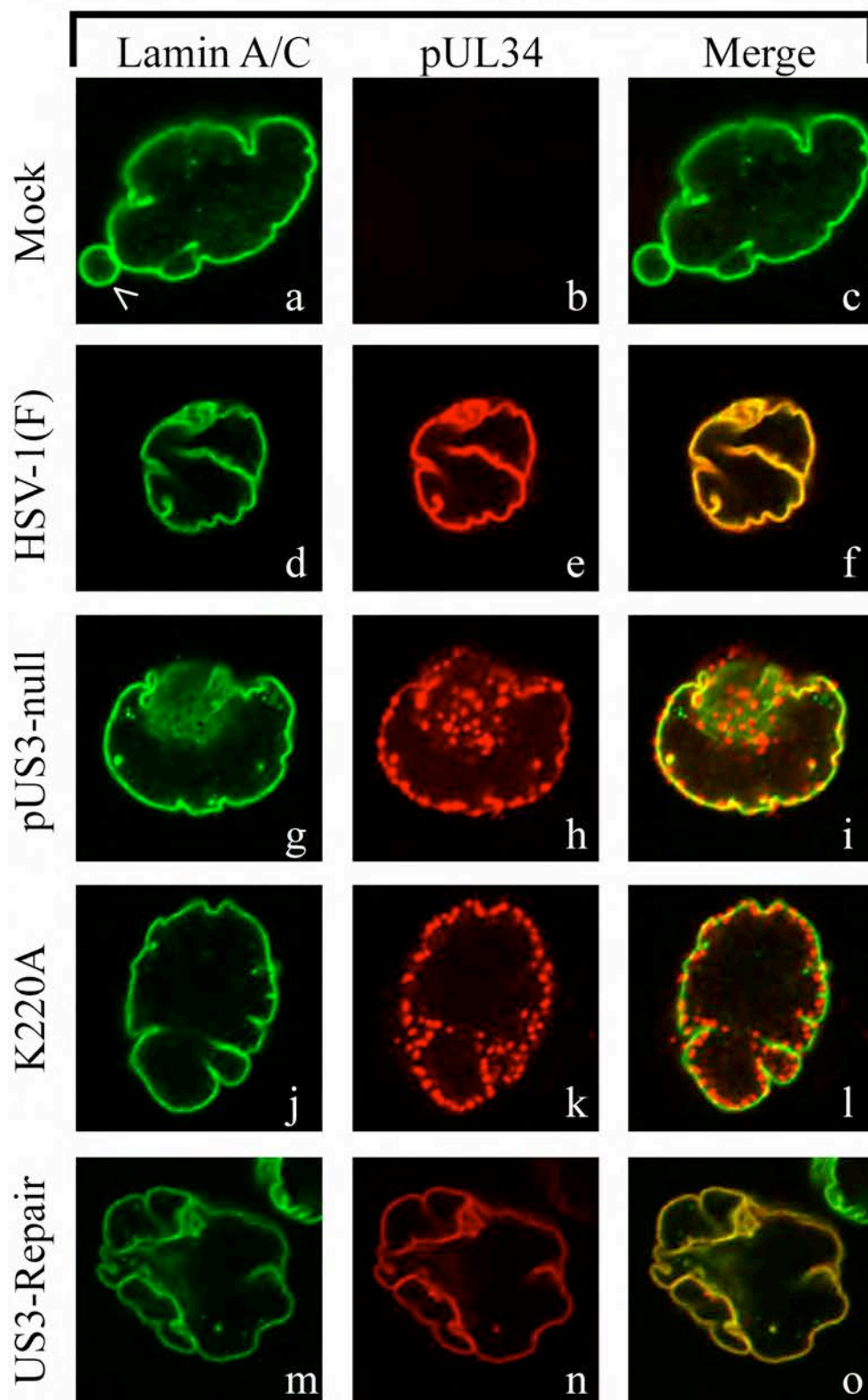
MCF-7

Emerin | pUL34 | Merge

Mock

a | b | c

HSV-1(F)

d | e | f

zoom

US3-null
(vRR1202)

g | h | i

US3-kinase dead
(vRR1204)

j | k | l

US3-null Repair
(vRR1202Rep)

m | n | o

Figure 5.6. Disruption of lamin A/C in BT-549 and MCF-7 cells in uninfected and HSV-1(F) pUS3 mutants. Shown are digital confocal images of optical sections near the center of the nuclei of **A)** BT-549 or **B)** MCF-7 cells. Cells were uninfected (a-c) or infected with WT HSV-1(F) (d-f), US3-null (vRR1202) (g-i), US3 kinase dead-K220A (vRR1204) (j-l), or US3-null-homologous-Repair (vRR1202Rep) (m-o) with ten PFU/cell for 16 hr. The cells were then fixed and stained with monoclonal antibodies against lamin A/C and pUL34. Lamin A/C is represented in green and pUL34 in red.
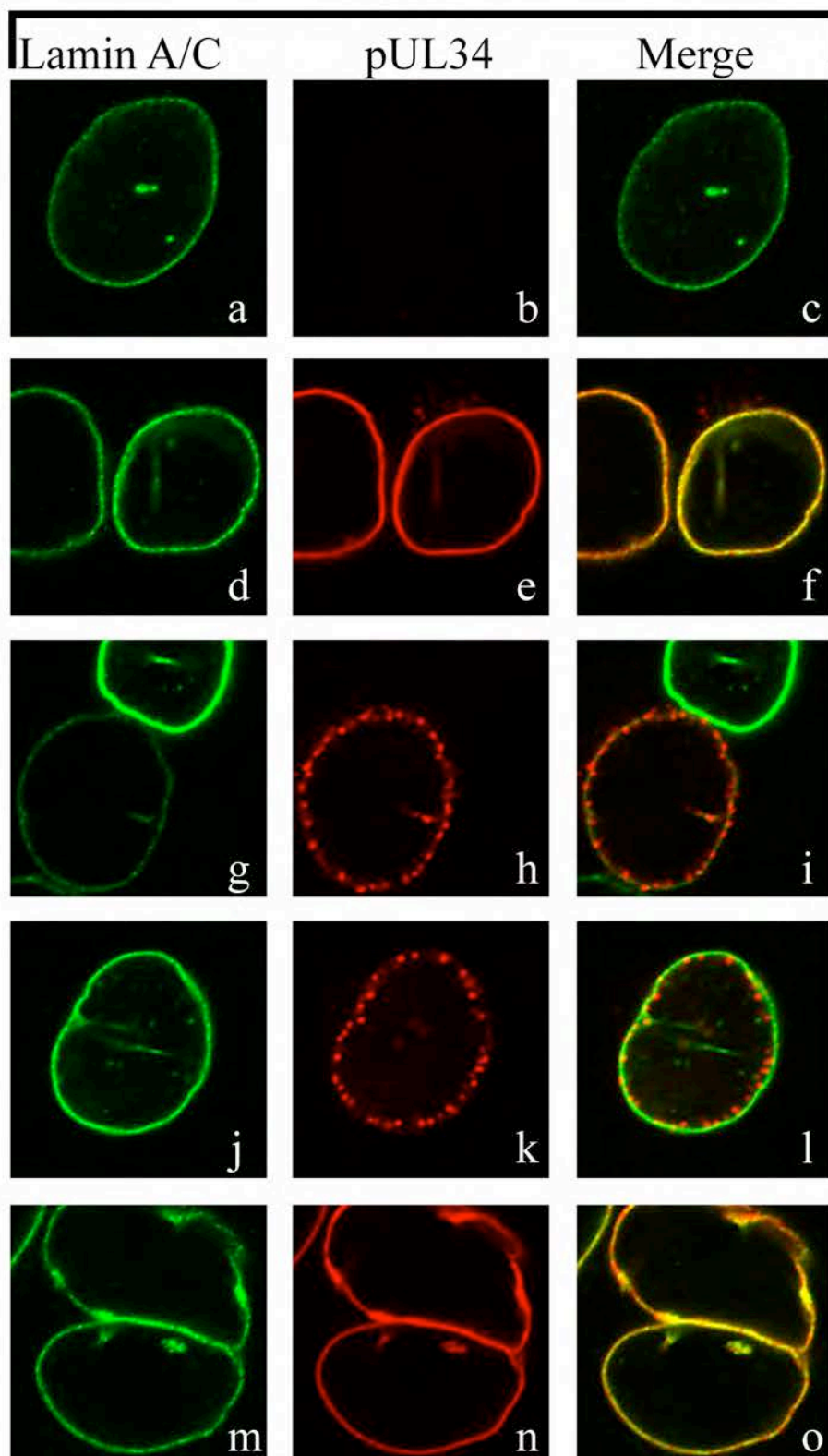
A. BT-549

	Lamin A/C	pUL34	Merge
Mock	a	b	c
HSV-1(F)	d	e	f
pUS3-null	g	h	i
K220A	j	k	l
US3-Repair	m	n	o

B. MCF-7

Lamin A/C	pUL34	Merge

Figure 5.7. Predicting cellular pUS3 complementing kinase(s) using the NCI-60 database. Shown is a graph of the fold changes in mRNA levels of different kinases in MCF-7 and BT-549 cells. The fold change in mRNA is compared the mean of all 60 NCI cell lines. Except for PKA-G, at least three independent experiments were included into the analysis. mRNA values were determined from the NCI-60 website (http://dtp.nci.nih.gov/mtweb/index.jsp). The error bars indicate standard deviation from the mean fold change from the mean of all cell lines.
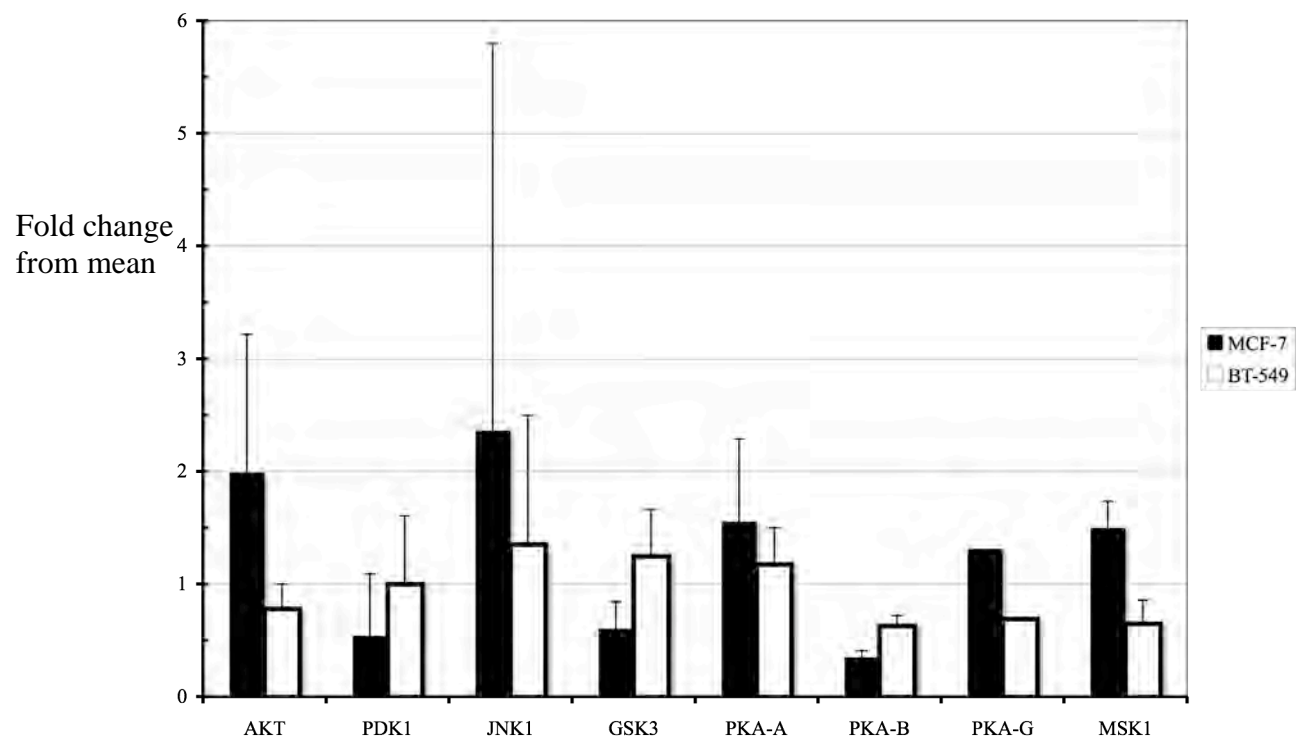
Figure 5.8. Model of double defect in primary envelopment and de-envelopment. A) Normal egress intermediates. Capsids are shown in the nucleus without an envelope. They then undergo primary envelopment at the INM and are in the perinuclear space briefly as enveloped particles. The perinuclear envelope fuses with the ONM, and then deposits an unenveloped capsid in the cytoplasm. The capsids then are trafficked to the Golgi where they undergo secondary envelopment, and are released as enveloped mature virions. **B)** Defect in primary envelopment. If a block is placed on primary envelopment, the following defects in capsid maturation are observed. Capsids are formed in the normal ratio in the nucleus and they are packaged with DNA, but few are found in the cytoplasm or outside the cell. An example of a primary envelopment block would be removal of pUL34 or pUL31. **C)** Defect in de-envelopment at the ONM. When de-envelopment at the ONM is blocked by removal of pUS3 or its kinase activity, capsids are formed in the nucleus and proceed through primary envelopment to the perinuclear space. However, they accumulate in this space and fewer capsids are found elsewhere in the cell due to the decreased efficiency of de-envelopment. **D)** Double defect in primary envelopment and de-envelopment. If both primary envelopment and de-envelopment of capsids were blocked, then capsids would enter and leave the per-nuclear space at a slower rate. This rate change may look via TEM the same as the example in **C** depending on the degree of inhibition at each step.
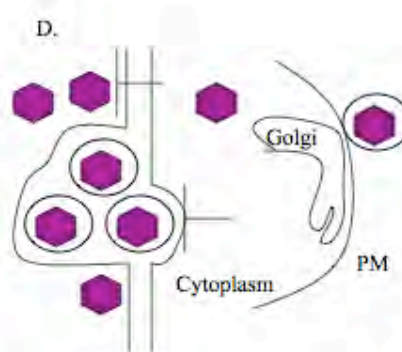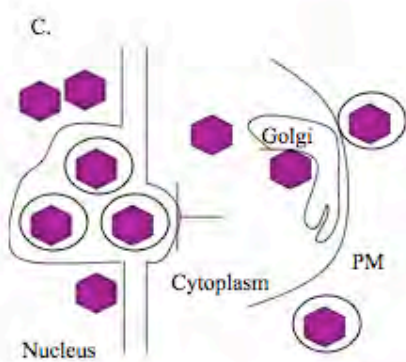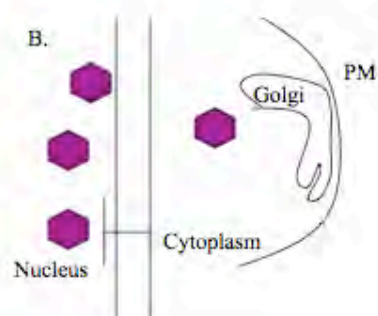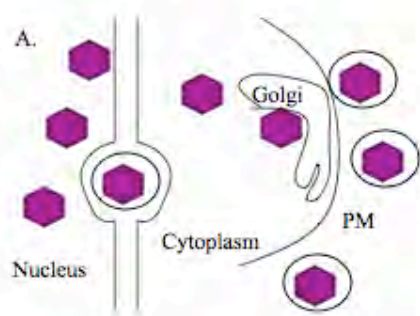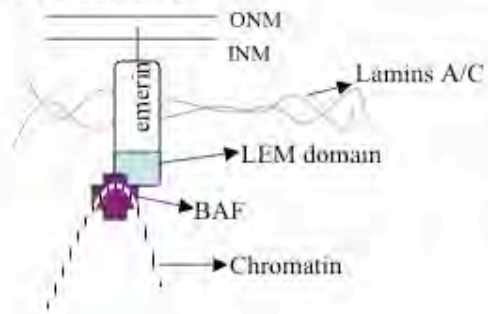
Figure 5.9. Model of emerin disruption in BT-549 cells. A) Uninfected cells emerin is attached to the INM via the TM domain and interactions with lamin A/C. Emerin is bound to BAF (purple) via the LEM domain. **B)** BT-549 cells do not express at all or insufficent levels of a cellular kinase that can be recruited in a pUL34-dependent mechanism for emerin phosphorylation. The pUL34-dependent cellular kinase may also function redundantly with pUS3. Infection with WT HSV-1 leads to direct pUS3-dependent phosphorylation of emerin at serine 49, which disrupts BAF binding. pUS3 also directly phosphorylates lamins with disrupts emerin-lamin A/C binding. **C)** Upon US3-null infection of BT-549 cells, emerin is not phosphorylated on a residue that is critical to primary envelopment of the capsid because emerin is still bound to its lamina-binding partners. The interactions between lamin A/C and emerin are maintained, and capsids are retained within the nucleus. **D)** One alternative hypothesis to this model is that BT-549 cells express a mutant emerin that cannot be relocalized because the mutant emerin cannot be phosphorylated by the pUL34-dependent cellular kinases.

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A. Un-infected

ONM
INM
Lamins A/C
emerin
LEM domain
BAF
Chromatin

B. WT HSV-1

UL34-dependent phos site
emerin
p
p
p
US3 phos site
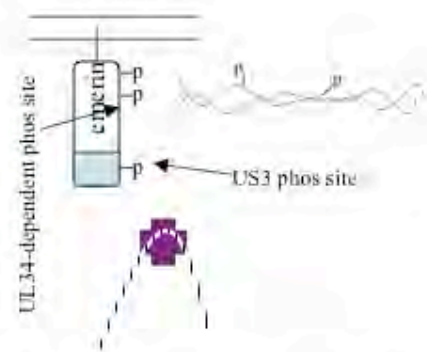
C. pUS3-null HSV-1
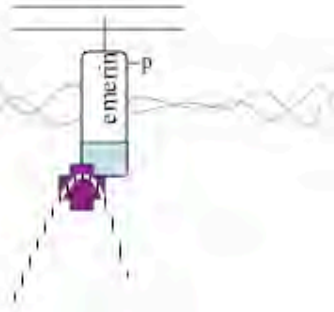
emerin
p

D. Alternative-Mutant emerin and pUS3-null

emerin
p
S/T>A at UL34-site
emerin
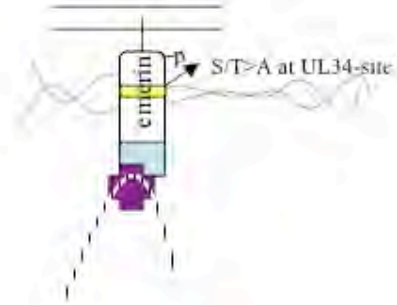
CHAPTER VI

GENERAL DISCUSSION

The nuclear envelope is a multifunctional structure that above all else regulates the movement of macromolecular compounds between the nucleus and cytoplasm. It is composed of the inner and outer nuclear membranes (INM and ONM), which are separated by the perinuclear space (for a detailed review see Gruenbaum 2005). In addition to their role in maintaining the integrity of the nucleus, lamins and LAPs regulate nuclear disassembly/reassembly during mitosis, the cell-cycle, gene expression, and apoptosis. The structure and functions of the lamina are being intensively researched because mutations in the lamina components lead to a variety of genetic diseases whose pathogenesis is not understood. The lamina is of increasing interest to virologists because viruses manipulate its structure and function of the lamina components in order to allow nuclear entry or egress, to facilitate genome integration and perhaps to regulate gene expression and the cell cycle. This thesis has focused on the mechanisms that herpes simplex has developed to overcome, manipulate, and parasitize the components of this structure. Understanding how HSV-1 induced phosphorylation of emerin regulates emerin localization and function, may lead to a greater understanding of emerin function in uninfected and EDMD cells.

Evidence for HSV-1 tailored lamina disruption

Completely disassembled-emerin. The nuclear lamina, both lamins and LAPs, are disrupted during HSV-1 and other herpesvirus infections (20, 34, 36, 89, 142, 145, 180, 181, 188, 190, 191, 223, 242, 249). The data presented thus far suggests infection-induced disruption of nuclear lamina components falls into threes classes: (i) completely disrupted, (ii) localized or microdomain disruption, and (iii) unknown or untested. Data presenting in this thesis and by others in the field would suggest that emerin is part of the completely disrupted class (Figure 3.8 A and 5.4 A) (142, 186). The hypothesis of

tailored disruption of the nuclear lamina suggests the virus has developed a sophisticated mechanism of manipulating the many different proteins that compose the nuclear lamina.

WT HSV-1(F) infection induces emerin hyperphosphorylated (142). This is dependent on pUL34 and pUS3 as well as cell type (Figure 2.1, 2.3, 2.4, and 5.4). Although levels of emerin hyperphosphorylation can vary due to cell type, it appears that all the emerin in the lamina is phosphorylated upon WT infection (Figure 2.1, 2.3, 3.8, and 5.4). Emerin itself is not required for infection with HSV-1 (Figure 2.5) but it is unknown if phosphorylation of emerin is required for herpesvirus growth. In a WT infected cell, although emerin is still localized to the NE in blebby patches, via FRAP, it is significantly more mobile within the membrane suggesting disconnection from other nuclear lamina-binding partners such as lamin A/C (142). Additional support for complete disruption is observed in an US3-null infection when extreme nuclear lamina disruption can easily be observed (20, 142). Under these conditions, emerin and lamin A/C no longer co-localize, as they do in uninfected cells (43, 142). These data all support the model that the HSV-1-mechanism of emerin disruption entails complete emerin detachment from at least lamin A/C.

Emerin phosphorylation sites and dependency of phosphorylation for infection. pUL34 and pUS3 are both essential in WT levels of emerin hyper-phosphorylation during infection (Figure 2.1). pUS3 direct phosphorylation of emerin had not been demonstrated however; there is a pUS3 consensuses sequence in emerin (212, 226). The pUL34-dependent component of emerin phosphorylation was dependent on a cellular kinase (Figure 2.3 and 2.4). Envelopment complex proteins across *Herpesviridae* have been implicated in the recruitment of PKC isoforms to the nuclear envelope (180, 191, 206). The sensitivity of pUL34-depenent emerin phosphorylation to Rottlerin treatment suggests that a cellular kinase sensitive to Rottlerin is responsible for emerin phosphorylation (RttSK) (Figure 2.4). At its conception, Rottlerin was considered a specific inhibitor of PKC delta, however, new data suggests non-specific cellular kinases

(non-PKC isoforms) are also inhibited by Rottlerin (253). This pUL34-dependent cellular kinase is likely not a PKC isoform, due to the insensitivity of phosphorylation to PKC inhibition by BIM I treatment (Figure 3.8 B and C). More work should be done to determine which emerin residues are modified upon infection, which are dependent on the expression of pUL34 and/or pUS3, and which kinases mediate those post-translational events.

The LEM domain of emerin contains a pUS3 consensus site that is phosphorylated by PKA (215, 226). The PKA phosphorylation disrupted emerin-BAF association (226). It remains is unknown if pUS3 directly phosphorylates serine 49 of emerin or if this is an essential event in the disruption of the nuclear lamina prior to nuclear egress. Regardless, data presented in this thesis suggest a model presented in (Figure 6.1). If serine 49 were mutated to alanine (S49A) or glutamic acid (S49E) would either inhibit pUS3-dependent phosphorylation or mimic respectively. The mock-infected S49A cell should demonstrate a smooth, round NE. The S49E would be blebby and wrinkled and resembled WT infected cell emerin (Figure 6.1). US3-null infected of S49A and S49E expressing cells should not change the localization of either mutant emerin unless this is not the pUS3-dependent site or the pUL34-dependent phosphorylation site creates an intermediate phenotype for the S49A mutant. Alternatively, infection with a double pUS3-pUL34-null virus would be extremely informative in these experiments; however, several attempts to create this virus have been unsuccessful. It is also possible that in the absence of pUS3, a negative regulator of lamina regulation, extreme emerin disruption will be observed regardless of mutation (20). WT infection of the mutant cells could result in multiple possible outcomes due to the pUL34-dependent component phosphorylation site. It is still unknown which residue is phosphorylated and which kinase mediates this modification. Therefore, WT infection of S49A cells have two possible outcomes: (i) round, smooth or (ii) some wrinkling,

blebbing. WT infection of S49E cells will be either: (i) indistinguishable from mock infected or (ii) extremely blebby and wrinkled (Figure 6.1).

Emerin phosphorylation is an important topic of this thesis and for human health. Mutations in emerin and other lamin components are one cause of a class of diseases known as laminopathies (255). The results from both BT-549 cells and Rottlerin treated cells suggest that phosphorylation of a pUL34-dependent site(s) of emerin is sufficient to mediate emerin disconnection from the lamina in cultured cells (142) (Figure 3.8 and 5.4) (Model Figure 5.8). To determine whether phospho-mutant emerin mutants can directly interact with known binding partners involved in emerin retention at the INM, interactions with lamin A/C and BAF could be measured using GST pull-down assays. This targeted approach would evaluate the molecular mechanism of phospho-dependent emerin binding with the known lamina interaction partners BAF and lamin A/C, whose binding mediates assembly and maintenance of emerin in the nuclear lamina (Figure 6.1) (43, 69, 146). Therefore, mutation of individual pUS3- or pUL34-dependent phosphoacceptor sites should still allow infection-dependent disconnection of emerin from the lamina. This disconnection should be reflected in altered emerin localization and increased FRAP mobility in infected cells as we have seen before (142). Alanine substitutions of all pUS3- or pUL34-dependent phosphorylation sites, on the other hand, should allow emerin to remain connected to the lamina in infected cells resulting in localization pattern similar to uninfected cells (Table 6.1). S/T>A emerin with all infection-dependent phosphoacceptors mutated will be less mobile in FRAP analysis than WT emerin after HSV-1 infection because it cannot be disassembled from the lamina via phosphorylation. This mutant will also retain interactions with BAF and lamin A/C.

This thesis has attempted to determine if phosphorylation of emerin and other lamina components is required for nuclear egress of HSV (Chapter III). The S/T>A and S/T>E emerin mutants should differ in their effects on viral growth. Mutant emerin with all-infection-dependent phosphoacceptors substituted with alanines, and which cannot be

modified, may be significantly inhibit viral growth because emerin's association with the lamina cannot be disrupted via phosphorylation. If this is true, then for the individual substitutions, we expect that viral growth will be correlated with the ability of that emerin mutant construct to be disconnected from the lamina during infection. This outcome would suggest the hypothesis that inhibition of viral growth is due to inhibition of nuclear egress. This hypothesis could then be tested by electron microscopic examination of infected mutant emerin-expressing cells (225, 232, 236).

What is the cellular kinase? Emerin becomes hyperphosphorylated upon infection with herpesviruses (142, 186) (Figure 2.1). pUS3 is required for part of the WT level hyperphosphorylation, while pUL34 is required to recruit a cellular kinase (142) (Figure 2.3 and 2.4). Rottlerin, a small molecule inhibitor, can inhibit the pUL34-dependent cellular kinases HSV-1 induced emerin and lamin B phosphorylation (Figure 2.4) (142, 207). In spite of these data, the pan-PKC inhibitor, BIM I, had no effect on emerin hyperphosphorylation (Figure 3.8 C) (144). This suggests that PKC isoforms are not involved in emerin modification but kinases sensitive to Rottlerin are involved in emerin hyperphosphorylation (Figure 2.4 and 3.8 C). Unfortunately, we were unable to identify the cellular kinase responsible for the pUL34-dependent phosphorylation event(s), however the likely candidates include: AKT, PKA, MSK, PDK1, and JNK1 (253). These cellular kinases are sensitive to Rottlerin and contain a consensus sequence in human emerin. Identification of the phosphorylated residues should allow identification of the kinase responsible.

Local disruption-lamin A/C. In contrast to emerin, lamin A/C is disruption is not complete. Rather, lamin A/C disruption appears to fall into the classification of microdomain disruption. This phenotype is especially noticeable in US3-null infected cells where large holes are opened up in the lamina (142). All of the emerin is reorganized into puncta, but not all of the lamin A/C is disrupted ((142) and Figure 4.5). The contrasts in mechanism can also be observed in the kinases utilized to disrupt lamina

components. In HEp-2 cells RttSKs do not regulate lamin A/C relocalization (Figure 4.5) but do regulate emerin relocalization in pUS3-kinase-dead infected cells (Figure 3.8 A). Lamin A/C and emerin are both substrates of emerin but only ten-percent of the lamin A/C in the lamin is phosphorylated upon infection while it appears that almost 100% of emerin is phosphorylated (20, 142, 188, 189). These data suggest that HSV-1 has multiple mechanisms for disrupting the multiple players in the lamina.

Enhanced associated with lamina coupled with de-modification-LAP2. LAP2 alpha and beta were both de-modified during WT HSV-1 infection, however, unlike emerin and lamins, this de-modification did not required the expression of pUL34 or pUS3 (Figure 2.2). While the exact modification is unknown, it is likely de-phosphorylation due to the size shift in SDS-PAGE. Both LAP2 alpha and beta are reversibly phosphorylated during mitosis in a manner that regulates their association with lamins and chromosomes (53, 73, 275). This novel discovery that infection induces de-modification creates a third class of infection induced lamina disruption. The third class is divided from emerin (the first) and lamins (the second) in that it is de-modified and its association with the lamina is actually enhanced upon infection (Figure 2.2 and Bjerke, unpublished). These data would suggest that the virus has developed multiple mechanisms to deal with the different lamina components. One hypothesis for the differential disruption by the virus could be due to the different functions of these proteins. It is possible that HSV-1 has developed a mechanism to disrupt emerin functions while retaining LAP2 functions.

As described previously, LAPs, especially LAP2alpha, have non-structural functions such as regulating gene expression though pRB and controlling the cell cycle (193, 198). LAP2beta is important in regulating gene expression by interacting with the N-terminus of HA95, inhibiting the transcription activity of E2F, and interacting with HDAC3, all of which are interactions that are predicted to cause transcriptional repression (59, 172, 173, 204, 254). Like all LEM domain proteins, LAP2 alpha and beta

have important roles in chromatin structure and organization by binding to BAF (87, 92, 246). It is possible that herpesvirus have developed a mechanism of co-opting LAP2alpha function control of the cell-cycle (193). This could also explain why the envelopment complex proteins were not involved in de-modification (Figure 2.2). In the future, the role of HSV-1 proteins such as ICP0 and ICP27 should be examined in LAP2 de-modification since they have previously been implicated in arresting the infected cell-cycle (63, 64, 106, 162).

What happens to the other LAPs? This thesis has focused primarily on emerin and LAP2 yet there are many other LAPs. Lamin B receptor (LBR) was the first LAP whose disruption was demonstrated upon herpesvirus infection (242). It remains unknown if MAN1, LAP1, LUMA, and other unlisted or un-discovered LAPs are affected by herpesvirus infections. It is also unknown if expression of envelopment complex proteins is required for disruption as it is with emerin or if the mechanism of de-modification is envelopment complex independent similar to LAP2 (142) (Figure 2.2). An uncharacterized phenotype and mechanism of disruption is also possible. The last layer of complexity is the cellular factors that influence these modification pathways that maybe differently regulated in different cell types as observed for emerin between HEp-2 and Vero cells and therefore lead to spectrum of modification phenotypes (142). It is possible that due to the multiple roles these proteins play (structural and signaling) that unlike emerin, deletion of one of these LAPs may lead to virus growth reduction. Therefore, HSV-1 growth should be tested in the absence of LAP2beta, as well as other LAPs, to determine if, unlike emerin, they are required for efficient single step growth or not.

Why are different kinases used-PKC redundancies in lamina disruption? The data from Chapter III and other work from the field suggests that cellular kinases such as PKC alpha and delta, but not PKC zeta, are required for phosphorylation of lamina components prior to envelopment of a herpesvirus capsid (191, 207). Inhibition

of PKC alpha or PKC delta individually, however was not sufficient to inhibit virus growth suggesting that PKC's may function redundantly for disruption of the lamina in infected cells (Figure 3.5). Direct testing of this hypothesis has not been rigorously evaluated. Also, each PKC isoforms in human cells is regulated individually (220). They are each transcribed from a different gene in every cell type may have a different expression level of the ten different PKC isoforms (220). Therefore, if pUL34 is required for recruiting PKC isoforms to disrupt the lamina, more work should be done to determine how the redundancy functions on the molecular level. Knockout mice have been created for PKC: alpha, beta I/II, gamma, delta, epsilon, zeta, and theta (26, 150-152, 182, 241, 261). Alternatively, shRNA or siRNA constructs could be designed to decrease individual PKC isoform expression. These constructs, individually and in all possible combinations, or knockout cells should be used to determine the mechanism of PKC redundancy in WT and US3-null HSV growth. Also, it would be interesting to determine what portion of pUL34 is necessary and sufficient to recruit PKC isoforms to the NE (191, 206). It would also be interesting to determine if the recruitment of PKCs occurs via a direct or indirect interaction with pUL34. These data would not only provide more information about how nuclear lamin disassembly occurs in herpesviruses, but also on how PKC isoforms may or may not be able to function redundantly.

<div align="center">Viral and cellular kinase redundancy</div>

Significance of cell type. There are cell type differences in the degree of hyperphosphorylation, however all the emerin in each cell is modified and the same viral genes are required (Figure 2.1 and 5.4). Emerin is hyperphosphorylated in HSV-1 infected cells as demonstrated by the decrease in emerin mobility in SDS-PAGE that is reversible by phosphatase treatment (142). Emerin hyperphosphylation in BT-549 and Vero cells is extreme (Figure 5.4 B and Figure 2.1), while in HEp-2 (Figure 2.1 A) was limited to at max three species. SNB-19 (Figure C) cells have a hyperphosphorylation between the two extremes. Regardless of the limited phosphorylation (HEp-2) or

extreme (Vero), WT infection of emerin lead to wrinkled, blebby localization suggesting that levels of hyperphosphorylation of emerin were not important but rather critical phosphorylation events(s) (142). In BT-549 cells, US3-null infection lead to a smooth even distribution of emerin along the nuclear rim, suggesting it was bound to its nuclear lamina binding partners (Figure 5.4 Ag). Interestingly, emerin from US3-null infected cells was still hyperphosphorylated (Figure 5.4 B). Together these data suggest that, at least in BT-549 cells, hyperphosphorylation is not sufficient to disconnect emerin from the NE. A specific residue, although unknown at this time, must be phosphorylated. This lack of disconnection could due to lack of expression of a pUL34-recruitable kinase or expression of mutant emerin (Figure 5.9). It would be of interest to determine what molecular signal determines emerin-lamin A/C interactions or emerin-NE retention during HSV infection. It is also imperative to the herpes egress and EDMD fields that the identity of the infection–induced phosphorylated residue on emerin is identified and if phosphorylation of that residue on emerin does indeed detach emerin from binding to lamin A/C.

Lamin A/C localization also appears to be regulated in a cell type dependent manner. Lamin A/C in BT-549 uninfected cells resembles progeria mutant LMNA and LMNC (264). The belbs and undulations that are present in both cells are strikingly similar (264). Lamin A/C localization in uninfected BT-549 cells also resembles from HEp-2 US3-null infected cells (Figure 5.6 Aa) (142). Although the mechanism of the disrupted nuclei has yet to be determined, it seems reasonable to conclude that disrupted protein-protein interactions within the NE are likely responsible for the phenotype. Interestingly tumors are graded based on the localization of lamin B and emerin (32). Cancer cells are graded from low to high grade, according to the appearance and amount of nuclear shape pleomorphism (32). The BT-474 cells, similar to BT-549, anti-emerin confocal images also demonstrate blebs and wrinkles suggesting that the deformed nuclear pleomorphism is a common feature of cancerous cells and could be used to test

US3-null growth and emerin re-localization (32). Also, it is also unclear yet if the defect in US3-null growth in BT-549 cells is due to a defect in emerin disruption (Figure 5.9), nuclear egress coupled with reduced de-envelopment (Figure 5.8), or aberrant lamin ratio, but it seems possible that if the LMNA and emerin are already mislocalized, and pUS3 is missing, HSV-1(F) capsid primary envelopment may be additively impaired or altered (245). It is also possible that the BT-549 cells express a mutant emerin that cannot be phosphorylated by a viral or viral-recruited kinase thus blocking lamina disruption (Figure 5.9). Regardless, this cell line makes a great system for studying pUS3-function but these data also suggest that perhaps it could provide new data on the dynamics of the NE in progeria, X-linked EDMD, AD-EDMD, FPLD, and breast cancer cells.

US3, PKC and RttSK redundancy in protein translation. Chapter III and Chapter IV both demonstrate that the small molecule inhibitors used in this thesis inhibit protein translation. (i) Uninfected Vero and HEp-2 cells are dependent on PKC activity for protein translation (Figure 3.4 and 4.4) (283). (ii) RttSKs are required for both HSV-1 infected and uninfected protein translation (Figure 4.4) (253, 283). (iii) WT HSV-infected cell translation is not dependent on PKC activity (Figure 3.4 and 4.4). This finally finding suggests that HSV infection protects the infected cell protein synthesis machinery form the effect of PKC inhibition by BIM I treatment. PKC isoforms have been identified mostly as positive regulators of translation in indirect signaling through Erk, MAPK, or mTOR signaling pathways (2, 3, 104, 136, 233, 283). Unfortunately due to the severe protein translation inhibition associated with Rottlerin treatment, few conclusions could be made about Rottlerin-treated infected cell envelopment related data (Figure 4.1, 4.2, and Table 4.1).

Global protein translation did not appear to be additionally inhibited in BT-549 cells (Figure 5.2 A). Certain bands were of different intensities between WT infected and US3-null infected BT-549 and MCF-7 cells (Figure 5.2 A). This would suggest that specific proteins are translated at different levels in the different cells in the different

viruses. While the difference was not large, if the particular protein has an essential function in viral growth, this minor defect could be compounded. The essential proteins tested (VP5, pUS11, pUL34, gC) did not appear to have accumulation defects in US3-null infected cells (Figure 5.2 B). It would be interesting to determine if the pUS3-dependent phosphorylation of TSC2 and indirect inactivation of 4E-BP1 occurs in BT-549 cells (40).

Role of PKC and RttSK in pUL34 accumulation. Rottlerin appears to be a potent viral translation inhibitor for almost all genes tested except for pUL34 (Figure 4.3). When compared to another, leaky-late gene such as VP5, treatment with Rottlerin at five hpi results in a huge decrease in the accumulation of VP5 but almost no change in pUL34 (Figure 4.3 compare lanes two and three). Viral genes also seem immune to BIM I treatment (Figure 3.4). This suggests that the mechanism for protein translation inhibition is: (i) different for the two small molecule inhibitors and (ii) there is something unique about either pUL34 stability as a protein or (iii) translation of this mRNA occurs via a mechanism that is unlike that of the other tested viral proteins. pUS11 has been demonstrated to bind to UL34 mRNA and negatively regulate its accumulation (230). It is possible that in Rottlerin treated cells, pUS11 levels are sufficiently reduced to relieve any pUS11-mediated repression. This would have to be directly tested by performing an electrophoresis mobility shift assay (EMSA) in Rottlerin and vehicle treated cells. The combination of pUL34 mutant(s), pUL34-null, pUS11-null, and WT HSV-1 infections should allow for the mechanistic unraveling of pUL34 stability in Rottlerin treated cells.

Translation inhibition: PKC redundancy with pUS3. In Chapter IV the question of pUS3 redundancy with PKC and RttSK during protein translation was addressed (Figure 4.4). pUS3 was recently attributed with a new role in regulating protein translation (40). pUS3 appears necessary for the inactivation of 4E-BP1 by phosphorylation of TSC2 (40). The TSC2-silencing via pUS3 phosphorylation allowed the translation complex to assemble (40). In Figure 4.4 A, the hypothesis was tested that

in HEp-2 cells there is functional redundancy between pUS3 and PKC for efficient translation of viral genes late in infection. However in Vero cells (Figure 4.4 B), the redundancy is no longer observed. The loss of redundancy could be due to the lack of expression of a cellular protein in Vero cells. Rottlerin treatment inhibits translation regardless of pUS3 expression and cell type (Figure 4.4 A and B). This would suggest that (i) the two small molecule inhibitors function in different pathways (ii) pUS3 and PKC are in the same pathway in HEp-2 cells and (iii) RttSK inhibit pUS3-dependent and –independent translation.

Herpesviruses are known activators of the mTOR pathway (4, 28, 183, 280, 281). BIM I is a known inhibitor of 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) and S6K (p70 ribosomal protein S6 kinases), two signaling kinases upstream of the mTOR pathway (283). It therefore seems possible that viral protein translation is blocked in the presence of BIM I and absence of pUS3 is due to the inability to activate the mTOR pathway. This hypothesis however would have to be directly testing by evaluating the activation of different mTOR components in BIM I-treated US3-null infected cells or testing HSV-1 infectivity (and translation) in MEFs that lack components of the mTOR pathway.

In conclusion, the history of cellular and molecular biology shows clearly and repeatedly that the most encouraging news one can receive when studying a mysterious cellular process is that there is a virus that makes use of that system and alters it. The data presented in this thesis would suggest that HSV-1 alters the nuclear lamina as well as cellular protein translation. Easy genetic manipulation of HSV-1, and well-developed tools for assessing HSV-1 replication often make it easier to study a cellular protein's function in the context of infection than to determine the function in uninfected cells.

Figure 6.1. Model of mutations in emerin-serine 49. Schematic drawing of possible phenotypes of mutations made to Serine 49 in the LAP emerin. Emerin is drawn to demonstrate the transmembrane (TM) domain, lamin A/C binding domain, and LEM domain. The C-terminus of emerin contains the TM-domain and anchors the protein into the INM. The LEM domain binds to BAF (purple cross), which can bind to DNA, (black and white line) to anchor chromatin to nuclear periphery. Serine 49 is in the LEM domain (blue), within the BAF binding sequences. If this were changed to glutamic acid (S49E) this would mimic phosphorylation (red line). Conversely, if mutated to alanine (S49A) would block phosphorylation (yellow line). S49E should not be able to bind BAF, and S49A should always be bound to BAF. The green images below are to model the localization of emerin in confocal microscopy in a optical section near the center of the nuclei. Mock-infected S49E cells would have wrinkled blebby nuclei, while S49A cells would have round smooth nuclei. US3-null infected cells would look the same as mock infected. HSV-1(F) infected S49E cells would have extreme emerin disruption. While S49A HSV-1(F) infected would either be round and smooth or have a small, minor amount of disruption due to pUL34-dependent phosphorylation.
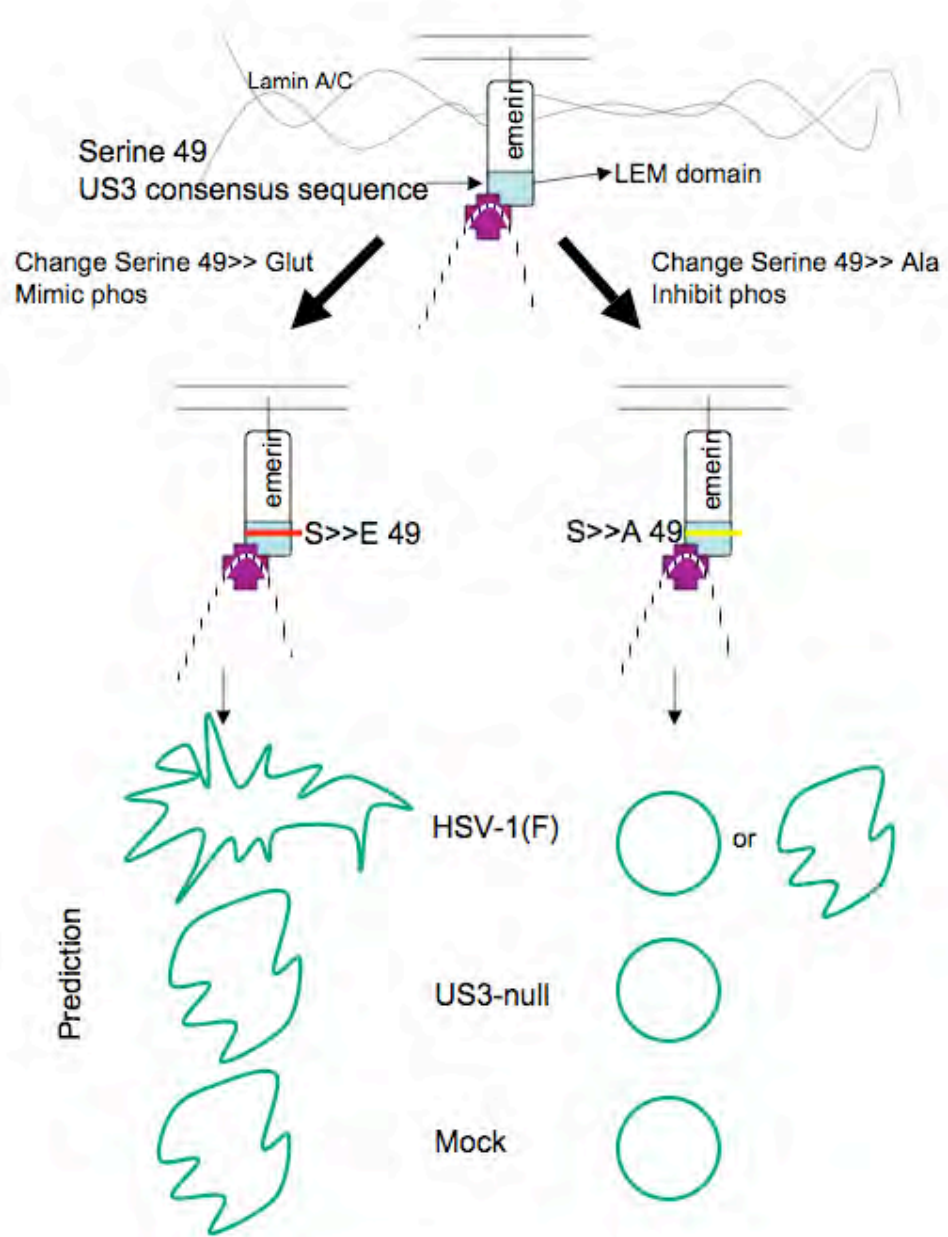
Table 6.1. Predictions of phospho-emerin mutants. Emerin is a highly phosphorylated protein due to the 53 serine and theronines. To determine the requirement of specific residue phosphorylation for protein-protein interaction disruption, HSV-1(F) viral growth, and emerin localization in the nuclear lamina, the specific residues that are phosphorylated during HSV-1(F) infection should be identified. This table describes the possible outcomes of mutating the identified residues to alaines or glutamic acids to prevent or mimic phosphorylation, respectively. These data would allow for rigorous evaluation of the aforementioned hypotheses.

Infection	Mock			HSV-1		
Emerin	WT	S/T»A	S/T»E	WT	S/T»A	S/T»E
Confocal localization	Smooth nuclear rim	Smooth nuclear rim	Similar to WT/HSV-1	Wrinkled and blebby nuclear rim	No change from mock/ S/T»A	Indistinguishable from mock/ S/T»E
FRAP	Baseline mobility	Same as mock/WT	Increase above mock/WT- Similar to WT/HSV-1	Significant increase in mobility over mock/WT	No change from S/T»A/mock	Same as mock/ S/T»E
Lamin A/C and BAF binding	Bind	Bind	No binding	No binding	Bind	No binding
Viral Growth				WT levels	Reduced	WT levels

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