Ubiquitin Targets and Molecular Mechanisms of Herpes Simplex Virus 1 Infection in Adult Sensory Neurons

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ABSTRACT

Herpes simplex virus 1 (HSV-1) is a double-stranded DNA virus, often acquired during childhood, that currently infects more than 50% of the human population. The symptoms of infection are herpetic lesions that frequently appear throughout a host's life in response to stress in the orofacial or genital region. As a pathogen, HSV-1 replicates rapidly in epithelial cells, but it is also capable of infecting neurons where it can pursue a lytic or latent infection. Latency is a state of viral quiescence where the virus can persist indefinitely yet remain poised to reactivate. Latency is unique to herpesviruses and key to HSV's success, but the molecular mechanisms that govern this state are unclear. A virus-encoded E3-ubiquitin ligase, Infected Cell protein 0 (ICP0), is often correlated with latency establishment but is detected in opposition to the state of latency. During lytic infection, ICP0 has many biological roles but primarily catalyzes the addition of ubiquitin to target substrate, marking proteins for degradation or altering their function. This ubiquitination ability allows ICP0 to alter the intracellular environment making neurons conducive to lytic or latent HSV-1 infection. ICP0's neuron-specific targets, however, are unknown, representing a significant gap in knowledge. Through the studies presented in this dissertation, we identified some of the neuron-specific ubiquitination targets of ICP0 in neurons. We utilized primary adult sensory neurons of the dorsal root ganglia and HSV-1 viral strains KOS, wild-type virus encoding a fully functional ICP0, and HSV-1 n212, encoding a truncated ICP0 protein, to illuminate the mechanisms involved in establishing and maintaining HSV latency. By using adult primary neurons and functional HSV-1 strains with and without ICP0, we were able to show that ICP0 regulates host and viral proteins during the initial onset of neuronal infection. We also show that based on neuronal conditions set forth before HSV-1 initial infection, host proteins will influence HSV-1 viral proteins to repress viral gene expression, thereby promoting the establishment of latency.

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GENERAL PUBLIC ABSTRACT

Herpes simplex virus (HSV-1) is a virus, often acquired during childhood, that more than 50% of people have. Those who are infected with HSV-1 often have cold sores that appear in response to stress on the face or on the genitals. As a virus, HSV-1 replicates around the eyes, nose, and mouth but can also infect neurons where it can continue to replicate or establish latency. Latency is when the virus is inside the neurons but is unnoticeable and can reappear in response to stress. The state of latency is unique to herpesviruses and key to the success of HSV-1, but scientists are unsure of how it works. A protein made by the virus, Infected Cell Protein 0 (ICP0), is often correlated with the state of latency but is often present when the virus is not latent. ICP0 does a lot to support HSV-1, but it primarily destroys proteins that prevent the virus from replicating. By destroying proteins that prevent HSV-1 replication, ICP0 can help the virus make more viruses. The proteins that are destroyed by ICP0 are currently unknown, which represents a significant gap in knowledge. Through the research conducted in this dissertation, we identified some of the proteins that ICP0 destroys in neurons. We utilized neurons from the dorsal root ganglia and HSV-1 viral strain KOS, which encoded a functional ICP0, and n212, which encodes a nonfunctional ICP0, to study the mechanisms used by the virus to infect neurons. By using HSV-1 viruses with and without ICP0, we were able to show what proteins ICP0 destroys during infection in neurons. We were also able to show that HSV-1's ability to establish latency is dependent on how the neurons handle the initial onset of infection. Overall, a combination of host and viral proteins coordinates the virus's ability to establish latency and persist within a host.

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1) INTRODUCTION

Unlike most epidemics in history, the herpes virus epidemic has lasted for centuries as a silent pestilence worldwide. In a subtle sequence of events, viruses in Herpesviridae, one of the most prominent viral families, infects not just humans but almost every other animal species, from bivalves (1) and frogs (2) to non-human primates (3). Humans have eight herpes viruses that readily infect, establish latency, and cause reoccurring diseases (4). Once infected with a herpes virus, the infection will remain for the host's life.

As of writing this dissertation, more than 50% of the United States population has herpes simplex virus 1 (HSV-1) (5). In addition, we are currently amid an epidemiological shift in the presentation of HSV-1. HSV-1, which previously dominated as the most common cause of oral herpetic lesions, is now emerging as the most common cause of new genital lesions in young adults, usurping herpes simplex virus 2 (HSV-2) as sexual practices change (6, 7).

Herpes simplex virus 1

Clinical presentation and complications

Orofacial lesions and recurrence

HSV-1 is often acquired during childhood via direct contact of herpetic lesion exudate or infected bodily fluids; this can be through the sharing of beverages, kissing, of close physical contact. Although a seronegative host may encounter infectious virions, symptoms may not arise for up to 2 – 3 weeks after the first contact. Often the symptoms of the first outbreak are the most severe and in addition to the characteristic herpetic lesions at or near the site of initial infection, infection can present with accompanying symptoms such as fever, sore throat, headache, muscle aches, swollen lymph nodes. Lesions can take days to weeks to heal and are most contagious after the lesions rupture, releasing a clear exudate of infectious virions, and exposing a sensitive red wound beneath. This wound will develop a yellow crust and heal, often without leaving a scar. This Initial replication event, in most cases, will be follow by reoccurring events triggered by stress-inducing stimuli.

HSV-1 infection is most commonly recognized and known by its reoccurring herpetic lesions that appear in the orofacial mucosal. Sensations of burning, itching, and tingling, can be felt six to 24hrs prior, at or near the site of lesion development. This sensation results from the virus replicating and migrating down axon terminals, causing irritation and neuronal destruction. Over 2 - 3 days, small, clustered lesions will appear, filling with fluid and eventually coalescing into a sizeable fluid-filled blister. Between 4-14 days, the blister will mature and ultimately rupture, releasing a semi-clear exudate. The exudate release creates a painful red wound followed by the development of a yellow crust. At this point, the lesion is the most contagious, as the exudate is the release of intact mature HSV-1 virions. The red spot will heal as the lesion heals, and HSV-1 will reestablish latency until another reactivation stimulus is encountered (8).

Neonatal herpes

Neonatal herpes is the transmission of HSV-1 or HSV-2 during gestation, birth, or shortly after birth (9, 10). As the virus replicates within the neonate, unchecked by an immature immune system, it can cause severe complications, neurological damage, and death. HSV-1 infection in neonates varies in severity depending on the means of acquisition. In the USA, HSV transmission to neonates can occur in utero (5%), peripartum (85%), or postnatally (10%) (10). In utero, transmission occurs when the virus crosses the placenta infecting the fetus. Symptoms indicative of HSV infection will arise, such as active lesions, hyper/hypopigmentation, neurologic manifestations, microcephaly, and intracranial calcifications (11). Most neonatal HSV infections occur during peripartum when newborns contact active lesions while passing through the birth canal (12).

Herpetic keratitis and encephalitis

HSV-1 can cause more severe complications in some individuals. HSV-1 is one of the leading causes of blindness, a direct result of a condition known as herpetic keratitis. Herpetic keratitis results from HSV-1 infecting and replicating in the cornea of the eye, but HSV-1 can spread to all layers, including the conjunctiva, cornea, retina, and eyelids (13). Symptoms of herpetic keratitis include general ocular pain,

photophobia, tearing, redness, and inflammation (14). Ocular infections result from direct inoculation into the eye or reactivation if the virus follows the ocular branch of the trigeminal ganglia (TG) instead of the more common mandibular or maxillary branch (15).

Herpetic encephalitis is caused by HSV-1 replication within the brain, causing an altered mental state, seizures, edema, and inflammation (16). Affecting 1-4 cases per 1,000,000, herpetic encephalitis is a life-threatening complication with mortality at 30% with treatment and 70-80% without treatment (17, 18). The pathology of herpetic encephalitis is not well understood; it seems to be strain-specific and can result from primary infection or reactivation (19, 20).

Therapeutics

Therapeutic options for HSV infections are limited to acyclovir (ACV) and its derivatives. Acyclovir is an antiviral that is useful against many herpesviruses (21). ACV is administered as a topical cream, taken as a tablet, a suspension, or in emergencies, administered intravenously. ACV is 10-20% bioavailable and must be phosphorylated by the host cell (22), where it has a higher affinity for the herpes thymidine kinase than the host thymidine kinase (23). Once phosphorylated, ACV is incorporated into the viral genome during replication, functioning as a guanosine analog and restricting DNA synthesis so new viral progeny cannot be produced.

Molecular biology of HSV-1

Localization of HSV-1

Historically, HSV-1 has predominantly localized to the TG that innervates the face during primary infection (24). Due to changes in sexual practices over the last few decades, sexual transmission of HSV-1 has been increasingly more common, which localizes the latent virus to the DRG that innervates the genitalia (6, 7). The TG is the collection of cell bodies belonging to the 5th cranial nerve in humans. It is situated within Meckel's cave (25), in the dense petrosal area between the temporal bones below the brain. The TG has three branches (V1-V3) that innervate the face and respond to chemical, mechanical, and nociceptive

stimuli. V1 (Ophthalmic Nerve) innervates the forehead, V2 (Maxillary Nerve) innervates the upper lip, mucosa, and teeth, and V3 (Mandibular Nerve) innervates the lower lip, mucosa, and teeth, and has motor function over the lower mandible (26). The ganglia consist of 20,000 – 35,000 neurons supported by more than 100-fold non-neuronal cells (27).

DRGs are located at the intersection of the dorsal root nerve and the spinal nerves receiving afferent input from the skin, neck, bone, and visceral organs (28). Like the other ganglia of the peripheral nervous system (PNS), DRGs are a heterogeneous population of about 15,000 neurons per ganglion, consisting of mechanoreceptors, thermoreceptors, and nociceptors (28, 29). DRGs are located symmetrically along the vertebral column extending from the intervertebral foramen triangulated by the vertebral disk and the preceding and succeeding vertebra. Their paravertebral location also excludes them from the protection of the blood-brain barrier (30).

The TG and DRG have similar neuronal populations (26), but the reactivation of HSV-1 differs for each ganglion (31).

HSV-1 replication cycle

The HSV-1 replication cycle begins with viral entry into epithelial cells, specifically those composing the mucosal epithelium or damaged keratinocytes. HSV-1 can utilize dynamin, cholesterol (32), membrane fusion, or receptor-mediated endocytosis (33) to access host cells. Predominantly, HSV-1 viral entry is mediated by engaging with multiple host cell receptors for membrane fusion. The HSV-1 virion contains more than a dozen viral entry receptors, four of which, glycoproteins B, D, H, and L (gB, gH, and gL), are required for envelope fusion (34). From the host's perspective, glycosaminoglycans on the end of proteoglycans (specifically heparan-sulfate) are preferred for viral entry (35, 36). Membrane fusion leaves the lipid membrane behind, and once inside, the viral nucleocapsid and tegument proteins are transported retrograde to the nucleus (37). Tegument proteins such as virion host shutoff protein (VHS), viral protein 16 (VP16), and infected cell protein 0 (ICP0) (38) are released into the cytoplasm to facilitate viral success through mechanisms such as degradation of cellular mRNA and ubiquitination of host proteins. The virion,

with viral nucleic acid enclosed, is translocated retrograde to the host cell nucleus using kinesins and dynein motors (37). Once docked at a nuclear pore, the nucleocapsid will inject linear double-stranded DNA into the nucleus to initiate viral transcription and replication. In epithelial cells, HSV-1 viral transcription follows a temporal cascade of gene expression in epithelial cells (39). Genes are sequentially transcribed in classes known as immediate-early (α), early (β), and late (γ) genes. Immediate-early genes control the host's response to viral infection and initiating early viral gene transcription. Early genes are those that regulate viral genome replication. Late genes are structural and essential for the maturation and egress of new viruses.

Completing the temporal cascade results in a complex maturation and egress cascade with a double-envelopment process. Late gene translation produces two primary groups of viral proteins: (1) capsid proteins that protect the viral DNA and (2) glycoproteins that protrude through the viral envelope and facilitate entry into new host cells. The capsid protein mRNAs are translated in the cytoplasm and transported into the nucleus forming empty capsid cages (40). The envelope glycoproteins are translated on ribosomes and eventually dot the cytoplasmic membranes, including but not limited to the endoplasmic reticulum (ER), Golgi, and early endosomes (41, 42).

Newly synthesized viral genomes are packaged into empty capsids, to protect viral DNA, with the assistance of seven virally encoded proteins (43). The packaging of viral capsids with viral DNA prompts the budding of the capsids through the inner nuclear membrane into the perinuclear space of the nucleus. This process is called primary envelopment and results in singly enveloped viral capsids within the perinuclear space (44). With the aid of a nuclear egress complex (NEC), the pre-enveloped capsids bud through the outer nuclear membrane leaving the nuclear envelopment behind and releasing a naked capsid into the cytoplasm (44-46). Once in the cytoplasm, the capsid is saturated with viral proteins such as VP16, VHS protein, ICP0, and ICP4, filling the tegument (38). The virion then buds into cytoplasmic membranes, such as the cis-face of the Golgi, early endosomes, and ER, where it acquires a new envelope studded with glycoproteins to facilitate entry into new host cells (41, 42). The now mature virion is double-enveloped

inside a cellular vesicle, which is then transported to the cell surface, where it is secreted into the extracellular medium as a single mature enveloped virion (47).

The secretion of mature virions from the initial epithelial infection allows HSV-1 to spread to the neuronal axons that innervate near the site of infection. This initiates the neuronal phase of infection, where virions infect the peripheral nervous system. Using a combination of methods mentioned above for viral entry, newly synthesized virions are internalized and transported retrograde into the neurons soma where gene transcription is initiated in neurons. Like epithelial cells, many virions undergo a complete temporal cascade of gene expression resulting in the complex double-envelope cascade and demise of the host neurons. However, during the initial onset of viral infection (primary-lytic), a decision is made to pursue a lytic infection or establish latency. During the establishment of latency, the temporal cascade is interrupted and the viral genome adopts an episomal conformation (48), entering a state of viral quiescence. The molecular mechanisms that facilitates this decision if unclear, but it only occurs in a small population of neurons (49). During this quiescent state, α, β, and γ genes are heterochromatinized (50, 51), significantly reducing their expression, while the Latency Associated Transcript (LAT) region is transcribed as RNA but not translated (52, 53). The state of latency can be maintained indefinitely and is characterized by a lack of readily detectible lytic genes and the expression of LAT (54).

Latency is key to the success of HSV-1, and allow the virus to persist within a host and reactivate in response to stress-inducing stimuli. Once stress0inducing stimuli are encounters, the temporal cascade will be initiated from those latently infected neurons, triggering a reactivation event. Through an unclear cascade of events and signaling pathways, viral α, β, and γ genes will be sequentially transcribed and translated to produce mature virions. These virions travel anterograde from the neuronal nucleus and soma to the synaptic bouton, depositing infections virions near naïve epithelial cells. The epithelial cell infection will restart at or near the site of initial infection, often producing a characteristic herpetic lesion. This periodic recrudescence of HSV-1 lesions and shedding perpetuated the infection cycle of HSV-1 within a host and increases the change of transmission to a naïve host. The ability of HSV-1 to establish latency and

periodically reactivate is key to its success as a viral pathogen. Understanding the viral mechanisms as they occur in neurons is essential to losing the knowledge gap of HSV-1.

Immediate-early proteins

The immediate early proteins of HSV-1 are critical to the viral establishment within the host. Collectively these proteins establish the foundation of HSV-1 infection in both epithelial cells and neurons. Functionally each of these proteins exhibit characteristics of Intrinsically Disordered Proteins (IDP), where they lack a stable native conformation and adapt rigidity based on their interactors (55, 56). This attribute allows these proteins to rapidly shift functionality, establish complex protein-protein interaction, interact with signaling cascades, and engage in DNA-RNA binding (57). Each immediate-early protein is unique and confers attributes that allow HSV-1 to modulate gene expression and alter the intracellular environment. These proteins are first expressed during initial infection and immediately following a reactivation stimulus (39). For most, deletion from the genome is detrimental to the virus solidifying their necessity for HSV-1 pathogenesis. Here I provide a brief overview of the HSV-1 immediate-early proteins and some essential functions they exhibit upon infection.

ICP0

Like all IE proteins, infected cell protein 0 (ICP0) has multiple functions that have been identified in non-neuronal cells. Unlike some of the other immediate-early proteins, deletion of ICP0 from the genome is not detrimental to HSV-1 but viral gene expression is less coordinated or efficient without it. In non-neuronal cells, ICP0 has a role in restricting the immune response (58), regulating gene expression (59), increasing viral transcription (60), and decreasing host gene expression (61). ICP0's diverse functionality is achieved without directly binding nucleic acid but through complex protein-protein interactions often dependent its E3 ubiquitin ligase ability. In neurons, however, many of ICP0's functions are unknown, and its neuron specific targets are unclear. Studying ICP0 in neurons is pivotal to understanding HSV-1 neuronal pathogenesis the mechanisms that govern latency and reactivation.

ICP4

Infected cell protein 4 (ICP4) is a viral transcription factor that activates immediate-early and early genes. Packaged within mature viral capsids, expressed during the initial onset of infection, and immediately following reactivation (38), ICP4 has critical functions supporting viral pathogenesis. Its deletion from the genome is detrimental to the virus. ICP4 has been shown to function in coordination with ICP0, in which both proteins can increase viral transcription contributing to the efficiency of viral establishment during initial infection (60). ICP4 can also downregulate viral gene expression by repressing some viral promoters (62).

ICP22

Infected cell protein 22 (ICP22) can regulate host and viral gene expression by modulating the phosphorylation status of RNA Polymerase II (RNA Pol II). RNA Pol II exists in two forms, phosphorylated and unphosphorylated, controlled by one of six general transcription factors through the formation of the preinitiation complex (63, 64). The mechanisms by which ICP22 interferes with the preinitiation complex formation are poorly understood.

ICP27

Infected cell protein 27 (ICP27) is involved in repressing host gene expression, contributing to the host shutoff protein synthesis, inducing viral gene expression (65), and preventing apoptosis (66). Its functions are exerted through its association with DNA and RNA (67). The mechanisms that influence the increase and decrease in gene expression are poorly understood.

ICP47

Infected cell protein 47 (ICP47) is heavily involved in preserving viral mRNA and controlling the intrinsic host antiviral response. It can block RNA splicing early in infection and later aids in shutting mRNA from the nucleus to the cytoplasm. To stop the intrinsic antiviral response, it interferes with the Major

Histocompatibility Complex (MHC), preventing it from binding to the Antigen-dependent transporter, thus presenting empty MHC to immune cells (68, 69). ICP47 can directly contribute to the evasiveness of HSV-1 by blocking the CD8+ T cell response (70).

Gap in knowledge

Despite the advances in HSV-1 research, much remains to be clarified. Do the molecular mechanisms that occur in epithelial cells persist in neurons? How does HSV-1 establish latency? Is latency active or passive? Why does HSV-1 not establish latency in non-neuronal cells? Is latency the lack of molecular machinery or neuron-specific mechanisms that grant this ability? Is latency truly a silent phase of infection, or is it a state of reduced gene expression below the threshold of detection for conventional techniques?

I aim to decipher some of these critical questions through my research. I study HSV-1 in primary neurons and attempt to illuminate the mechanisms that govern HSV-1 pathogenesis.

Study 1

Study 1 focuses on the ubiquitination ability of Infected Cell Protein 0 (ICP0) in adult sensory neurons during acute productive infection. ICP0, an immediate-early E3 ubiquitin ligase, can hijack the last and defining step of the ubiquitin-proteasomal degradation pathway. By interacting with host E2-ubiquitin enzymes, ICP0 can catalyze the transfer of ubiquitin to its target substrate using its Really Interesting New Gene (RING) finger domain. In epithelial cells, ICP0 has been shown to ubiquitinate host proteins such as p53 (71), USP7 and SP100 (72) in support of productive infection, but the neuron-specific ubiquitination targets remain unknown. Identifying the neuron-specific ubiquitin targets of ICP0 will illuminate the viral mechanisms permitting neuronal HSV-1 infection and help identify targetable proteins for developing new antivirals. We hypothesized that ICP0 ubiquitinates neuron-specific proteins that restrict viral infection during initial onset; in doing so, ICP0 facilitates productive infection in neurons. We utilized liquid chromatography with tandem mass spectrometry (LC-MS/MS) to identify ubiquitinated proteins within

adult sensory neurons infected with HSV-1 KOS and HSV-1 n212, a nonfunctional ICP0 truncation mutant, to compare to uninfected control neurons.

We identified High Mobility Group I/Y (HMG I/Y) and Transactive Response DNA binding protein 43 (TDP43) through our LC-MS/MS databases analysis. Both proteins exhibited increased ubiquitination in the presence of functional ICP0 and showed changes in their protein profile during the first 10 hours of productive HSV-1 infection. This study revealed that ICP0 has a unique protein profile in neurons compared to non-neuronal cells. ICP0 also inversely correlated with HMG I/Y during productive infection and seemed to suppress TD43 below the level of uninfected neurons.

Hypothesis: ICP0 ubiquitination targets differ in HSV-1 infected adult sensory neurons from those previously observed in non-neuronal cells.

> Aim 1**:** Establish a clear and concise protein profile for ICP0 during initial productive infection to determine the best time to immunoprecipitate for ICP0 ubiquitination targets.
>
> Aim 2: Identify potential neuron-specific targets of ICP0 through downstream molecular-based assays to determine if ICP0 ubiquitinates them.

Study 2

Study 2 focused on the ubiquitination targets of ICP0 during reactivation of HSV-1 in adult sensory neurons. Unlike acute infection where HSV-1 must hijack a naïve cell, during reactivation the viral genome is already established within the nucleus. Latency is essential to viral persistence within a host, and although the molecular mechanisms are unclear, ICP0 is hypothesized to be intricately involved. ICP0 has not been previously detected during latency (73), but is one of the first viral proteins to appear after a reactivation inducing stimulus (74). The ability of ICP0 to function immediately post-reactivation stimulus would be hindered by delays in transcription and translation suggest different mechanisms may govern latency and reactivation in neurons. We hypothesize that ICP0 ubiquitinates host derived molecular repressors that maintain HSV-1 latency to start reactivation in adult sensory neurons. To identify ICP0 mediated

ubiquitination targets during HSV-1 reactivation, we immunoprecipitated proteins from primary adult sensory neurons latently infected with HSV-1 KOS (WT-ICP0), HSV-1 n212, (non-functional, truncated ICP0), and an uninfected control after 1 week of latency establishment and reactivated by neurotrophic factor deprivation. Immunoprecipitated proteins were identified by LC-MS/MS and comparative analysis to identify ICP0 mediated ubiquitination targets.

From our LC-MS/MS data we determined that β – catenin protein levels decreased in HSV-1 KOS infected samples. β – catenin is the master regulator of the Wnt signaling cascade that controls growth and development in many cells. It is not known to interact directly with ICP0 but, through previous data generated in our lab, we have observed a reduction in β – catenin during HSV-1 reactivation. Upon further exploration, we determined that ICP0 did not increasingly ubiquitinate β - catenin but reduced the overall protein level during the establishment of latency. This observation was further substantiated by a distinct inverse relationship between β – catenin, and ICP0 during the 7-day period allotted for the establishment of latency in neuronal cultures. We looked to identify a link between ICP0 and β – catenin that could affect the establishment of latency. Through primary literature, we pinpointed immediate-early viral protein ICP4 and HMG I/Y, previously studied in Study 1, as a potential link between ICP0 and β – catenin (75, 76). Charting the protein profile of β – catenin, ICP0, HMG I/Y, and ICP4 during the establishment of latency for seven days following infection of sensory neurons, we observed an inverse relationship with ICP0 compared to ICP4, HMG I/Y, and β – catenin. We hypothesize that β – catenin upregulates HMG I/Y, which combines with ICP4 to repress ICP0 gene expression. This complex interplay between host and viral proteins suggests a novel mechanism for the HSV-1 latent lytic switch with β – catenin as a key indicator of viral latency.

<u>Hypothesis: ICP0 ubiquitinates host proteins that repress viral transcription and maintain latency in adult sensory neurons.</u>

<u>Aim 1:</u> Identify ICP0 neuron-specific ubiquitination targets during reactivation of HSV-1 in adult sensory neurons using LC-MS/MS.

Aim 2: Confirm the ubiquitination of the target proteins by ICP0 using a combination of immunoblots and immunoprecipitation of neurons infected with HSV-1 KOS and HSV-1 n212.

Aim 3: Identify the involvement of the ubiquitinated protein in latency and reactivation of HSV-1 in adult sensory neurons.

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2) PERSPECTIVES OF HERPES SIMPLEX VIRUS 1 INFECTED CELL PROTEIN 0 (ICP0) IN NEURONS

Harrell TL and Bertke AS

Abstract

Infected Cell Protein 0 (ICP0) is an immediate-early protein encoded by herpes simplex virus 1 (HSV-1). As an immediate-early protein and E3-ubiquitin ligase, ICP0 is upregulated during initial infection and immediately following a reactivation stimulus. ICP0's primary function is to hijack the ubiquitin proteasomal system to modulate and destroy vital cellular proteins. Its presence is essential for efficient HSV-1 replication and it is implicated in almost every aspect of HSV-1 pathogenesis, yet information regarding its expression patterns, protein interactions, and functional domains remains unclear, especially in neurons. This review explores known facts about ICP0's available domains, expression patterns, and neuron-specific mechanisms. We conclude with insight on future directions that could further illuminate the neuron-specific mechanisms of ICP0 and general pathogenesis of HSV-1 in neurons.

Introduction

Herpes simplex virus 1 (HSV-1) is a large double-stranded DNA virus that currently infects more than 50% of the global population (1). HSV-1 infection is defined by the occasional recrudescence of cold sores in the orofacial region (2). On the surface, HSV-1 herpetic lesions appear harmless but, in some cases, can cause life-threatening encephalitis (3), meningoencephalitis (4), and blindness through herpetic keratitis (5). Despite the commonality of HSV-1 infection and potential severity, the molecular mechanisms that govern its pathogenesis remains unclear.

HSV-1 is an *Alphaherpesvirus* (6), similar to Herpes Simplex virus 2 (HSV-2), the causative agent of genital herpes. HSV-1, like other *Alphaherpesviruses*, are classified as such because they exhibit neurotropism for sensory neurons, where the viruses establish latency, and possessing a rapid replicative ability utilizing immediate-early transactivator proteins (7, 8). The neurotropic preference of HSV-1 is

essential to its success as a pathogen, but it exhibits a dual cell replication cycle that adds an extra layer of complexity.

Viral replication begins in epithelial cells, typically orofacial or genital mucosa, where HSV-1 rapidly produces mature virions that bud from the infected cells (9). The mature viral progeny enter axon terminals that innervate the initial infection site, initiating infection within sensory neurons (10). Once internalized, virions are transported retrograde to the neuron soma (11). After migrating through the soma, HSV-1 will localize to the nucleus and undergo a lytic infection, involving a temporal cascade of gene expression and viral replication, or establish latency, a state of viral quiescence. HSV-1 can only establish latency in neurons where it can remain in a state of viral quiescence until stress-inducing stimuli prompts the initiation of the temporal cascade of gene expression (11). This ability to establish latency and stay in a quiescent state is critical to HSV-1 success among the human population.

During lytic infection, the virus replicates through a temporal cascade of sequential expression of immediate-early (IE), early (E), and late (L) genes that culminate in the production of mature HSV-1 virions (12). In epithelial cells, HSV-1 follows a temporal cascade of gene expression consisting of immediate-early, early, and late genes. Immediate-early (IE) genes modulate the intracellular host environment to be more conducive to viral infection and initiate transcription of viral early and late genes. Early (E) genes mediate the synthesis of new viral DNA strands, with further manipulation of the host environment. Late (L) genes are predominantly structural, forming the viral capsid and coordinating viral DNA packaging into progeny virions. Each classification of proteins is essential, but the immediate-early proteins are critical to establishing infection and ensuring a permissive host environment.

Infected cell protein 0 (ICP0) is an immediate early viral transactivator that mediates HSV-1 pathogenesis (13). As an immediate-early protein, ICP0 is one of the first proteins to be expressed during primary-lytic infection and reactivated-lytic infection (14). ICP0 has multiple functions, but the majority of functional studies have been performed in non-neuronal cells. Therefore, its neuron-specific mechanisms are largely

unknown. Here, we review known facts about ICP0 and reported neuron-specific facts to cultivate different perspectives on ICP0 functionality and HSV-1 infection in neurons.

ICP0 functional domains and functions

ICP0 was discovered in 1976 as an immediate-early peptide (15, 16). The protein consists of 775 amino acids, suggesting western blot localization at ~75kDa, but ICP0 appears at ~118kDa, possibly due to post-translational modification (17). The protein is encoded twice within the genome, but only one copy is sufficient for efficient replication, and deletion of both copies from the genome does not prevent viral replication (18). Despite such peculiarities, much of ICP0's function is known through amino acid sequence analysis and molecular-based biological approaches involving disrupting its functional domains.

ICP0 encodes multiple functional domains that all contribute to its promiscuous protein-protein interactions (Fig. 2.1). ICP0's most studied domain is the RING (Really Interesting New Gene)-finger domain, which is the basis of its classification as a protein. There are ~600 mammalian proteins that contain RING-finger domains (19) functioning in cell cycle modulation, cell homeostasis, and gene expression. RING-finger domains specifically enable proteins to catalyze the last step of the ubiquitin proteasomal degradation pathway. ICP0 has exhibited E3-ubiquitin ligase functionality by mediating the transfer of ubiquitin to target proteins *in vitro* and *in vivo* (20). The exact mechanism and regulation of ICP0's ubiquitin ligation, however, is unclear. RING E3-ligases often function as scaffolds (21), transferring ubiquitin chains that are formed sequentially from ubiquitin-activating (E1) and ubiquitin-conjugating (E2) enzymes. The ligation step coordinated by an E3-ligase is coordinated by two zinc ions evenly spaced with cysteine and histidine residues buried within the active site (22). The zinc ions form a cross-brace with the ubiquitin donating E2 and the target while the E3 serves as the mediator; E3-ligases do not form a catalytic intermediate with ubiquitin (21).

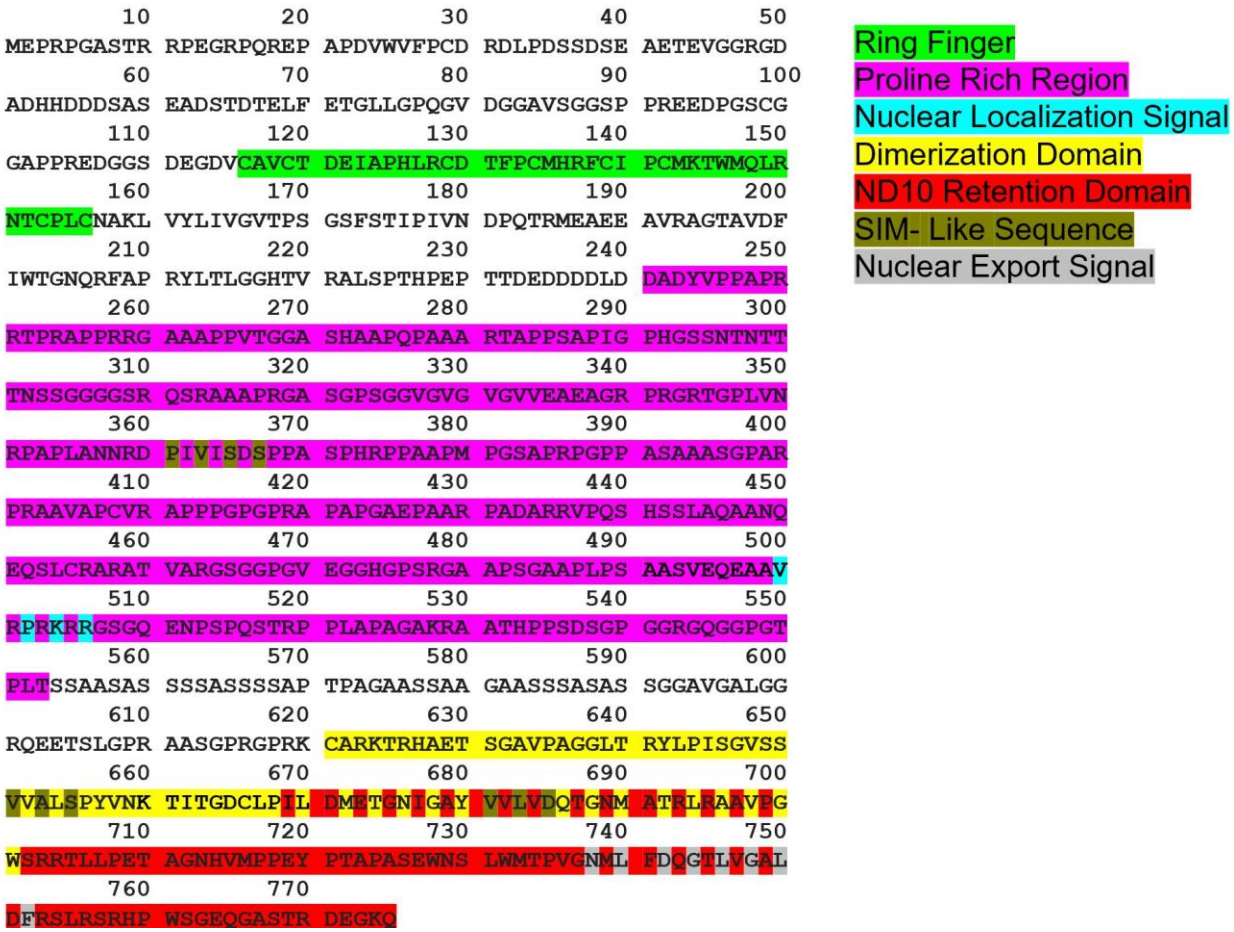

Figure 2.1: ICP0 functional domains.

Graphical representation of ICP0 functional domains color coded to display location within the ICP0 amino acid sequence. Reference sequence HSV-1 strain 17.

Located within the center of the ICP0 amino acid sequence is the Proline-Rich Region (PRR), situated between amino acids 241-553. This region comprises 40% of ICP0's overall amino acid sequence and is important for protein-protein interactions and signaling (23). PRRs are associated with proteins that lack a modular structure and favor entropy (24, 25). ICP0's amino acid sequence exhibits regions of disorder (Fig. 2.2) and expected globular domains (Fig. 2.3) when assessed using Globplot (26). Such proteins often function as dynamic signaling molecules and are classified as intrinsically disordered proteins (IDP) (27). The PRR is defined by an increased presence of the amino acid proline that permits additional mobility and a transient three-dimensional structure (25). The transient structure allows the protein to form signaling

complexes called signalosomes (28) that integrate seemingly unrelated proteins based on specific recognition codes (28-30). The interacting proteins combine as molecular biosensors integrating multiple signaling cascades (31, 32). Upon the arrival of various stimuli, the complex becomes activated, eliciting a downstream effect such as chromatin remodeling, gene transcription, and alterations in cell signaling (33). By nature, PRR-containing proteins are versatile and can adapt to the variations of intracellular signaling events often mediated by posttranslational modifications (34). ICP0 is known to respond to changes in biological stimuli facilitating viral reactivation and to interact with key signaling molecules (35, 36). Although HSV-1 ICP0 is loosely mentioned as an IDP, the ability to dock, the large PRR, and a lack of stable structure permitting shape changes (37) support this classification.

Figure 2.2: ICP0 regions of protein instability and disorder.

Regions of instability and disorder were determined by the Russell/Linding definition and highlighted in blue. Amino acid regions of ICP0 instability 2-62, 72-115, 170-178, 221-330, 339-398, 405-430, 462-488, 505-578, 584-621, 632-641, 711-726, 759-772. Reference sequence HSV-1 strain 17.

meprpgastr rpegrpqrep apdvwvfpcd rdlpdssdse aetevggrgd
adhhdddsas eadstdtelf etgllgpqgv dggavsggsp preedpgscg
gappredggs degdvCAVCT DEIAPHLRCD TFPCMHRFCI PCMKTWMQLR
NTCPLCNAKL VYLIVGVTPS GSFSTIPIVN DPQTRMEAEE AVRAGTAVDF
IWTGNQRFAP RYLTLGGHTV ralspthpep ttdeddddld dadyvppapr
rtprapprrg aaappvtgga shaapqpaaa rtappsapig phgssntntt
tnssggggsr qsraaaprga sgpsggvgvg vgvveaeagr prgrtgplvn
rpaplannrd pivisdsppa sphrppaapm pgsaprpgpp asaaasgpar
praavapcvr apppgpgpra papgaepaar padarrvpqs hsslaqaanq
eqslcrarat vargsggpgv egghgpsrga apsgaaplps aasveqeaav
rprkrrgsgq enpspqstrp plapagakra athppsdsgp ggrgqggpgt
pltssaasas sssassssap tpagaassaa gaasssasas sggavgalgg
rqeetslgpr aasgprgprk cARKTRHAET SGAVPAGGLT RYLPISGVSS
VVALSPYVNK TITGDCLPIL DMETGNIGAY VVLVDQTGNM ATRLRAAVPG
WSRRTLLPET agnhvmppey ptapasewns lwmtpvgnml fdqgtlvgal
dfrslrsrhp wsgeqgastr degkq

Figure 2.3: ICP0 globular domains.

ICP0 Globular domains as determined by Globplot. Globular domains are located between amino acids 116 - 220 and 622 - 710. Reference sequence HSV-1 strain 17.

Additional domains in ICP0's amino acid sequence influence its subcellular location and binding partners, indicative of its broader functions in HSV-1 pathogenesis. ICP0 appears in the cytoplasm and nucleus of HSV-1 infected cells, although the mechanism and time vary depending on cell type. To translocate to the nucleus, ICP0 encodes a classical monopartite nuclear localization signal (NLS) with the sequence V-R-P-R-K-R-R between amino acids 500 to 506. Classical NLS signals are recognized by Importin α/β, which work in coordination to transport substrate through a nuclear pore and into the nucleus. Once inside the nucleus, the Importin α/β complex is destabilized by RanGTP and the substrate is released into the nucleus (38). HSV-1 proteins, including ICP0, have been shown to require various combinations of importins to enter the nucleus. ICP0 has been shown to utilize importin α1 and α3 but not importin α4. Replication of HSV-1 and the nuclear localization of ICP0, and ICP4, are reduced without importin α1, suggesting the importance of this nuclear import pathway (39).

The amino acids responsible for nuclear to cytoplasmic localization of ICP0 are less clear and have been identified between amino acids 738 and 752 (40, 41). The presence of a nuclear export sequence is not

always clear as proteins can mask the sequence, and it can be modified through post-translational modification to coordinate export via a series of signaling, transport molecules, and protein-protein interactions (42, 43). Host and viral proteins likely mediate the mechanism by which ICP0 is transported out of the nucleus. ICP0 is known to interact with cyclin D3 and localize to the cytoplasm, often in coordination with HSV-1 late gene expression (44). In contrast, treatment with Leptomycin B for the inhibition of CRM1 (Chromosomal Maintenance 1), a major nuclear export receptor, did not influence ICP0 cytoplasmic localization but amino acid mutations within another HSV-1 immediate early protein, ICP27, did alter cellular localization (40). Taken collectively, the mechanisms that ICP0 uses to localize to the cytoplasm are not dependent on one pathway but seem transient during HSV-1 infection.

ICP0's ND10 retention and Sumo-like domain are not as well understood. Both are hypothesized to enable an association with Nuclear Domain 10 (ND10) bodies. ND10 bodies are membrane-less nuclear structures consisting of multiple protein components, implicated in cellular functions such as DNA damage response/repair (45), inflammation (46), oncogenesis (47), antiviral mechanisms (48), immune signaling (49), gene regulation (50), cell cycle progression (51), and apoptosis. They were described as interchromatin structures with ten dots associated with transcriptionally active regions within the nucleus (52). Since their discovery, they have been linked to multiple disease states and biological functions, but the exact function and biological relevance are unclear. ICP0 has been shown to localize to and mediate the degradation of ND10 components (53), but its direct implication on HSV-1 pathogenesis is subject to debate. ND10 bodies have been loosely characterized in some cells, HeLa cells (54), and deemed non-existent in others, such as neurons (55). The research into their importance has been described as enigmatic and difficult to interpret. As a result, more research is needed to understand their overall significance and their interaction with ICP0.

Expression regulation of ICP0

Similar to the analysis of ICP0's functional domains, which provides information about its mechanics, analysis of its gene promoter can give information about its expression. ICP0 is encoded twice within the

HSV-1 genome, located antisense to the Latency Associated Transcript (LAT) (Fig. 2.4). The importance of dual encoding is unclear, but only one copy is sufficient for rapid gene transcription. The promoters are seemingly identical and contain the same regulatory regions that influence gene expression. Studies support that viral and host proteins play a role in ICP0's gene expression, and different mechanisms likely influence expression from viral and host perspectives. Understanding the two primary regulatory sequences controlling its spatiotemporal expression has yielded some results in understanding ICP0's expression in neurons. The two core promoter sequences most characterized for ICP0 expression are the TATA Box and the TAATGARAT sequence.

Figure 2.4: HSV-1 genome organization.

Schematic of ICP0 gene in relation to the Latency Associated Transcript (LAT) gene within the HSV-1 genome. ICP0 promoter DNA nucleotide sequence is provided with highlighted TAATGARAT sequence (yellow) and TATA Box (red). Reference sequence HSV-1 strain 17.

The ICP0 TATA box is located less than 100bp upstream of the transcription start site and is a part of the core ICP0 promoter. TATA boxes are the most well-characterized promotor sequences, present in 24% of human genes (56). Within the core promoter, it functions as the primary binding site of RNA transcription

factors such as RNA Pol II and TFIIX (A, B, D, E, and F) (57), which collectively compose the basal transcription complex. This promoter sequence only provides low transcription levels and requires other regulator sequences for increased transcription (58).

The TAATGARAT sequence can be found throughout the promoters of the HSV immediate-early proteins. This sequence appears to be unique to HSV-1 and functions as a combinatorial sequence for gene regulation and expression patterns (59, 60). The TAATGARAT sequence within ICP0's promoter is analogous to the octamer control sequences (ATGCAAT) located in many eukaryotic genes (61). These sequences control when, where, and how much of a gene under its control is expressed. These sequences are recognized by octamer proteins Oct-1 and Oct-2, which are POU homeodomain transcription factors that control key genes such as housekeeping and stress-related genes (62, 63). Oct-1 is ubiquitous (64), while Oct-2 is expressed solely in B-cells and neuronal tissue (65). During HSV-1 infection, Oct-1 and Oct-2 have shown in-depth involvement in the expression of ICP0, although binding is relatively poor. Oct-1 forms a complex with VP16 on the TAATGARAT sequence, increasing the expression of ICP0. Oct-2 has an inhibitory effect on ICP0 expression (59, 66).

ICP0 in neurons

In addition to functional domains and regulatory promoter sequences, the host cell type is critical to understanding the molecular mechanisms of ICP0. Upon infection, HSV-1 localizes to peripheral ganglia, specifically the sensory trigeminal and/or dorsal root ganglia, and sympathetic superior cervical ganglia. Unlike HSV-1 infection in non-neuronal cells, neurons respond differently to infection and may or may not be permissive to different outcomes of HSV-1 infection (67-69). Furthermore, only 1/3rd of the neuronal population in peripheral sensory ganglia are permissive for HSV-1 lytic infection, and even smaller percentages will be conducive to latency establishment and reactivation (67). Due to these neuron-specific differences, ICP0 exhibits different functionality to ensure viral success. Using historical HSV-1 literature focused on HSV-1 infection in neurons, we can begin to delineate some of the functions and patterns of ICP0 that contribute to viral success and persistence.

Acute infection

ICP0's presence is not limited to the immediate early gene expression during neuronal HSV-1 infection. When mature virions fuse with the axon terminals during initial infection, 100-200 tegument ICP0 proteins enter the cytoplasm with the nucleocapsid (70). ICP0's presence in the cytoplasm is often disregarded, but one of its functions involves curtailing the intrinsic neuronal antiviral response. Peripheral neurons produce some interferons during HSV-1 infection but are largely dependent on the surrounding cells' inflammatory molecules (71, 72). As a result, they respond to extracellular inflammatory molecules by transcribing and post-translationally modifying antiviral proteins such as STAT1 (73, 74). Cytoplasmic ICP0 is essential to evading this early localized response to viral infection at the axon terminal by repressing host defenses such as disruption of NF-kB, interfering with Pattern Recognition Receptors (PRR), and restricting the response of neurons to extracellular interferon α/β and γ (75, 76).

Expressed ICP0 from the viral genome is produced within the nucleus via transcription of HSV-1 DNA into mRNA. In non-neuronal cells, HSV-1 follows a temporal cascade of α, β, and γ genes. In neurons, HSV-1 can alter the progression of gene expression based on necessity and progression to latency. For ICP0, this occurs by utilizing the aforementioned regulatory sequences of the ICP0 promoter and protein-protein interactions to modulate gene expression. In neurons, ICP0 mRNA expression gradually increases over the first 8hrs of infection and plateaus between 16-24 hours post inoculation (hpi) (Harrell, Chapter 3). After translation, ICP0 localizes to the nucleus but does not accumulate as observed in non-neuronal cells (77). In neurons, ICP0 is more dispersed, localizing to both the nucleus and cytoplasm in significantly lower amounts when compared to non-neuronal cells. The reduced ICP0 expression could be due to the absence of ND10 bodies within neurons, contributing to the concentrated localization of nuclear ICP0 as observed in non-neuronal cells, or due to auto-ubiquitination and degradation in the proteasome (Harrell, Chapter 3).

Latency

The partial consensus dogma of HSV-1 pathogenesis is that associated lytic proteins, including ICP0, are not expressed during latency. Latency is a state of viral quiescence where mature virions are absent, lytic gene transcripts are repressed, and the Latency Associated Transcript (LAT) accumulates (78). This presumption, however, promotes an ideology that latency is a purely passive process and disregards any viral involvement beyond LAT. Approaching the paradigm from a different perspective can shed light on the neuronal and viral-mediated mechanisms contributing to latency and illuminate the potential mechanisms that maintain it.

At some point in neuronal HSV-1 infection, a decision is made to commit to a lytic infection or establish latency. The molecular mechanism of this decision remains unclear, but there is evidence that it occurs between immediate-early and early gene expression involving both viral and host factors. Proenca et al. showed that 35% of ganglionic neurons exhibit ICP0 promoter activation before the establishment of latency using a Cre-recombinase system under the control of the ICP0 promoter (79). Similar experiments with Cre-recombinase under the control of the late glycoprotein C (gC) promoter yielded significantly lower results suggesting an incompatibility with latency establishment and cell survival (80). The expression of ICP0 before the establishment of latency is not entirely unusual because ICP0 can modulate heterochromatic markers established on the HSV-1 genome (81).

An interesting aspect of ICP0 in neurons is the activity of its promoter and the presence of ICP0 mRNA during latency. ICP0 is encoded within the HSV-1 genome on either side of the Unique Long (U_L) segment antisense to the LAT gene. During latency, the HSV-1genome circularizes (82) and becomes associated with trimethylated histones, H3K27me3 and H3K9me3, which occupy most of the genome (83, 84), preventing transcription factors and RNA polymerase from accessing the viral DNA. The LAT region, however, remains in a euchromatic state and is continuously transcribed (85). The LAT region partially encompasses ICP0, but the viral genome outside of the LAT region is heterochromatinized during latency

(86). The partially heterochromatinized state of the ICP0 gene has been used to support the hypothesis that ICP0 is not expressed during latency, suggesting a markedly passive process.

A common misconception of epigenetics is that it is passively maintained once established. Chromatinized genes are modulated by histone acetyltransferases (HATs) and histone deacetylases (HDAC) that coordinate gene expression with writers, readers, and eraser proteins (87). This fact, however, omits the necessity of regulators that maintain the chromatinized state of genes. Although established in a condensed state, heterochromatin requires transcription factors and positive feedback loops to sustain genetic confirmation (88). The maintenance of such genes are controlled by transcriptional activators that are expressed at low levels and function as background mediators (89).

Detection of ICP0 at the protein level during latency has yet to be reported; however, there are signs of ICP0's influence during latency when comparing wild-type viruses to ICP0 mutants. Raja et al. reported decreases in H3K27me3 and H3K9me3 in latently infected TG neurons when comparing HSV-1 n212, which expresses a truncated nonfunctional ICP0, and an HSV-1 n212 rescuant that expressed a functional ICP0 (81). In the same report, ICP0 was proposed to influence LAT expression, although additional reports suggest LAT may be influencing the expression of ICP0 (81, 90). Perhaps ICP0 may function on the protein level at minute levels, implying latency is a viral-induced quiescent state.

Reactivation

It is well established that the reactivation of HSV-1 is heavily associated with stress, but the extent and the exact mechanism are subject to debate. ICP0-null mutants exhibit discoordination and lack of efficiency in gene expression, supporting the necessity of ICP0 for an efficient viral response (91). This observation, however, does not clarify ICP0's role in reactivation but indicates that without ICP0, reactivation is possible, although hindered. Thus, ICP0's role in reactivation remains unclear as it may function before the reactivation stimuli, post reactivation stimuli, or aid in diminishing the stimuli supporting latency.

Generally, three central mechanisms have been utilized to induce HSV-1 reactivation from experimental latency: suppression of the immune system through the use of immune-suppressing drugs such as dexamethasone (Dex) (92), chromatin modulation through the use of histone deacetylase inhibitors such as trichostatin A (93), and alteration of intrinsic cell signaling through a technique such as deprivation of neurotrophic factors (94). These cascades conclude with the transcription of host genes that modulate, reduce, or exacerbate the external stimuli through the transcription of immediate-early and early host genes, not to be confused with the viral immediate early and early (α IE and Eβ) genes of HSV-1. Neurons are highly responsive to extracellular stimuli and rapidly react by upregulation of host genes and transcription factors. The most studied immediate-early neuronal genes are c-fos (95) and zif268 (96). When induced, these genes are rapidly transcribed, translated, and localized to the nucleus and upregulate early neuronal genes containing TPA-response (TRE) elements and cyclic AMP (C-amp) response elements (97). The HDAC treatment and neurotrophic deprivation can also induce many of the genes upregulated by c-fos and zif268 (96). The complexity of the neuronal signaling cascades in response to dexamethasone, HDAC and neurotrophic factor deprivation are outside the scope of this review, but ICP0 has been implicated in each of these responses (98), although ICP0's direct role is still unclear.

Conclusion

ICP0 is an intriguing protein that has the subject of investigation for decades. Despite intense research, many of its neuronal mechanisms and neuron-specific protein interactors remain unknown. Although research into the molecular mechanisms of ICP0 and HSV-1 continues, alternative approaches should be pursued, and the accepted dogmas about latency should be challenged. As explained throughout this review, the molecular mechanisms for ICP0, and HSV-1, are markedly different in neurons compared to what is observed in non-neuronal cells. This can be directly attributed to neuron-specific mechanisms that make viral infection within neurons unique, not only from other cell types but also within distinct neuronal populations. The constant expression of neurotrophic factor signaling, heightened environmentally stimulated gene expression, continuous maintenance of connectivity, and robust synaptic transmission are

just some of the mechanisms that define neurons and cannot be replicated in non-neuronal cells. HSV may depend on a variety of these characteristics for persistence within a host. ICP0, and HSV-1, have historically been studied in non-neuronal cells of various types, and differences in cell-specific mechanisms add extra complexity to data interpretation for alternative models. Focusing on neurons can diminish some ambiguity in conclusions and progress the field to unlocking the secrets of ICP0 and HSV-1.

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3) HERPES SIMPLEX VIRUS 1 (HSV-1) INFECTED CELL PROTEIN 0 (ICP0) TARGETS OF UBIQUITINATION DURING PRODUCTIVE INFECTION OF PRIMARY ADULT SENSORY NEURONS

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Abstract: Herpes simplex virus 1 (HSV-1) enters sensory neurons with the potential for productive or latent infection. For either outcome, HSV-1 must curtail the intrinsic immune response, regulate viral gene expression, and remove host proteins that could restrict viral processes. Infected Cell Protein 0 (ICP0), a

virus encoded E3 ubiquitin ligase, supports these processes by mediating the transfer of ubiquitin to target proteins to change their location, alter their function, or induce their degradation. To identify ubiquitination targets of ICP0 during productive infection in sensory neurons, we immunoprecipitated ubiquitinated proteins from primary adult sensory neurons infected with HSV-1 KOS (wild-type), HSV-1 *n*212 (expressing truncated, defective ICP0), and uninfected controls using anti-ubiquitin antibody FK2 (recognizing K29, K48, K63 and monoubiquitinated proteins), followed by LC-MS/MS and comparative analyses. We identified 40 unique proteins ubiquitinated by ICP0 and 17 ubiquitinated by both ICP0 and host mechanisms, of which High Mobility Group protein I/Y (HMG I/Y) and TAR DNA Binding Protein 43 (TDP43) were selected for further analysis. We show that ICP0 ubiquitinates HMG I/Y and TDP43, altering protein expression at specific time points during productive HSV-1 infection, demonstrating that ICP0 manipulates the sensory neuronal environment in a time-dependent manner to regulate infection outcome in neurons.

Keywords: HSV; Human Herpes Virus; alphaherpesvirus; ICP0; mass spectrometry; primary neurons; HMG I/Y; High Mobility Group Protein I/Y; TDP43; TAR DNA binding protein 43.

Introduction

Herpes simplex virus 1 (HSV-1) is a double-stranded DNA virus that infects more than 50% of the global population (1). HSV-1 infections can cause recurring orofacial and genital lesions (2), resulting in pain, itching, and discomfort for the host (3), as well as stress and anxiety in the affected individual. In some instances, however, HSV-1 can cause additional complications such as severe skin manifestations, herpetic keratitis (4), and life-threatening encephalitis (5). The recurrence pattern, frequency, and chances of more severe complications vary significantly between individuals. Treatment options are limited to a guanosine analog, acyclovir and its derivatives, which can be instrumental in reducing the severity or frequency of HSV-1 recurrences, but an HSV-1 infection cannot be cured and individuals remain infected for life.

The pathogenesis of HSV-1 is complex, with an infection cycle that begins in epithelial cells and progresses to peripheral sensory and autonomic neurons (6). In epithelial cells, HSV-1 follows a temporal cascade of

gene expression consisting of immediate-early, early, and late genes. Immediate-early (IE) genes modulate the intracellular host environment to be more conducive to viral infection and initiate transcription of viral early and late genes. Early (E) genes mediate the synthesis of new viral DNA strands, with further manipulation of the host environment. Late (L) genes are predominantly structural, forming the viral capsid and coordinating viral DNA packaging into progeny virions. Completion of this temporal cascade results in the production of mature virions and the demise of the host cell. In neurons, however, the temporal cascade can be altered depending on the neuronal phenotype. Peripheral sensory ganglia contain a heterogenous population of neurons that respond to different stimuli and neurotrophic factors; they also differentially regulate HSV-1 infection (7-9). In some neurons, HSV-1 will progress through a productive infection while in others, the virus will establish latency, a period of viral quiescence in which the viral genome will persist indefinitely and may reactivate in response to various stress-inducing insults. The mechanisms that regulate the decision to undergo productive infection or establish latency after entry into neurons are unclear.

HSV-1 expresses five IE proteins that contribute to the early stages of productive infection. Each IE protein antagonizes different aspects of the host cell, with molecular redundancy and some functional overlap. ICP0 and ICP22 modify the intracellular host cell environment by ubiquitinating host proteins (10) and altering the physiology of the nucleus (11), respectively. ICP47 inhibits the transporter associated with antigen processing (TAP) protein complex, preventing MHC class I presentation of HSV-1 antigens to immune cells (12, 13), contributing to viral evasion. ICP4 and ICP27 interfere with host cell gene expression to curtail host antiviral responses while increasing viral gene expression (14-16). Collectively the IE proteins modulate host cell functions at multiple levels to establish the foundation necessary for HSV-1 infection, although whether these proteins function to promote productive infection or the establishment of latency upon viral entry into sensory neurons is not clear.

ICP0 and its functions have been extensively studied in non-neuronal cells. Through experiments conducted in HeLa Cells, fibroblasts, and Vero cells, ICP0 has been shown to downregulate proinflammatory

mechanisms (17) and toll-like receptor signaling (18), interfere with cell cycle regulation (19), increase viral transcription (20), and decrease viral genome silencing (21, 22). ICP0 also has the ability to activate promoters of IE, E, and L genes (20) without binding DNA or RNA directly. However, it is unknown if these functions identified in non-neuronal cells translate to mature neurons, where HSV-1 establishes latency. ICP0 is classified as an E3 ubiquitin ligase, containing a ubiquitin ligase domain associated with a RING (Really Interesting New Gene) finger domain (23). As such, ICP0 can direct the last step of the ubiquitin cascade, catalyzing the addition of ubiquitin moieties to target substrates. Ubiquitination facilitates downstream effects such as degradation by the proteasome or redirecting a target protein for another function. ICP0 has been shown to interact with an array of proteins, such as the deubiquitinating enzyme ubiquitin-specific peptidase 7 (USP7) (24) and cellular E2 ubiquitin-conjugating enzymes UbcH5a and UbcH6a (25) to regulate its ubiquitination functions. The proteins known to be ubiquitinated in the presence of ICP0 in non-neuronal cells include essential cellular proteins such as p53 (26), Sp100, USP7 (24), and Schlafen 5 (22) but in neurons, where HSV-1 can undergo either productive or latent infection, the ubiquitination targets of ICP0 remain unknown.

To identify sensory neuron-specific ubiquitin targets of HSV-1 ICP0 during productive infection, we utilized an anti-ubiquitin antibody (FK2) to immunoprecipitate ubiquitinated proteins from primary adult sensory neurons infected with either wild-type HSV-1 KOS or HSV-1 *n*212, which expresses a truncated, defective form of ICP0. Identification of the ubiquitinated proteins by mass spectrometry and comparison between KOS-infected, *n*212-infected and uninfected neurons identified neuronal proteins specifically targeted for ubiquitination by ICP0, providing insight into neuron-specific molecular mechanisms regulated by ICP0 during HSV-1 infection. We selected two of these proteins, High-Mobility Group Protein I/Y (HMG I/Y) and TAR-DNA Binding Protein 43 (TDP43), for further study.

Results

ICP0 protein profile is biphasic in primary adult sensory neurons

ICP0 expression patterns and protein levels have been determined previously through sparsely collected time points using a variety of non-neuronal cell types and modified in vitro assays (19, 27-29). Although essential information has been obtained using these methods, mechanisms in non-neuronal cell types likely differ from those in neurons, where HSV-1 manipulates the environment to either proceed through productive infection or establish latency and yet remain poised to reactivate. To determine the ideal time to perform mass spectrometry to identify potential ICP0-mediated ubiquitin targets, we needed to produce a clear and concise protein expression profile for ICP0 during productive infection in primary adult sensory neurons. To compare ICP0 expression between non-neuronal and neuronal cells, Vero76 cells and cultured primary adult dorsal root ganglion (DRG) neurons were inoculated with HSV-1 KOS, and independent samples of each were collected incrementally over 24 hrs, beginning at time 0 (T0), immediately upon inoculation (Fig 3.1). In Vero76 cells, ICP0 was first detected above 0 hr levels as early as 1 hour post inoculation (hpi) and steadily increased until 24 hpi with minor fluctuations (Fig. 3.1A). ICP0 protein expression was significantly greater in Vero76 cells compared to expression in neurons at 10 hpi, and 16-24 hpi ($p < 0.05$, note the Y-axis scale difference between Vero cells and neurons). In contrast, ICP0 was detected above 0 h levels 30 minutes post inoculation (pi) in adult sensory neurons and increased to a distinct minor peak 3 hpi. ICP0 protein levels subsequently decreased until 8 hpi, after which ICP0 increased through 24 hpi, the last time point we analyzed (Fig. 3.1B). This biphasic protein profile of ICP0 appears to be unique to neurons, suggesting dynamic mechanisms and the possibility of multiple functions at different times during productive HSV-1 infection. To identify proteins ubiquitinated by HSV-1 ICP0 early during productive infection, we selected the 8-hour time point, following the first ICP0 protein peak, since these early events likely contribute to the decision between productive infection vs. establishment of latency.

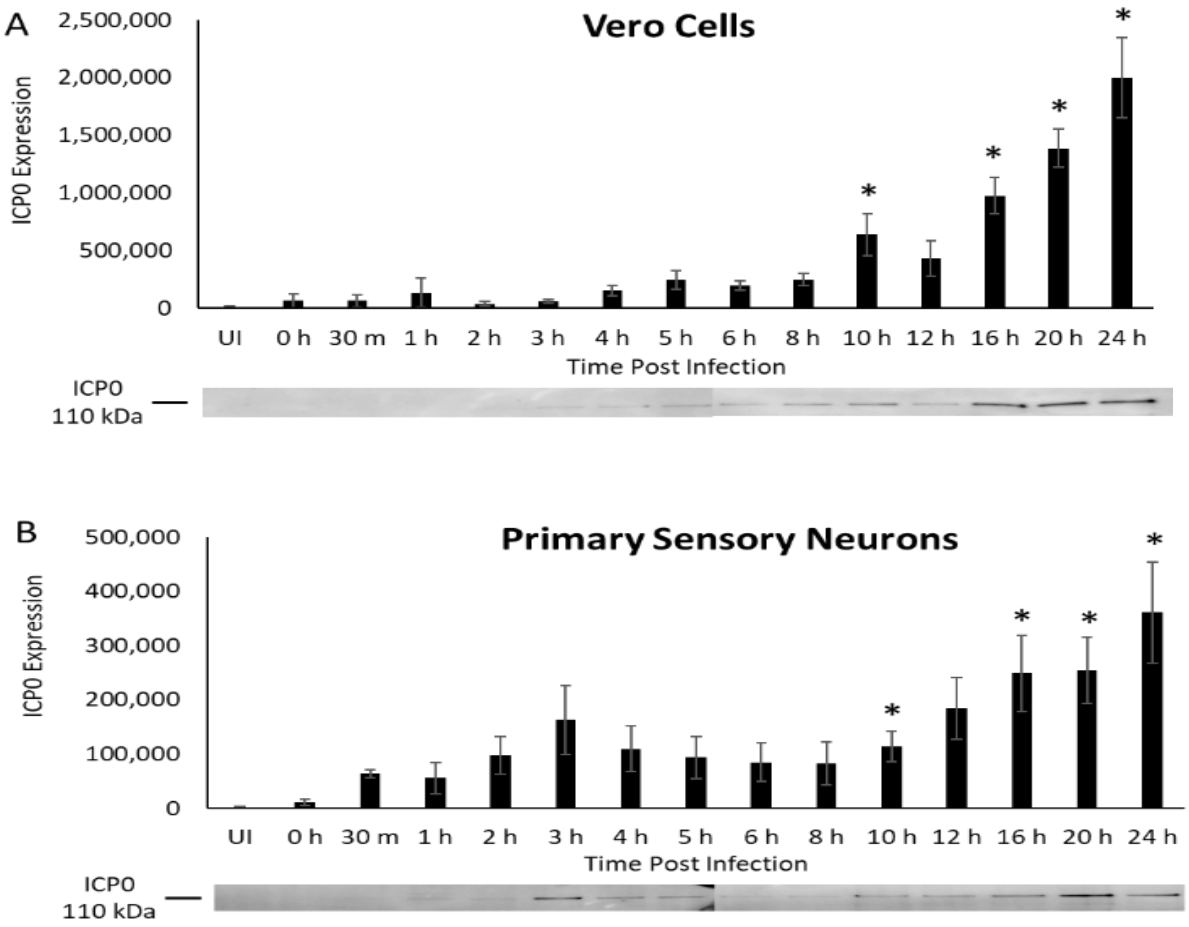

Figure 3.1. Protein profile of ICP0 in Vero 76 cells and primary sensory neurons

Protein expression profile of ICP0 in Vero 76 cells (A) and primary adult sensory dorsal root ganglion (DRG) neurons (B), based on densitometry analysis normalized to total protein using TCE (n=4 independent immunoblots). UI = uninfected neurons, 0 h = immediately upon inoculation. Error bars = SEM. Asterisks = statistically significant difference between Vero76 cells and primary sensory neurons ($p < 0.05$). Representative western blots are below each graph.

Mass spectrometry analysis of proteins ubiquitinated by ICP0

ICP0 engages in complex protein-protein interactions to modify the cellular environment during productive infection (30). Some of ICP0's interactions catalyze the transfer of ubiquitin to target proteins for proteasomal degradation (31), while others stabilize ICP0 (32). Several protein interactors, such as Sp100 (33), PML (33), and USP7 (34) have been identified in U2OS cells, human embryonic lung fibroblasts, and other non-neuronal cell types but proteins targeted by ICP0 for ubiquitination in neurons remain unknown. To identify proteins that ICP0 selectively ubiquitinates in primary adult sensory neurons, we utilized a comparative mass spectrometry approach, illustrated in Figure 3.2, using primary adult DRG sensory neurons infected with HSV-1 KOS, which expresses fully functional ICP0, and HSV-1 *n*212, which expresses a non-functional ICP0 fragment due to a nonsense linker inserted at codon 212 built in a KOS background (35). Proteins ubiquitinated by ICP0 would only be identified in KOS-infected neurons but would not be ubiquitinated, or would be ubiquitinated at a lower rate, in uninfected samples or HSV-1 n212-infected samples because ICP0 is absent or non-functional, respectively.

Primary cultured DRG neurons from 6-week-old mice were infected with KOS or n212 in the presence of MG132, a cell-permeable proteasome inhibitor, to prevent degradation of any proteins ubiquitinated before collection (Fig 3.2). Uninfected neurons were maintained in parallel and also treated with MG132. HSV-1 infection was allowed to progress for 8 hrs before total protein from each condition was collected in a non-denaturing buffer with proteasome inhibitor MG132, deubiquitinase inhibitor PR619, and protease/phosphatase inhibitors to protect the conjugated ubiquitin moieties on target proteins. Equal amounts of protein from each sample were incubated overnight with magnetic beads conjugated to FK2 antibodies that recognize K29, K48, and K63 poly-ubiquitination chains and mono-ubiquitinated proteins (36). Immunoprecipitated samples were subjected to nano liquid chromatography tandem mass spectrometry (LC-MS/MS) to generate datasets of putative proteins ubiquitinated in each condition.

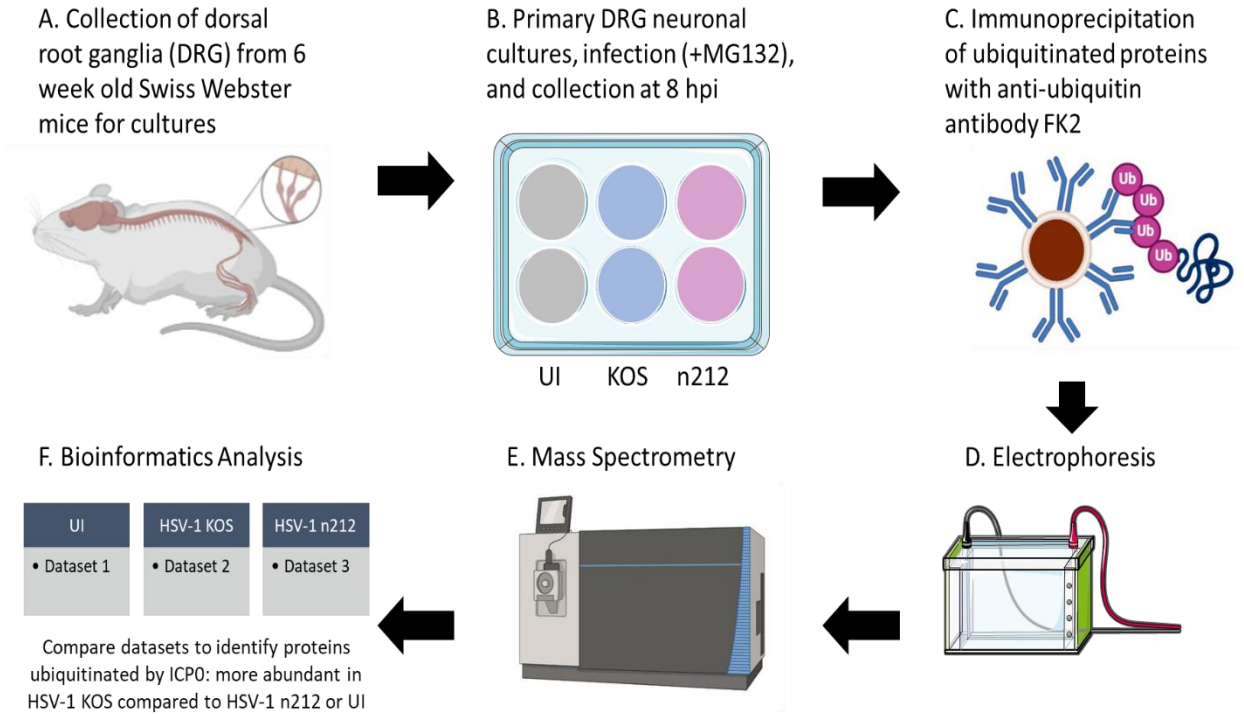

Figure 3.2. Procedure for LC-MS/MS

Schematic representation of the sample preparation for LC-MS/MS. (A) Primary adult dorsal root ganglion (DRG) neurons were resected from 6wk old Swiss Webster mice and cultured at a minimum of 40,000 neurons per well. (B) DRG neurons were treated with MG132 and infected with HSV-1 KOS or HSV-1 *n*212, or left uninfected (UI) for 8 hrs. (C) Equal amounts of total DRG protein were mixed with FK2 antibodies covalently bound to magnetic Dynabeads to immunoprecipitate ubiquitinated proteins from each sample. Proteins were eluted from beads. (D) Protein eluate was separated on a 10% SDS-PAGE gel, excised into 10 equal size bands, and digested in-gel with trypsin. (E) Peptides were analyzed by nano LC-MS/MS. (F) Data were analyzed and compared.

Proteins were identified by at least 2 unique peptides and screened based on MASCOT score (≥ 50) and Exp-q score ≤ 0.05 for acceptable confidence in the protein identification. Based on these screening criteria, we classified the ubiquitinated proteins in each condition (KOS, *n*212, UI) for function and signaling pathway affiliation using Reactome (Fig. 3.3A). Identified proteins mapped to biological pathways such as the cell cycle, cellular response to stimuli, immune system, cellular metabolism, and signal transduction, suggesting broad impacts on cellular processes during HSV-1 infection of neurons. To identify those proteins ubiquitinated by ICP0, we compared peptide spectrum matches (PSM) of each protein and focused on those proteins with at least a 1.25-fold increase in KOS-infected samples when compared to HSV-1 *n*212 or uninfected samples. In total, 169 unique host proteins and 17 viral proteins were identified, with 30 host and 15 viral proteins specifically ubiquitinated by ICP0 (identified only in KOS-infected neurons) (Fig. 3.3B). We also identified 46 proteins in common between KOS- and *n*212-infected neurons, 26 of which were ubiquitinated at least 1.25-fold higher in KOS-infected compared to n212-infected neurons. Of the 17 common proteins identified in both uninfected (UI) and KOS-infected neurons, only one was more highly ubiquitinated by ICP0 (1.86-fold higher in KOS-infected neurons) but 13 were more highly ubiquitinated in uninfected neurons compared to KOS-infected neurons, and 20 unique proteins were also identified only in uninfected (UI) neurons, suggesting that the presence of the virus somehow inhibits ubiquitination of several host proteins.

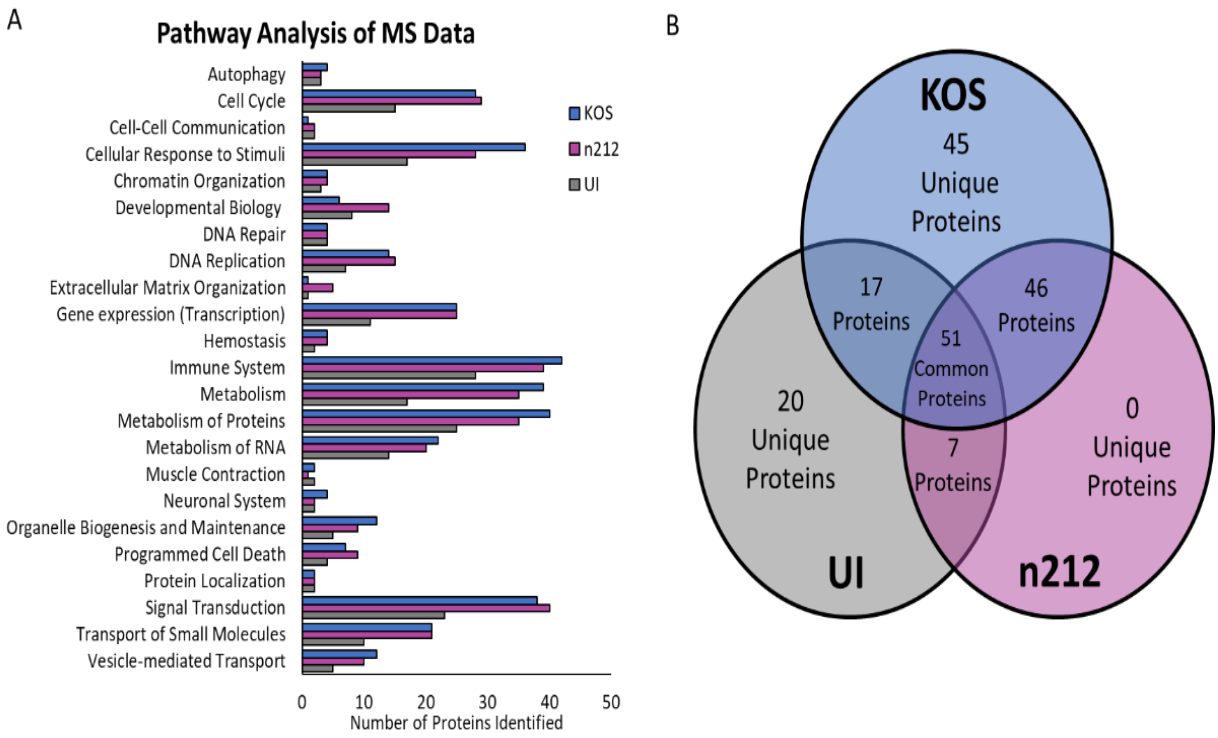

Figure 3.3. Bioinformatic analysis of LC-MS/MS identified proteins

Protein classification of LC-MS/MS identified proteins from neurons (A) infected with HSV-1 KOS (wild type), *n*212 (expressing truncated ICP0), or uninfected neurons were classified based on known function through pathway analysis using Reactome. (B) Statistical analysis identified 169 unique proteins: 45 unique to KOS-infected neurons, 20 unique to uninfected (UI) neurons, and proteins common to two conditions or all three.

Both viral and host proteins were identified in our LC-MS/MS analysis of ubiquitinated proteins, providing insight into potential ICP0 ubiquitination targets as well as neuronal viral mechanics (Table 1). ICP0 was identified in HSV-1 KOS-infected neurons, suggesting that ICP0 targets itself for ubiquitination early during productive infection in neurons. Other viral proteins were also detected only in KOS-infected neurons, including major viral transcription factor ICP4, tegument protein VP16, transcriptional regulator ICP22, and envelope glycoprotein B. These viral proteins were not identified in *n*212-infected neurons

suggesting that these proteins are selectively ubiquitinated by ICP0 during wild-type infection (Table 1. Viral Proteins). Thymidine kinase (TK) and the large subunit of ribonucleoside-diphosphate reductase were also ubiquitinated at significantly higher levels in KOS-infected compared to *n*212-infected neurons. As many of these viral proteins are important during productive infection, identification as ubiquitination targets in KOS-infected neurons suggests that ICP0 may be attempting to inhibit productive infection, at least in some sub-populations of the sensory neurons.

The identified host proteins exhibited a broad range of functions, subcellular locations, and potential relevance to HSV-1 pathogenesis (Table 1. Host Proteins). Proteins such as cell cycle exit and neuronal differentiation protein 1, histone H1.2, 14-3-3 protein epsilon, and β-synuclein were enriched in KOS-infected neurons when compared to *n*212-infected and uninfected neurons, suggesting selective targeting by functional ICP0. Our focus, however, centered on transcription factors involved in gene expression regulation, gene repression, and neuron-specific functions that could be relevant to HSV-1 pathogenesis. Of the identified proteins, High Mobility Group Protein I/Y (HMG I/Y) and Trans-Activation Response (TAR) DNA-Binding Protein 43 (TDP43) fit these criteria. HMG I/Y is a member of the HMG superfamily consisting of three primary classifications: HMGA, HMGB, and HMGN (37, 38). The HMGA family, with which HMG I/Y is associated, utilizes AT-hook to alter the structure of DNA by binding inside the minor groove (39), making DNA more or less accessible to transcription factors and DNA binding proteins (40). HMG I/Y has previously been shown to interact with TAATGARAT sequences within the HSV-1 genome, increasing viral gene expression (41), but its ubiquitination status in relation to HSV-1 has not been determined. TDP43 has not been linked to HSV-1 but was first identified for its role in binding to Human Immunodeficiency Virus (HIV) Trans-Activation Response (TAR) elements and repressing viral replication (42). More recently, TDP43 has been shown to regulate spatiotemporal and tissue-specific gene expression, RNA polymerase pausing, and RNA splicing (43), impacting more than 30% of the cell transcriptome (44). HMG I/Y and TDP43 are essential for neuronal gene expression and RNA metabolism, respectively. Given the previously reported role of these proteins in viral replication, we selected these

proteins for further study to determine if their ubiquitination is mediated by ICP0, and their roles in HSV neuronal pathogenesis.

	Accession	Protein	Gene	UI	KOS	n212	KOS/ UI	KOS/ n212	KOS:UI (*p* value)	KOS:n212 (*p* value)
Viral Proteins	P03176	Thymidine kinase	TK	0.0	12.5	3.0	NC	4.17	0.0016	0.0136
	P08543	Ribonucleoside-diphosphate reductase large subunit	RIR1	0.0	112.0	3.5	NC	32.00	0.0249	0.0266
	P10221	Inner tegument protein	UL37	0.0	58.5	0.0	NC	NC	0.0018	0.0018
	P04296	Major DNA-binding protein	DBP	0.0	90.0	0.0	NC	NC	0.0202	0.0202
	P04294	Alkaline nuclease	UL12	0.0	41.0	0.0	NC	NC	0.0279	0.0279
	P10211	Envelope glycoprotein B	gB	0.0	22.0	0.0	NC	NC	0.0670	0.0670
	P04485	Transcriptional regulator ICP22	ICP22	0.0	21.0	0.0	NC	NC	0.0955	0.0955
	P06492	Tegument protein VP16	UL48	0.0	21.0	0.0	NC	NC	0.1196	0.1196
	P08392	Major viral transcription factor ICP4	ICP4	0.0	28.0	0.0	NC	NC	0.2222	0.2222
	P06491	Major capsid protein	MCP	0.0	46.0	0.0	NC	NC	0.2421	0.2421
	P08393	E3 ubiquitin-protein ligase ICP0	ICP0	0.0	36.0	0.0	NC	NC	0.2865	0.2865
	P04488	Envelope glycoprotein E	gE	0.0	6.5	0.0	NC	NC	0.4226	0.4226
Host Proteins	Q60900	ELAV-like protein 3	Elavl3	0.0	21.0	0.0	NC	NC	0.0023	0.0023
	P61027	Ras-related protein Rab-10	Rab10	0.0	20.0	0.0	NC	NC	0.0025	0.0025
	A0A1B0GS70	Proteasome endopeptidase complex	Psma1	0.0	15.5	0.0	NC	NC	0.0250	0.0250
	P54775	26S proteasome regulatory subunit 6B	Psmc4	0.0	21.5	0.0	NC	NC	0.0255	0.0255
	Q9JKC6	Cell cycle exit and neuronal differentiation protein 1	Cend1	0.0	12.0	0.0	NC	NC	0.0572	0.0572
	P49312	Heterogeneous nuclear ribonucleoprotein A1	Hnrnpa1	0.0	19.0	0.0	NC	NC	0.0628	0.0628
	P17095	High mobility group protein HMG-I/HMG-Y	Hmga1	0.0	12.5	0.0	NC	NC	0.4226	0.4226
	P43274	Histone H1.4	H1-4	0.0	72.5	21.5	NC	3.37	0.2593	0.3874
	P15864	Histone H1.2	H1-2	0.0	74.5	23.0	NC	3.24	0.3050	0.4444
	Q91ZZ3	Beta-synuclein	Sncb	0.0	40.0	18.0	NC	2.22	0.0056	0.0258
	Q8R0B4	TAR DNA-binding protein 43	Tardp	0.0	29.0	15.5	NC	1.87	0.0105	0.0995
	Q3THW5	Histone H2A.V	H2az2	0.0	41.0	23.5	NC	1.74	0.0450	0.1917

*Complete list of identified proteins in supplemental figure (S1)

Table 1. Virus and host proteins identified by LC MS/MS as ubiquitinated.

HMG I/Y and TDP43 exhibit increased ubiquitination in the presence of functional ICP0

Ubiquitination of target proteins occurs through the addition of ubiquitin moieties on lysine residues on the target proteins (45). To validate that ICP0, HMG I/Y, and TDP43 are ubiquitinated by ICP0 during HSV-1 productive infection, we inoculated primary DRG neuronal cultures with KOS or *n*212. After 8 hrs of infection, neurons were collected in a non-denaturing buffer with MG132 and PR619 to prevent proteasomal degradation and de-ubiquitination of proteins while in solution. Ubiquitinated proteins were subsequently immunoprecipitated using the FK2 antibody, used previously for mass spectrometry, followed by immunoblots using antibodies specific for HMG I/Y and TDP43. We also immunoblotted for ICP0 since

ICP0 has been previously reported to self-ubiquitinate in cell free assays and we detected ICP0 as a ubiquitinated protein in KOS-infected neurons by mass spectrometry, but not in neurons infected with *n*212; (46). Total protein input (InP), supernatant from the immunoprecipitation (S), and the eluate from the FK2-conjugated beads (IP) were loaded onto SDS-PAGE gels and probed for each protein of interest using protein-specific antibodies.

For our immunoprecipitation experiments, ICP0 was detected in the total protein input (InP) of KOS-infected neurons (~118 kDa), and the truncated ICP0 was detected in *n*212-infected neurons (~37 kDa) (Fig. 3.4A). However, no ICP0 protein was detected in the supernatant, suggesting that ICP0 had been captured by the anti-ubiquitin FK2-conjugated beads, but no detectable patterns of mono-ubiquitination or poly-ubiquitination were observed in the IP. HMG I/Y appeared at ~20 kDa in total protein (InP) samples and at ~37 kDa in IP samples (Fig. 3.4B). HMG I/Y was not detected in the supernatant, demonstrating successful immunoprecipitation with FK2, and exhibited increased band density in HSV-1 KOS-infected samples. This shift in size is consistent with a change of approximately 17 kDa, supporting the addition of two ubiquitin moieties (8.6 kDa per ubiquitin) (47). A faint band was also detected at ~80 kDa in KOS-infected neurons, suggesting a small portion of HMG I/Y was poly-ubiquitinated, as well (Fig. 3.4B). TDP43 was detected as a doublet at 43 and 50 kDa in InP samples and a portion of the 50 kDa band was detectable in the supernatant (Fig. 3.4C). These data indicate the likely presence of post-translationally modified forms of TDP43 in total protein (InP) samples, besides ubiquitination. In IP samples, TDP43 exhibited characteristic patterns of poly-ubiquitination with increased smear density above 50 kDa in KOS-infected samples. These results support that HMG I/Y and TDP43 are ubiquitinated by ICP0 during the first 8 hrs of productive HSV-1 infection in sensory neurons, but ubiquitinated ICP0 was not detected in this assay.

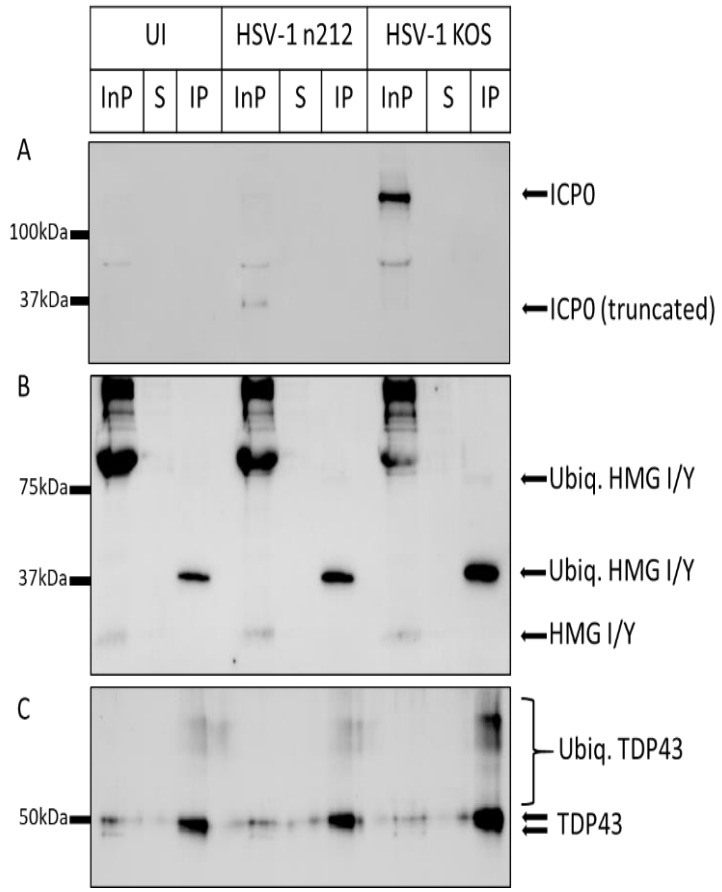

Figure 3.4. Immunoprecipitation of ICP0, HMG I/Y, and TDP43 at 8 hrs post infection

Ubiquitination of ICP0 (A), HMG I/Y (B), and TDP43 (C). Immunoprecipitation was conducted 8 hpi using Dynabeads conjugated to anti-FK2 antibodies that specifically targets K29, K48, K63, and mono ubiquitinated proteins. Total protein input (InP), supernatant from immunoprecipitation (S) and immunoprecipitated eluate (IP) from uninfected (UI), HSV-1 *n*212-infected, and HSV-1 KOS-infected neurons were probed with antibodies targeting each protein of interest.

Productive HSV-1 infection alters HMG I/Y and TDP43 proteins profiles in sensory neurons

To determine if HMG I/Y and TDP43 are targeted for degradation, mediated by ICP0, during productive HSV-1 infection, we analyzed the protein profile of each protein during productive infection with either KOS or *n*212 in the absence or presence of MG132 to inhibit the proteasome, preventing the degradation of ubiquitinated proteins. If HMG I/Y or TDP43 is degraded during productive infection, mediated by ICP0, we would observe a decrease in the respective protein only in samples infected with KOS without MG132. In comparison, HMG I/Y or TDP43 would exhibit an increase in protein in KOS-infected samples treated with MG132 since the proteasome could not degrade the ubiquitinated proteins. These protein changes should not occur in *n*212-infected neurons because this truncated form of ICP0 is defective.

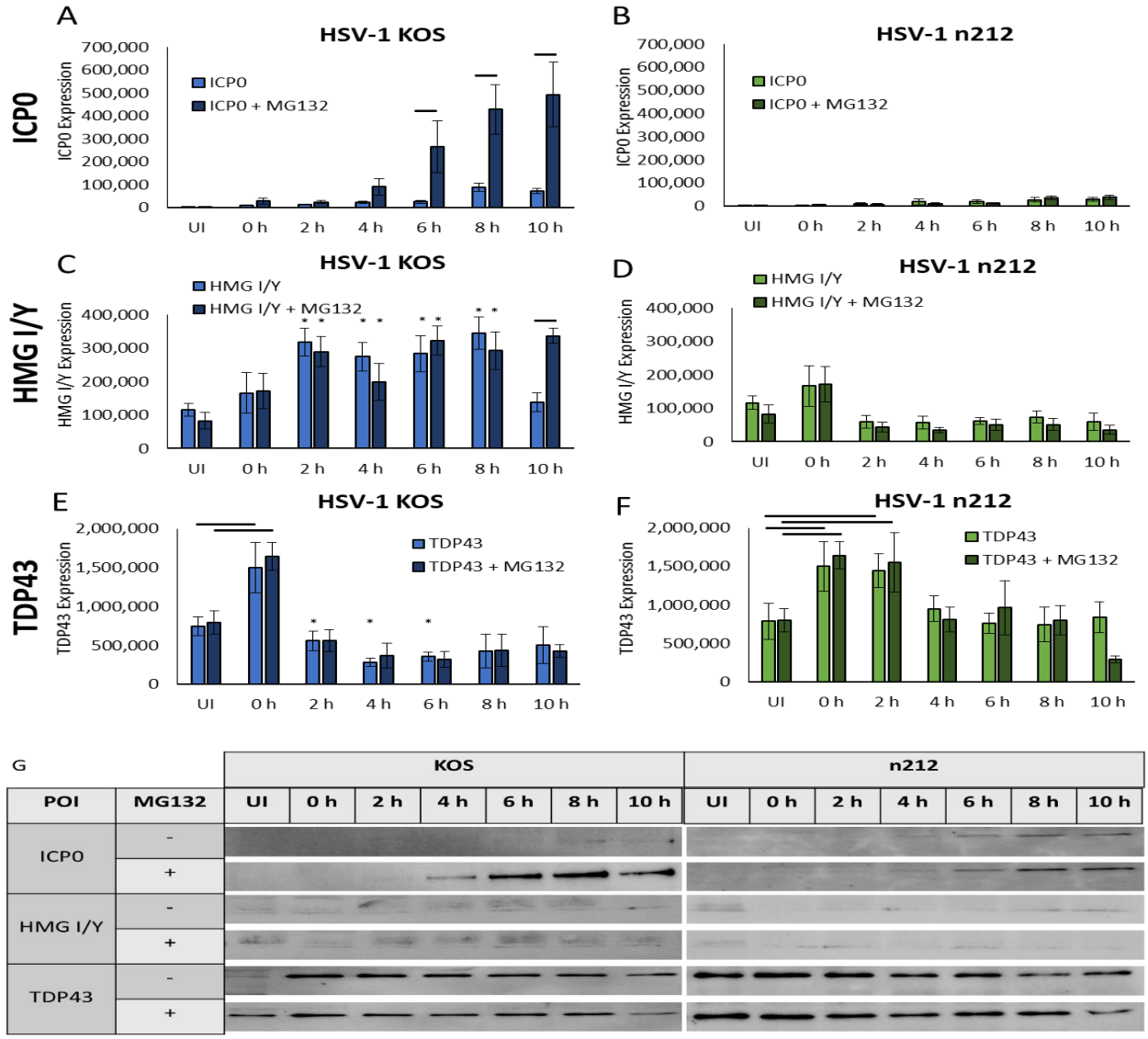

Figure 3.5. 10 hr Time course of ICP0, HMG I/Y, and TDP43 during the acute HSV-1 infection of adult sensory neurons

Protein expression of ICP0 (A-B), HMG I/Y (C-D), and TDP43 (E-F) during 10 hrs of HSV-1 KOS and HSV-1 n212 productive infection in primary adult DRG neuronal cultures, with or without MG132 to inhibit proteasomal degradation. Neurons were assessed every 2 hrs by western blot (n=3), analyzed by densitometry, and normalized to total protein visualized with TCE. Representative western blots are shown (G). Error bars = SD. Horizontal bars indicate statistical significance between MG132 treated and untreated (A, C) or between 0 h and UI (E, F) ($p < 0.05$). Asterisks indicate statistical significance between KOS- and *n*212-infected neurons ($p < 0.05$).

Primary adult DRG neuronal cultures were infected with KOS or n212, with or without MG132. Neurons were collected in 2 hr increments for 10 hrs and immunoblotted for ICP0, HMG I/Y, or TDP43. Protein bands from three independent blots were quantified using densitometry, normalized to total protein visualized with 2,2,2-trichloroethanol (TCE), and presented as bar graphs (Fig. 3.5A-F) with representative immunoblot images (Fig. 3.5G). ICP0 was detected at 0 hpi in KOS-infected neurons with and without MG132, indicating that ICP0 was present at low levels within the inoculating virus (Fig. 3.5A), which is consistent with previous reports that ICP0 is present within the tegument of purified HSV-1 (48). ICP0 protein expression levels in KOS-infected neurons without MG132 remained relatively low, but neurons treated with MG132 contained significantly greater quantities of ICP0 by 6 hpi ($p < 0.05$), demonstrating proteasomal degradation of ICP0 during productive infection (Fig. 3.5A). In contrast, the truncated ICP0 expressed by *n*212 remained low and relatively consistent for neurons that were treated or untreated with MG132 (Fig. 3.5B), demonstrating that the truncated ICP0 is not well-expressed in adult sensory neurons and is not degraded by the ubiquitin-proteasome pathway during productive infection in neurons.

HMG I/Y was detected in uninfected neurons, increasing slightly but not significantly in response to inoculation (0 h compared to uninfected, Fig. 3.5C and D), which was likely recognized as a transient stressor to the neurons. In KOS-infected neurons, HMG I/Y increased significantly compared to uninfected neurons by 2 hpi and remained high in both MG132 treated and untreated neurons ($p < 0.05$, Fig. 3.5C). At 10 hpi, HMG I/Y protein level remained high in KOS-infected neurons treated with MG132 but was significantly decreased in untreated neurons, returning to uninfected levels ($p = 0.55$ compared to uninfected, Fig. 3.5C), showing that HMG I/Y was degraded by the proteasome at this time point when ICP0 was present. In *n*212-infected neurons, HMG I/Y returned to uninfected levels by 2 hpi, suggesting that the impaired productive infection kinetics of *n*212 did not influence the overall level of HMG I/Y in adult sensory neurons and protein expression remained similar to uninfected neurons ($p = 0.18$-0.30, Fig. 3.5D). Treatment with MG132 had no significant effects on HMG I/Y in *n*212-infected neurons, compared

to untreated neurons, showing that HMG I/Y is not degraded by the proteasome when a non-functional ICP0 is expressed.

TDP43 protein was also detected in uninfected neurons and increased in response to virus inoculation at 0 h for both KOS and *n*212 infection (Fig. 3.5E and F). In contrast to HMG I/Y, however, the initial increase in TDP43 levels was statistically significant compared to uninfected neurons ($p < 0.05$), and resolved within 2 hrs to levels similar to uninfected neurons in KOS-infected neurons, remaining stable and consistent for both MG132 treated and untreated neurons throughout the 10 hr time period (Fig. 3.5 E). In n212-infected neurons, TDP43 remained high a little longer, for at least 2 hrs ($p < 0.05$). TDP43 then decreased to levels comparable to uninfected neurons by 4 hpi, remaining consistent through 8 hpi in neurons with and without MG132 (Fig. 3.5F). At 10 hpi, in neurons treated with MG132, TDP43 decreased slightly but not significantly to a level below what was observed in uninfected samples. This decrease was not observed in n212-infected neurons without MG132, in which the ubiquitin-proteasome is free to degrade ubiquitinated proteins. Although TDP43 is ubiquitinated by ICP0, it does not appear to be degraded in the proteasome, since MG132 had no significant effects on TDP43 protein expression. However, TDP43 protein expression was maintained at a significantly lower level during productive HSV-1 KOS infection between 2 and 6 hrs post infection ($p < 0.05$, indicated by asterisks) when compared to *n*212 infection, suggesting that HSV-1 infection results in reduced TDP43 expression early during productive infection when wild-type ICP0 is expressed.

HMG I/Y is increasingly ubiquitinated and degraded by ICP0 between 8 and 10 hpi

HMG I/Y protein levels increased and remained high during the first 8 hpi in neurons infected with KOS, but decreased significantly at 10 hpi in KOS-infected neurons without MG132, which inhibits the proteasome (Fig. 3.5C). The decrease suggested that in the presence of ICP0, HMG I/Y was degraded in the proteasome at 10 hpi. Our validation assay for ubiquitination was performed at 8 hpi and showed that only a small portion of HMG I/Y was ubiquitinated (faint band ~80 kDa in Fig. 3.4B). To determine if ICP0 increasingly ubiquitinates HMG I/Y at 10 hpi beyond what we previously observed at 8 hpi, we infected

primary adult sensory neurons with KOS or *n*212 in the presence of MG132, as described previously, and allowed the infection to progress for 10 hpi. KOS-infected, *n*212-infected, and uninfected neurons were collected in a non-denaturing buffer with MG132 plus inhibitors and immunoprecipitated using the FK2 antibody. Total protein input (Inp) and IP samples were immunoblotted to visualize patterns of ubiquitin for HMG I/Y. HMG I/Y exhibited bold patterns of ubiquitination in KOS-infected neurons at 10 hpi (Fig. 3.6), correlating with the decrease in protein levels observed in KOS-infected neurons without MG132 at 10 hpi (Fig. 3.5C). These data show that HMG I/Y is increasingly ubiquitinated by ICP0 between 8 – 10 hpi in the presence of wild-type ICP0, resulting in proteasomal degradation by 10 hpi.

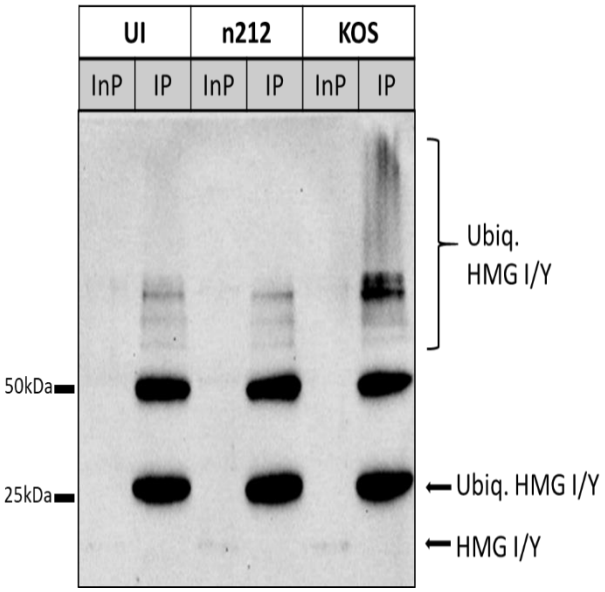

Figure 3.6. Ubiquitination of HMG I/Y 10 hrs post HSV-1 infection

Ubiquitination of HMG I/Y. Immunoprecipitation was conducted 10 hpi using Dynabeads conjugated to anti-FK2 antibodies that specifically targets K29, K48, K63, and mono ubiquitinated proteins. Total protein input (InP) and immunoprecipitated eluate (IP) from uninfected (UI), HSV-1 *n*212-infected, and HSV-1 KOS-infected neurons were probed with antibodies targeting HMG I/Y.

Discussion

HSV-1 entry into a neuron can result in productive infection or the establishment of latency, depending on the type of neuron and the physiological state of that neuron at the time of entry (7, 49-51). Previous studies have shown that host factors, such as chromatin mediators (52-56) and innate defense factors (22, 57-59), contribute to the establishment of latency but a mechanism for the decision between productive or latent infection following viral entry into neurons has yet to be fully defined. HSV IE protein ICP0 can

ubiquitinate host proteins to target them for proteasomal degradation, providing a mechanism by which HSV could remove host proteins detrimental to HSV infection. Therefore, we sought to identify proteins targeted for ubiquitination by ICP0 during productive infection, specifically in primary adult sensory DRG neurons, using a comparative mass spectrometry approach. We identified both host and viral proteins that were ubiquitinated by ICP0, as well as proteins that were ubiquitinated by both ICP0 and host processes. These proteins likely play key roles in early events during HSV-1 neuronal infection.

Ubiquitination of proteins is complex and can have multiple downstream effects depending on the type of ubiquitin moieties present on the target protein. Mono-ubiquitination typically has a low affinity for the proteasome but often alters the subcellular location or function of the target protein (60). Poly-ubiquitination, specifically K11 and K48, often results in degradation of the target protein by proteasomes (61), while K48 and K63 can also lead to phagolysosome autophagic degradation (62). These outcomes are further nuanced by functional overlap and mixed or branched polyubiquitin chains that could combine multiple ubiquitin moieties with elements of K11, K29, K48, and K63 chains. K29 chains, for example, are often found in combination with other ubiquitin moieties, and its role in protein functions remains unclear, in part due to a lack of sufficient antibodies to study its effects in isolation (63). We did not attempt to identify specific ubiquitin chains on the proteins we identified in this study, as ICP0 has been reported to stimulate the formation of complex ubiquitin chains *in vitro* (25), and the direct biological implications of this in neurons is unclear.

Previous reports have supported the ubiquitination of ICP0 in cell-free assays, suggesting this post-translational modification would facilitate its proteasomal degradation (46). In our studies, we identified ICP0 in our original mass spectrometry analysis using the anti-ubiquitin antibody FK2 to pull out ubiquitinated proteins, suggesting that it was indeed ubiquitinated during productive infection in sensory neurons. Although we did not observe patterns of ubiquitination for ICP0 by immunoblot, ICP0 was degraded by the proteasome, as shown by the use of a proteasome inhibitor. As a RING finger E3-ubiquitin ligase, ICP0, remaining true to classification, would be prone to auto-ubiquitination; however, auto-

ubiquitination is tightly controlled and more prevalent in the absence of other target substrate proteins (64-66). The lack of observable bands indicative of auto-ubiquitination could be the result of target/substrate abundance during the initial infection of HSV-1 (46, 66). In addition, ICP0 has been reported to rely on USP7 to protect itself from autoubiquitination while remaining active to target additional substrates during productive infection (46). The antibody binding epitope is also near the ubiquitination site. Thus, protein-protein interaction between ICP0 and other proteins or post-translational modifications could prevent the detection of ubiquitinated ICP0 by masking the antibody binding site. These observations do not eliminate the possibility of ubiquitin-independent mechanisms causing ICP0 degradation, as previously observed in human embryonic lung fibroblasts during early HSV-1 infection (67).

ICP0 has been shown to target key proteins such as Sp100 (33), other ND10 components (68), p53 (26), and Schlafen 5 (22), but we did not identify these proteins in our LC-MS/MS analysis. These differences in ICP0 ubiquitination targets are mostly likely due to differences in how primary sensory neurons regulate HSV-1 infection in comparison to non-neuronal cells. Any antiviral response that would likely induce apoptosis in infected non-neuronal cells must restrict viral replication and promote survival in neurons, as neurons are rather important and not easily replaced. We did, however, identify immediate-early, early, and late HSV-1 genes in KOS-infected samples, suggesting ICP0 mediates the ubiquitination and degradation of viral proteins. These proteins included all temporal classes of viral genes, which would be expected to be expressed in KOS-infected neurons but not necessarily ubiquitinated and targeted for degradation. ICP4, VP16, major DNA binding protein (ICP8), thymidine kinase, and even ICP0 are known to promote conditions for lytic infection, activate viral gene expression, and mediate viral replication (69-72). Structural components of the virus, including capsid proteins and envelope glycoproteins, were also identified. Taken together, these results suggest that ICP0 may actively target viral lytic proteins for ubiquitination early during infection of sensory neurons to induce an abortive infection, promoting the establishment of latency. Although this is contradictory to the accepted role of ICP0 stimulating viral

transcription, the identification of fifteen lytic viral proteins ubiquitinated by ICP0 suggests that ICP0 may have different functions in non-neuronal and neuronal cells, or in different sub-populations of neurons.

HMG I/Y is a transcriptional regulator that binds the minor groove of DNA, making it more accessible to RNA polymerase and transcription factors (38, 40). As an architectural transcription factor, HMG I/Y has no standard transcriptional function but is highly dependent on cofactors for its target specificity, serving dual functions as an activator of viral and host antiviral genes (73). Dependence of HMG I/Y on cofactors results in complex and diverse regulatory mechanisms at transcriptional (74) and translational levels by proteins, pseudogenes (75), and microRNAs, in addition to post-translational modifications. HMG I/Y has been shown to interact with promoters of HSV-1 genes, such as ICP4 and the latency-associated transcript (LAT) (76), as well as enhancing gene transcription of other viruses, including human papillomavirus (HPV) (77) and hepatitis B virus (HBV) (78). In contrast, HMG I/Y has been implicated in enhancing the expression of interferon genes that restrict viral transcription (79). This creates a complex interaction with HMG I/Y-regulated host genes and viral genes. In our studies, HMG I/Y expression initially increased in HSV-1 KOS-infected neurons but not in *n*212-infected neurons. HMG I/Y is reportedly sustained during HPV infection to aid in the coordination of gene expression (77, 80) and is directly upregulated by HBV X protein (78), so HMG I/Y may be necessary for some aspect of HSV-1 infection. HMG I/Y may have a transient enhancer and subsequent inhibitory role to HSV-1 in neurons depending on the viral proteins present, such as ICP4 (41). However, ubiquitination by ICP0 and degradation of HMG I/Y by 10 hours after infection could suggest a shift in viral mechanics, such that ICP0 attempts to abort a productive infection and facilitate establishment of latency.

In contrast to HMG I/Y, TDP43 expression was reduced in the presence of functional ICP0 during productive infection. Although TDP43 was ubiquitinated by ICP0, it was not degraded in the ubiquitin-proteasome, suggesting that TDP43 may have been functionally redirected or degraded through some other process. TDP43 is a nucleic acid binding protein that was first identified as an HIV viral repressor (42). More recently, TDP43 has been implicated in gene regulation (81), RNA metabolism, tissue-specific gene

expression (43, 82), and modulation of stress granules in response to stress (83). In normal homeostatic tissue, TDP43 modulates the stability and decay of host mRNA (43, 44), impacting up to 30% of the transcriptome (81). Its broad range allows it to influence many biological activities. For example, TDP43 stabilizes the mRNA of Ras-GAP SH3-domain binding protein 1 (G3BP1) (84), a key stress granule protein that promotes efficient activation of cyclic GMP-AMP synthase (cGAS), an important innate sensor directed against DNA viruses to activate a type-1 interferon response (85, 86). G3BP1 mRNA is reduced when TDP43 protein levels are low, as we found during productive infection in neurons infected with KOS, suggesting this could be a potential mechanism utilized by HSV-1 to control the host antiviral response.

This study presents unique insight into the manipulation of the neuronal environment of primary adult sensory neurons through ICP0-mediated ubiquitination of host cell proteins during HSV-1 infection. For productive infection in epithelial cells, ICP0 engages in dynamic protein-protein interactions with multiple proteins to enhance viral replication. These interactions are often dominated by ICP0's ubiquitination function to create a cellular environment conducive for the production of viral progeny. In neurons, ubiquitination of host proteins could facilitate either productive infection or the establishment of latency, from which it can later reactivate to cause recurrent disease and transmit to new hosts. Our results, showing that ICP0 ubiquitinates and facilitates degradation of key host proteins at specific times following infection, suggest an exquisitely complex manipulation of the neuronal environment. We did not detect proteins that were previously identified as ICP0 ubiquitination targets in non-neuronal cells, such as ND10 proteins or regulators of the interferon response, which would inhibit productive infection. We did, however, detect multiple HSV-1 proteins in our mass spectrometry screen, strongly suggesting that ICP0 actively targets its own productive cycle proteins for ubiquitination to potentially facilitate the establishment of latency in sensory neurons. Although further studies are needed to fully define the sequence of events orchestrated by ICP0 in neurons, the targets of ICP0 appear to be distinct from those in non-neuronal cells.

Materials and methods

Cells and Viruses. Vero76 cells (C1008, ATCC, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, MA USA) supplemented with 8% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). HSV-1 KOS (wild-type) and *n*212 strains (87) (both originally from the laboratory of Priscilla Schaefer) were generously provided by David Davido at the University of Kansas Department of Molecular Biosciences, Lawrence, Kansas, USA. HSV-1 *n*212 expresses a truncated ICP0 with a nonsense linker insertion at amino acid 212 on a KOS backbone (35). KOS was propagated and titrated on Vero76 cells and *n*212 on L7 cells (88) that express complementing ICP0, also kindly provided by David Davido.

Primary Adult Neuronal Cultures. Dorsal root ganglia (DRG) were resected from 6 wk old Swiss Webster mice (Hilltop Laboratories, Scottdale, PA, USA) and enzymatically digested using papain and collagenase/dispase (Worthington Biochemical, Lakewood, NJ, USA), followed by mechanical separation into single-cell suspensions. Neurons were counted and plated on Matrigel-coated (Corning, Silicon Valley, CA) cell culture plates at 3,000 - 80,000 neurons per well, depending on the assay (7). DRG neurons were maintained in Neurobasal A medium (Thermo Fisher Scientific, Waltham, MA, USA) with 1% penicillin-streptomycin (PS), 1X Glutamax, FUDR to deplete non-neuronal cells, and neurotrophic factors (nerve growth factor, glial cell-derived neurotrophic factor, neurturin; obtained from PeproTech, Cranbury, NJ, USA) (7). DRGs were allowed to acclimate to the culture plate for 3-4 days before experimental procedures. All studies were conducted in accordance with the Institutional Animal Care and Use Committee at Virginia Tech (protocol approved 2/8/2019).

Infection. Maintenance media was removed and DRG neuronal cultures were inoculated with 30 multiplicity of infection (moi) of HSV-1 KOS or *n*212 viruses in Neurobasal A medium (Thermo Fisher Scientific) for 1 hr. The viral inoculum was subsequently removed and replaced with NeuroComp media (Neurobasal A medium with 1% PS, Glutamax, and neurotrophic factors, but no FUDR) and maintained for the time periods indicated in the figures.

Antibodies. Primary antibodies included HSV-1 ICP0 (11060, Santa Cruz Biotechnology, Santa Cruz, CA, USA), TDP43 (GTX114210, GeneTex, Irvine, CA, USA and ab1044223, Abcam, Waltham, MA, USA), HMG I/Y (393213, Santa Cruz and ab168260, Abcam), and anti-ubiquitin FK2 (BML-PW8810-0500, Enzo Biochem, Farmingdale, NY, USA or Sigma Aldrich, St. Louis, MO, USA). Primary antibodies were visualized with secondary antibody goat anti-mouse or goat anti-rabbit IgG-HRP (31430 and 31460, Thermo Fisher Scientific).

LC-MS/MS. A total of 80,000 cultured DRG neurons per treatment were infected with HSV-1 KOS or HSV-1 *n*212, or were uninfected, and treated with MG132 (Cbz-Leu-Leu-Leucinal) to inhibit the ubiquitin-proteasomal degradation complex and preserve ubiquitinated proteins post-inoculation. The infection progressed for 8 hrs prior to protein harvesting in 125 µL non-denaturing lysis buffer (20mM Tris HCl pH8, 1% NP-40, 2mM EDTA) with MG132, PR619 (2,6-Diaminopyridine-3,5-bis(thiocyanate)) broad-spectrum deubiquitinating enzyme inhibitor to prevent the removal of ubiquitin moieties after collection, and Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Samples were incubated for 12 hrs with FK2 anti-ubiquitin antibody covalently conjugated to Invitrogen Dynabeads (Thermo Fisher Scientific), according to the manufacturer's protocol. Immunoprecipitated samples were rinsed 3X with non-denaturing buffer and resuspended in non-denaturing lysis buffer with MG132, PR619, and Halt Protease & Phosphatase Inhibitor. Samples were shipped overnight to MSBioworks (Ann Arbor, MI, USA) for LC-MS/MS analysis. Each sample was eluted in 70 µL 1.5X NuPage LDS Sample Buffer (Thermo Fisher Scientific) and boiled at 100 ºC for 15 min, followed by clarification via centrifugation. Half of each sample was processed by SDS-PAGE using a 10% Bis-Tris NuPage Mini-gel (Thermo Fisher Scientific) with an MES buffer system. A 2 cm gel space was excised into ten bands, washed with 25 mM ammonium bicarbonate and acetonitrile, reduced with 10 mM dithiothreitol at 60 ºC, alkylated with 50 mM iodoacetamide at room temperature, digested with trypsin at 37 ºC for 4 hrs, quenched with formic acid, and finally analyzed using a nano LC-MS/MS with Waters M-Class LC system interfaced to a Fusion Lumos mass spectrometer (Thermo Fisher Scientific). Each sample was analyzed for 5 hrs. The infection

was repeated a second time, using neuronal cultures performed on a different day and following the identical processes and protocols to generate a replicate set of mass spectrometry data.

Data processing. Raw data files from MSBioworks were downloaded by Virginia Tech Mass Spectrometry Incubator and reprocessed using Proteome Discoverer v. 2.5 (Thermo Fisher Scientific). Data files corresponding to all 10 bands of the same sample type were analyzed together. Searches using both Mascot (Matrix Science, Mount Prospect, IL, USA) and SequestHT (Thermo Fisher Scientific) were performed against the herpes simplex virus 1 reference proteome downloaded from UniProt, the mouse reference proteome downloaded from UniProt, and a database containing a list of common contaminant proteins provided with the Proteome Discoverer software, and the results merged using Proteome Discoverer. Search parameters included trypsin specificity with the possibility of two missed cleavages, precursor mass tolerance of ±10 ppm, fragment mass tolerance of ±0.1 Da, fixed modification of carbamidomethylation of Cys residues, and the following variable modifications: oxidation of Met, deamidation of Asn and Gln, formation of pyro-Glu when Gln was at the C-terminus of a peptide, acetylation of the protein N-terminus, and GlyGly characteristic of ubiquitin after trypsin cleavage at Lys and the protein N-terminus. The IMP-ptmRS node within Proteome Discoverer was utilized to validate the position and level of confidence for the GlyGly modification. The two repetitions were analyzed separately, and proteins identified in both replicates were included in the final comparative analyses.

Statistical Analysis. LC-MS/MS proteins were identified using a minimum of 2 unique peptide sequences and a 1% false discovery rate (FDR). Common contaminants and identified proteins with a MASCOT score < 50 ($p < 0.00001$ compared to average MASCOT score of each dataset) and an Exp-q value ≥ 0.05 were manually removed, and the resulting list was collated for comparison. The relative abundance for each protein was determined based on the peptide spectrum match (PSM) normalized to total spectra for each run provided by MSBioworks. Normalized PSM from each replicate were averaged within the respective treatment and compared to the other treatments. Proteins with a minimum of 1.25-fold increase in HSV-1 KOS samples, compared to both HSV-1 n212 and uninfected controls, were selected for downstream

biological justification. A literature search was performed on the selected proteins for previously reported functions to identify specific proteins with potential relevance to HSV-1 infection in neurons. Viral proteins that were identified by mass spectrometry were screened at a minimum MASCOT score of 30 ($p < 0.01$), since viral proteins were expected to be present at low concentrations compared to total protein.

Immunoblot. Neuronal cells were collected in 100 µL radioimmunoprecipitation assay (RIPA) buffer. Sample protein concentrations were quantified using Quick Start Bradford Dye Reagent (BioRad, Philadelphia, PA, USA) and Bovine Serum Albumin Standard Set (BioRad). A total of 15 µg of protein were loaded into a 10-12.5% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel with 1% 2,2,2-trichloroethanol (TCE) to measure total protein. Proteins were subsequently transferred to PVDF transfer membrane (Millipore, Burlington, MA, USA) and blocked in 5% milk buffer. Membranes were incubated with 5 - 8 µg of primary antibody for 4 hrs (HMG I/Y, TDP43) or overnight (ICP0) and 2.4 µg of HRP-conjugated secondary antibody for 2 hrs. Blots were imaged by chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Protein bands were analyzed by densitometry and normalized to total protein in each lane, based on TCE densitometry analysis.

Immunoprecipitation. Neuronal cells were collected in 125 µL non-denaturing lysis buffer (20mM Tris HCl pH8, 1% NP-40, 2mM EDTA) with MG132 (Cbz-Leu-Leu-Leucinal) to inhibit the proteasome, PR619 (2,6-Diaminopyridine-3,5-bis(thiocyanate)) broad-spectrum deubiquitinating enzyme inhibitor to prevent the removal of ubiquitin moieties after collection, and Halt Protease & Phosphatase Inhibitor (Thermo Fisher Scientific). Sample protein concentrations were quantified using Quick Start Bradford Dye Reagent (BioRad) and Bovine Serum Albumin Standard Set (BioRad). Immunoprecipitation was performed by covalently conjugating 5 µg of antibody to 1 mg Invitrogen Dynabeads (Thermo Fisher Scientific) using Dynabeads Antibody Coupling Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. A total of 15 µg DRG total protein was incubated with antibody-coupled Dynabeads for 12 hrs at 4ºC. Immunoprecipitated samples were rinsed with non-denaturing buffer and resuspended in a mixture of 25 µL non-denaturing buffer, 10 µL 4x Laemmli buffer (with 10% BME) (total volume of 35 µL) heated to

95ºC and loaded into a 10-12.5% SDS-PAGE gel for Western blot. Total protein as input protein control (InP), supernatant from antibody-bead conjugate supernatant (Sup), and antibody-bead eluate (IP) were loaded to ensure no antibody leeching and successful immunoprecipitation.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Host and Viral Proteins.

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Data Availability Statement: Mass spectrometry proteomics data were deposited to the ProtomeXchange Consortium via the PRIDE (89) partner repository, with the data set identifier PXD037767 and 10.6019/PXD037767.

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4) INFECTED CELL PROTEIN 0 (ICP0) MODULATES HOST CELL HOMEOSTASIS DURING LATENCY IN PRIMARY ADULT NEURONS

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Abstract

Herpes simplex virus 1 (HSV-1) is a successful viral pathogen known to infect most humans, causing blister-like lesions and cold sores in the orofacial region. The lesions are generally considered harmless, instigating anxiety and a burden of perceived stigmas, but infection is lifelong, and the lesions can recur in response to various stress-inducing stimuli. The variety of factors that contribute to the recurrence of herpetic lesions from a clinical perspective are generally well characterized (UV damage, immune suppression, tissue damage, etc.), but the molecular mechanisms that facilitate latency and reactivation remain unclear. HSV-1 latency, a state of viral quiescence and persistence, is hypothesized to be passive, requiring no viral transcription beyond the latency-associated transcript (LAT), and to be predominantly host-dependent. This hypothesis, however, is a gross oversimplification and understates the complexity of HSV-1, especially within sensory neurons. Here we present the detection and correlation of host and viral proteins during the establishment of latency that illuminate a potential mechanism by which HSV-1 mediates the establishment and maintenance of latency in primary adult sensory neurons.

Introduction

Herpes simplex virus 1 (HSV-1) is a ubiquitous pathogen that infects more than 50% of the human population (1). Infection is often acquired by young children via transmission of oral secretions and remains for the host's life, periodically recurring in response to UV damage, tissue damage, or stress (2). Symptoms are predominantly orofacial lesions, commonly referred to as cold sores, that cause itching, pain, and discomfort for the host (3). HSV-1 is also responsible for approximately 30% of newly diagnosed genital herpes cases. Clinically these lesions are considered a minor inconvenience rather than severe medical conditions warranting attention, but in rare instances, HSV-1 can cause severe symptoms. In the case of frequent reactivation, severe illness, or immune suppression, HSV-1 can cause herpetic keratitis (4) and life-threatening encephalitis (5), conditions that require immediate medical intervention.

On the molecular level, HSV-1 is complex involving a dual host cell replication cycle and establishment of viral latency that permits indefinite persistence within the host. After the internalization of mature virions, HSV-1 will follow a temporal cascade of gene expression in epithelial cells, typically of the oral or genital mucosa, to rapidly produce viral progeny. This initial replication is ultimately at the expense of the infected cells and produces the characteristic herpetic lesions associated with infection. The temporal cascade of gene expression involves three classes of viral genes, Immediate-early (IE), Early (E), and Late (L) genes, that ensure viral success (6). IE genes modulate the host cell environment, establishing the foundation for viral infection. They work to suppress the intracellular antiviral responses (7), alter chromatin dynamics (8), and initiate viral gene expression (9). E genes focus predominantly on viral gene expression and copying the viral genome. L genes are structural and facilitate the encapsulation of newly synthesized viral genomes to complete the formation of mature virions. The mature virions bud out of the host epithelial cell and infect the axon terminals of neurons that innervate the initial infection site, initiating the second phase of infection. The mature virions are transported retrograde to the neuron soma (10), where HSV-1 will establish a viral presence within the sensory ganglia, specifically the trigeminal ganglion (TG) (11) and/or dorsal root ganglion (DRG) (12). Once infection within the ganglionic neurons begins, HSV-1 can undergo a productive infection synonymous with epithelial cell infection or establish latency, a state of viral quiescence for indefinite persistence within the host.

The molecular mechanism that determines if HSV-1 will undergo a lytic or latent infection is the subject of much interest. Although currently unclear, HSV-1 latency is hypothesized to involve primarily host proteins, as viral proteins are reported not to be expressed during viral latency (13). The accepted dogma of HSV-1 latency involves Infected Cell Protein 0 (ICP0) and the Latency Associated Transcript (LAT). ICP0, a virally encoded E3-ubiquitin ligase (14), is one of the first viral proteins to appear during initial infection onset and immediately follows a reactivation stimulus. As an E3 ubiquitin ligase, ICP0's primary function is to catalyze the transfer of ubiquitin moieties from E2 ubiquitin ligases to target proteins marking them for degradation by the ubiquitin proteasomal degradation complex (15). This ability allows ICP0 to

support viral replication through complex protein-protein interactions while modulating host mechanisms that could hinder production of viral progeny. In non-neuronal cells, ICP0 has been shown to support viral replication by decreasing NF-kB signaling, increasing viral gene expression, downregulating inflammatory mechanisms, and ubiquitinating essential proteins such as USP7 (16) and p53 (17). In contrast, the LAT is a non-coding RNA predominantly expressed during latency (18). Its overall size is 8.3kb, but after splicing, it produces 1.5kb and 2.0kb introns that are stable and accumulate within infected cells (19). The purpose of LAT and its predominant expression during latency is unclear, but the expression dichotomy with ICP0 suggests a correlation.

We wanted to identify the neuron-specific targets of ICP0 during reactivation to clarify some of the mechanisms that govern HSV-1. Previously we showed the immunoprecipitation of ubiquitinated proteins using anti-ubiquitin FK2 antibody was sufficient to identify ICP0 targeted protein during productive infection. We again utilize this methodology with wildtype HSV-1 KOS and HSV-1 n212, a non-functional ICP0 truncation mutant, to generate LC-MS/MS datasets of proteins ubiquitinated by ICP0 during reactivation and to observe trends of the identified proteins. We have identified β – catenin as an important host protein through LC-MS/MS data trends and explored its role in establishing latency and reactivating HSV-1 from latency. We suggest that host proteins β – catenin and High Mobility Group Protein I/Y (HMG I/Y), and viral proteins ICP0 and Infected Cell Protein 4 (ICP4) may coordinate the establishment, maintenance, and reactivation of HSV-1 latency in adult sensory neurons.

Methods

Viruses: HSV-1 KOS (wile-type) and HSV-1 n212 (both originally from the laboratory of Priscilla Schaefer) were provided by David Davido at the University of Kansas Department of Molecular Biosciences, Lawrence, Kansas. HSV-1 n212 expresses a truncated ICP0 with a nonsense linker insertion at amino acid 212 on a KOS backbone. HSV-1 KOS was propagated and titrated on Vero76 cells and HSV-1 n212 were propagated and titrated on L7 cells, also provided by David Davido.

Primary Adult Neuronal Cultures: Dorsal Root ganglion (DRG) neurons were resected from 6wk old Swiss Webster mice (Hilltop Laboratories, Scottdale, PA, USA) and enzymatically digested using papain and collagenase/dispase (Worthington Biochemical, Lakewood, NJ, USA), followed by mechanical separation into a single cell suspension. Neurons were counted using a hemocytometer and plated on cell culture plates coated with Matrigel (Corning, Glendale, AZ, USA) at 3,000 – 100,000 neurons per well. DRGs were maintained in Neurobasal A media (Thermo Fisher Scientific, Waltham, MA, USA) with B27 serum supplement, 1% penicillin-streptomycin, Glutamax, and Floxuridine (FUDR), supplemented with neurotrophic factors (Nerve Growth Factor (NGF), Neurturin (NTN), and Glial Cell-Derived Neurotrophic factor (GDNF); obtained from PeproTech, Cranbury, NJ, USA). DRGs were allowed to acclimate for 3-4 days before experimental procedures. All studies were conducted in accordance with the Institutional Animal Care and Use Committee at Virginia Tech (protocol approved 2/8/2019)

Infection and Latency: Maintenance media was removed and DRG cultures were inoculated with 30 multiplicity of infection (moi) of HSV-1 KOS or HSV-1 n212 and incubated for 1 hr. with gentle rocking every 15 minutes. The viral inoculum was subsequently removed and replaced with NeuroComp media (Neurobasal A media with 1% penicillin-streptomycin, and Glutamax, supplemented with Nerve Growth Factor (NGF), Neurturin (NTN), Glial Cell-Derived Neurotrophic factor (GDNF) and 300μM Acyclovir). DRGs were subsequently incubated for seven days to establish latency. After latency establishment, neurobasal A media with Acyclovir was replaced with neurobasal A media with B27 serum supplement, 1% penicillin-streptomycin, Glutamax, and anti – neurotrophic factor antibodies to reactivate HSV-1. Reactivation was allowed to progress for 2 - 8 hrs before collection for downstream molecular-based assays.

Antibodies. Primary antibodies included HSV-1 ICP0 (11060, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Beta Catenin (ab32572, Abcam, Waltham, MA, USA), HMG I/Y (393213, Santa Cruz and ab168260, Abcam), HSV-1 ICP4 (69809, Santa Cruz), and anti-ubiquitin FK2 (BML-PW8810-0500, Enzo Biochem, Farmingdale, NY, USA or Sigma Aldrich, St. Louis, MO, USA). Primary antibodies were

visualized with secondary antibody goat anti-mouse or goat anti-rabbit IgG-HRP (31430 and 31460, Thermo Fisher Scientific).

LC-MS/MS: A total of 100,000 cultured DRG neurons per treatment were infected with HSV-1 KOS or HSV-1 n212, or were uninfected, and treated with Acyclovir to establish latency according to the methods outlined above. After 7 days, the maintenance media was removed and replaced with Neuro A media with B27 serum supplement, 1% penicillin-streptomycin, Glutamax, and anti – neurotrophic factor antibodies. The infection progressed for 2 hrs prior to protein harvesting in 125 µL non-denaturing buffer (20nm Tris HCL pH8, 1%NP40, 2mM EDTA) with MG132, PR619 (2,6-Diaminopyridine-3,5-bis(thiocyanate)) broad-spectrum deubiquitinating enzyme inhibitor to prevent the removed of ubiquitin moieties after sample collection, and Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Samples were incubated for 12 hrs with FK2 anti-ubiquitin antibody covalently conjugated to Invitrogen Dynabeads (Thermo Fisher Scientific), according to the manufacturer's protocol. Immunoprecipitated samples were rinsed 3X with non-denaturing buffer and resuspended in non-denaturing lysis buffer with MG132, PR619, and Halt Protease & Phosphatase Inhibitor. Samples were shipped overnight to MSBioworks (Ann Arbor, MI, USA) for LC-MS/MS. Each samples was eluted in 70 µL 1.5X NuPage LDS Sample buffer (Thermo Fisher Scientific) and boiled at 100 °C for 15 min, followed by clarification by centrifugation. Half of each sample was separated by electrophoresis using a 10% Bis-Tris NuPage mini-gel (Invitrogen)with an MES buffer system. A 2 cm gel space was excised into ten equally sized bands, washed with 25mM ammonium bicarbonate and acetonitrile, reduced with 10 mM dithiothreitol at 60 °C, alkylated with 50 mM iodoacetamide at room temperature, and digested with trypsin at 37 °C for 4 hrs, quenched with formic acid, and finally analyzed by nano LC-MS/MS with Waters M-Class LC system interfaced with Exploris 480 mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto a trapping column and eluted over a 75µm analytical column at 350nL/min. The column was heated to 55°C using a column heater. The mass spectrometer was in data-dependent mode, with the Orbitrap operating at 60,000 FWHM for MS and 15,000 FWHW for MS/MS. Each sample was run for 5 hrs.

Data processing. Raw data files from MSBioworks were downloaded by Virginia Tech Mass Spectrometry Incubator and reprocessed using Proteome Discoverer v. 2.5 (Thermo Fisher Scientific). Data files corresponding to all 10 bands of the same sample type were analyzed together. Searches using both Mascot (Matrix Science, Mount Prospect, IL, USA) and SequestHT (Thermo Fisher Scientific) were performed against the herpes simplex virus 1 reference proteome downloaded from UniProt, the mouse reference proteome downloaded from UniProt, and a database containing a list of common contaminant proteins provided with the Proteome Discoverer software, and the results merged using Proteome Discoverer. Search parameters included trypsin specificity with the possibility of two missed cleavages, precursor mass tolerance of ±10 ppm, fragment mass tolerance of ±0.1 Da, fixed modification of carbamidomethylation of Cys residues, and the following variable modifications: oxidation of Met, deamidation of Asn and Gln, formation of pyro-Glu when Gln was at the C-terminus of a peptide, acetylation of the protein N-terminus, and GlyGly characteristic of ubiquitin after trypsin cleavage at Lys and the protein N-terminus. The IMP-ptmRS node within Proteome Discoverer was utilized to validate the position and level of confidence for the GlyGly modification. The two repetitions were analyzed separately, and proteins identified in both replicates were included in the final comparative analyses.

Statistical Analysis. LC-MS/MS proteins were identified using a minimum of 2 unique peptide sequences and a 1% false discovery rate (FDR). Common contaminants and identified proteins with a MASCOT score < 60 ($p < 0.00001$ compared to average MASCOT score of each dataset) and an Exp-q value ≥ 0.05 were manually removed, and the resulting list was collated for comparison. The relative abundance for each protein was determined based on the peptide spectrum match (PSM) normalized to total spectra for each run provided by MSBioworks. Normalized PSM from each replicate were averaged within the respective treatment and compared to the other treatments. Proteins with a minimum of 1.25-fold increase in HSV-1 KOS infected neurons, compared to both HSV-1 n212 and uninfected controls, were selected for downstream biological justification. A literature search was performed on the selected proteins for

previously reported functions to identify specific proteins with potential relevance to HSV-1 infection in neurons.

Immunoprecipitation: Neuronal cells were collected in 125 µL non-denaturing lysis buffer (20mM Tris HCl pH8, 1% NP-40, 2mM EDTA) with MG132 (Cbz-Leu-Leu-Leucinal) to inhibit the proteasome, PR619 (2,6-Diaminopyridine-3,5-bis(thiocyanate)) broad-spectrum deubiquitinating enzyme inhibitor to prevent the removal of ubiquitin moieties after collection, and Halt Protease & Phosphatase Inhibitor (Thermo Fisher Scientific). Sample protein concentrations were determined using Quick Start Bradford Assay Dye Reagent (BIO-RAD, Philadelphia, PA, USA) and Bovine Serum Albumin Standard Set (BIO-RAD).). Immunoprecipitation was performed by covalently conjugating 5 µg of antibody to 1 mg Invitrogen Dynabeads (Thermo Fisher Scientific) using Dynabeads Antibody Coupling Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. A total of 15ug DRG total protein was incubated with antibody-bead slurry for 12 at 4°C. Immunoprecipitate proteins were rinsed 3x with a non-denaturing buffer and resuspended in a mixture of 25 µL non-denaturing buffer, 10 µL 4x Laemmli buffer (with 10% BME) (total volume of 35 µL) heated to 95ºC and loaded into a 10-12.5% SDS-PAGE gel for Western blot. Total protein as input protein control (InP), supernatant from antibody-bead conjugate supernatant (Sup), and antibody-bead eluate (IP) were loaded to ensure no antibody leeching and successful immunoprecipitation.

Immunoblots: DRGs were collected in 100 µL radioimmunoprecipitation assay (RIPA) buffer. Protein concentration was determined using Quick Start Bradford Assay Dye Reagent and Bovine Serum Albumin Standard Set (BIO-RAD, Philadelphia, PA, USA). A total of 15 µL was loaded into an 8 % sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel with 1% 2,2,2-Trichloroethanol (TCE) to measure total protein. Protein was transferred to PVDF transfer membrane (Millipore, Burlington, MA, USA) and blocked in 5% milk buffer. Membranes were incubated with primary antibody for 12 – 16 hrs before being washed 4x with TBST and re-blocked with 5 % milk buffer with secondary antibody for 1 hr. Blots were imaged by chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher

Scientific). Images were acquired on a ChemiDoc Imaging System (BIO-RAD). Protein bands were analyzed by densitometry and normalized to total protein in each lane, based on TCE densitometry analysis.

Digital Droplet Polymerase Chain Reaction (ddPCR). HSV-1 viral RNA was extracted from adult sensory neurons using TRI reagent (Thermo Fisher) and phenol-chloroform precipitation/extraction method. RNA was reverse transcribed using iScript (BIO-RAD) to produce cDNA. ICP0 and ICP4 transcripts were quantified using HSV-1 specific primers and EvaGreen QX200 Master Mix (BIO-RAD), normalized to 18S rRNA. Viral mRNA transcripts are reported as transcript copies per neuron.

Results

ICP0 is expressed at low levels following reactivation stimuli in primary adult sensory neurons

Previous reports about the necessity of ICP0 for reactivation have provided unclear and often conflicting results. It is generally accepted that ICP0 is required for efficient viral replication as ICP0-null viruses exhibit discoordination in viral gene expression and struggle to reactivate (20). This depiction, however, does not identify ICP0's specific role in reactivation, the time of initial expression, or the proteins targeted by ICP0 in support of viral reactivation. To determine the ideal time to identify ICP0 ubiquitination targets during reactivation of HSV-1 from latency, we first had to establish a clear and concise protein profile for ICP0 following a reactivation stimulus, using neurotrophic factor deprivation. Primary adult sensory dorsal root ganglion (DRG) neurons were inoculated with wildtype HSV-1 KOS (30 moi), treated with acyclovir (ACV), and incubated at 37°C without disturbance for seven days to establish latency. After seven days, DRG media with ACV was removed and replaced with media devoid of ACV and neurotrophic factors (NTFs), but containing anti-NTF antibodies to block the effect of residual NTFs. Deprivation of neurotrophic factors by this method induces viral reactivation and production of viral progeny (21). Independent samples were incrementally collected over 32 hrs at the following times: Time 0 (T0, immediately-post reactivation), 30 minutes, every hr for the first six hours, every 2 hrs for the next six hours, and every 4 hrs for the next 10 hrs (Fig. 4.1). ICP0 was first observed at T0, immediately upon the

removal of ACV media and in control neurons that were not deprived of neurotrophic factors, indicating that ICP0 protein is present in latently infected sensory DRG neurons. At 2 hpi, ICP0 levels increased slightly above what was observed at the T0 time point and decreased until 5 hpi. Slightly higher levels of ICP0 were observed at 6 hpi and subsequently decreased until 10 hpi. The fluctuations in expression continued until 32 hpi, the last time point collected. These data support basal yet cyclic expression of ICP0 following a reactivation stimulus. For ICP0 to participate in immediate viral reactivation, it would have to present at the time of the reactivating stimulus, and the effects exerted would have to be almost instantaneous. The delay associated with transcription and translation could potentially slow the progression of viral gene expression when gene transcription and promoter activation are critical. Therefore, we selected 2 hrs post reactivation to identify proteins immediately involved in viral reactivation.

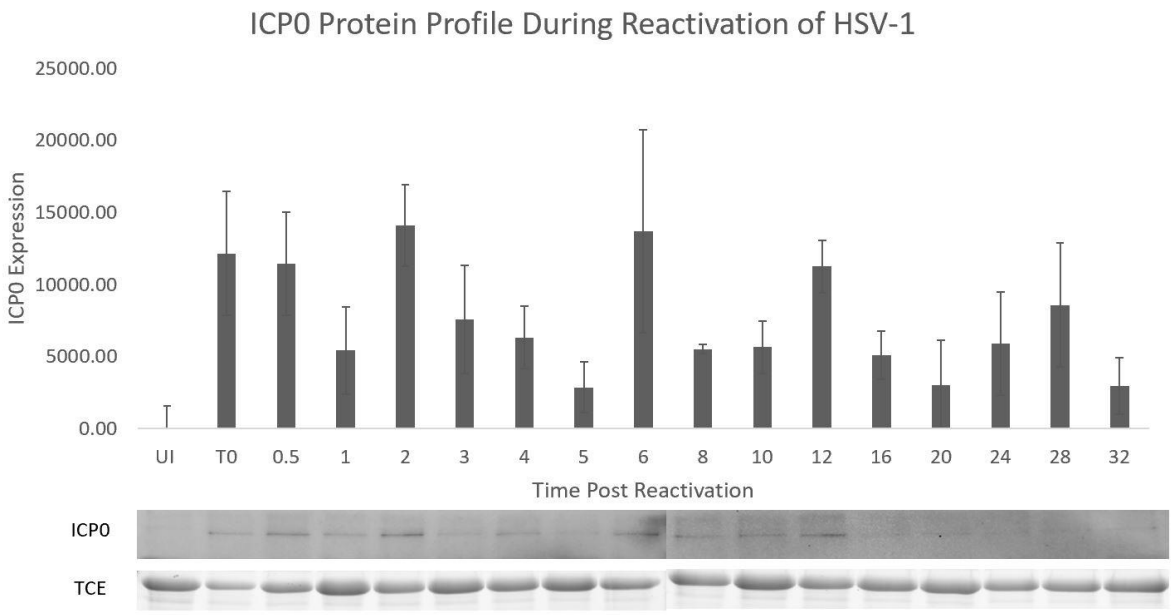

Figure 4.1. 32 hr protein profile of ICP0.

Protein profile of ICP0 32 hrs post neurotrophic factor deprivation. Bars represent the average densitometry reading of immunoblot images normalized to TCE, representing total protein. Standard error of the mean (SEM) is used to represent variability.

Mass spectrometry analysis of neuronal proteins ubiquitinated by ICP0 ubiquitinated during reactivation

Previously we showed the immunoprecipitation using FK2 antibodies conjugated to Dynabeads to selectively isolate ubiquitinated proteins was sufficient to identify proteins relevant to HSV-1 infection in primary adult sensory neurons. We again utilized this method, expanding our approach to observe trends within the LC-MS/MS data to better understand the neuronal changes that occur during latency and the reactivation of HSV-1. Adult dorsal root ganglion neurons were resected from six-week-old Swiss Webster mice, enzymatically digested into a single cell suspension, and plated in 12-well cell culture plates. After a 72 hr acclimatization period, DRGs were infected with HSV-1 KOS, a wildtype virus, or HSV-1 n212, which expresses a non-functional ICP0 fragment due to a truncation at the 212^{th} amino acid (22), for 1 hr with gentle rocking every 15 min. After the 1 hr adsorption period, the viral inoculum was removed and replaced with Neurobasal A media with glial-derived neurotrophic factor (GDNF), neurturin (NTN), nerve growth factor (NGF), and acyclovir (ACV) to establish latency. Neurons were incubated at 37°C, undisturbed, for 7 days to establish latency (Fig. 4.2).

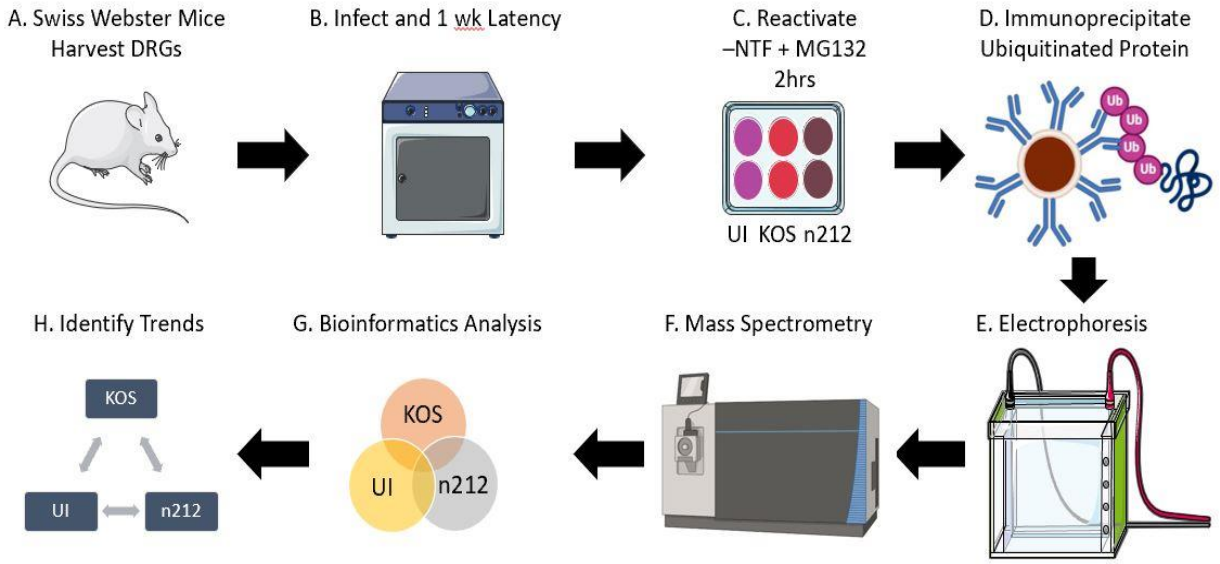

Figure 4.2. Schematic representation of the sample preparation for LC-MS/MS.

(A) Primary adult dorsal root ganglion (DRG) neurons were resected from 6wk old Swiss webster mice and plated at a minimum of 50,000 neurons per well. (B) DRG neurons were treated with MG132 and infected with HSV-1 KOS or HSV-1 n212 for 8hrs. (C) Equal amounts of total DRG protein was loaded onto magnetic epoxy Dynabeads with covalently attached FK2 antibodies to enrich for ubiquitinated proteins. (D) Protein beads were eluted in 1.5X LDS buffer and boiled at 95°C for 15 minutes. Protein eluent was run on a 10% SDS-PAGE gel, run 2cm, and excised into 10 equal size bands. Each band was excised and processed by in-gel with trypsin and analyzed by nano LC-MS/MS. (E) Data were searched using Mascot (Matrix Science) Mouse and Human Herpes databases with 1% protein and peptide FDR requiring at least 2 unique peptides per protein. (F) Datasets from each treatment were collated to identify proteins increasingly abundant in HSV-1 KOS infected neurons compared to UI and HSV-1 n212 infected neurons.

After the 7-day latency period, neuronal culture media used to establish and maintain latency was removed and replaced with Neurobasal A media supplemented with anti-neurotrophic factor antibodies and MG132, a cell-permeable proteasome inhibitor, but devoid of ACV and NTFs, to initiate viral reactivation. Reactivation was allowed to progress for 2 hrs before neurons were collected in a non-denaturing buffer supplemented with MG132 (a proteasome inhibitor), PR619 (a de-ubiquitinase inhibitor), and HALT protease phosphatase inhibitor cocktail. Equal amounts of protein were incubated overnight with Dynabeads conjugated to FK2, an anti-ubiquitin chain antibody (23), to immunoprecipitate proteins

conjugated to K29, K48, and K63 ubiquitin chains, and mono-ubiquitinated proteins. Immunoprecipitated proteins were subjected to LC-MS/MS to generate protein datasets for each of the three conditions (Uninfected, HSV-1 KOS, HSV-1 n212). Proteins were identified by 2 unique peptides and screened based on a MASCOT score 60 ≤ and an exp-q value ≤ 0.05 for an acceptable confidence interval. With these screening criteria, we identified more than 1000 proteins in each sample. Proteins were then classified based on functional characteristics using Reactome and mapped to pathways such as gene expression, protein metabolism, response to stimuli, and signal transduction (Fig. 4.3A). A total of 2,045 proteins remained with 1,643 UI proteins (422 unique), 1,257 n212 proteins (111 unique), and 1,414 KOS protein (163 unique) (Fig. 4.3B).

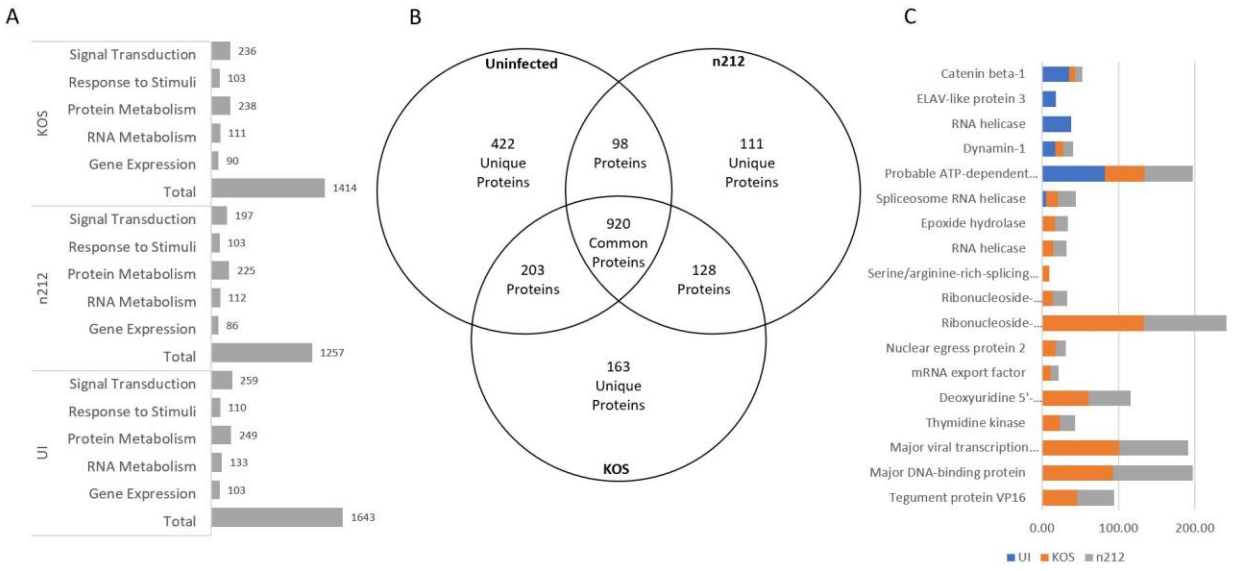

Figure 4.3. Analysis of protein identified by LC-MS/MS.

(A) Classification of uninfected (UI), HSV-1 n212 (n212), and HSV-1 KOS (KOS) neurons. (B) Number of proteins unique and common to each treatment after files were culled based on a MASCOT score ≥60 and experimental-q value ≤ 0.05. (C) Peptide spectrum match (PSM) protein trends relevant to HSV-1 reactivation. Horizontal bars are representative of PSMs

To identify proteins relevant to the early events during HSV-1 reactivation, we relied on the biological trends within the LC-MS/MS dataset (Fig. 4.3C) and published literature for justification. Unlike the initial onset of productive infection, where viral proteins must hijack a naive cell, reactivation is initiated from a viral genome already established within the host cell nucleus. Viral proteins that initially subjugate the host cells are not present. We, however, identified ICP0 immediately post-reactivation (T0) at the protein level suggesting lytic associated protein are present in low amount prior to a reactivation stimulus. As an E3-ubiquitin ligase, ICP0 catalyzes the addition of ubiquitin moieties to target proteins marking them for degradation (24). Ubiquitination of target proteins can be observed in LC-MS/MS data by a change in the overall protein abundance exemplified in total peptide sequence matches (PSM) for the target protein or the presence of glycine-glycine (gly-gly) remnants on lysine as ubiquitin is cleaved off by trypsin (25). We considered these phenomena in our mass spec data and additionally factored in the biological condition of latent HSV-1 before reactivation and treatment with MG132 to stop the ubiquitin-proteasome. Proteins reduced in infected neurons (KOS) compared to uninfected (UI) neurons could result from viral-mediated suppression of host proteins during latency. Proteins increasingly present in infected neurons could be upregulated by the virus or degraded by host mechanisms targeted against viral infection. Proteins increasingly present in HSV-1 KOS infected neurons, when compared to n212 infected neurons and controls, could be proteins ICP0, specifically, ubiquitinates immediately post-reactivation that could not be degraded due to the presence of MG132 inhibiting the proteasome. Proteins increasingly present in HSV-1 n212 infected neurons and controls, compared to HSV-1 KOS infected neurons, are those proteins potentially suppressed through mechanisms influenced by ICP0 during latency and are overall low in KOS infected neurons.

In our LC-MS/MS data, viral proteins were detected in similar amounts in HSV-1 KOS and HSV-1 n212 infected neurons suggesting host mediated degradation of viral proteins during reactivation, most likely the result of host cell anti-viral responses. Host proteins such as Dynamin-1 and ATP-dependent RNA helicase were comparably present in all treatments indicating continuous turnover within the cells irrespective of

viral infection. Proteins such as RNA helicase, ELAV-like protein 3, and β – catenin exhibited reduced presence in KOS and n212 infected neurons, when compared to controls, suggesting potential suppression during latency before reactivation. β – catenin, a protein previously studied in our lab, is a master regulator of the Wnt-ß-catenin signaling cascade and maintains a constant presence within cells to maintain homeostasis. In homeostatic cells, β - catenin is constantly transcribed and degraded by the β - catenin degradation complex consisting of multiple proteins such as Axin, Adenomatous polyposis coli (APC), Ser/Thr Kinase GSK-3, and E3-ubiquitin ligase B-TrCP (26, 27). During periods of stress, the degradation of β - catenin is suspended, and β - catenin localizes to the nucleus inducing the transcription of cell survival genes (28). β – catenin is reduced overall during latency and reactivation of HSV in primary adult sensory neurons (*Unpublished data*). The reduction observed through our current LC-MS/MS data suggests that the decrease in β – catenin is mediated by ICP0. As a result, we selected β – catenin to further investigate its relevance to HSV-1 latency and reactivation in primary adult sensory neurons.

β – catenin is reduced in HSV-1-KOS infected neurons when compared to HSV-1-n212-infected and uninfected dorsal root ganglion neurons

To validate the reduction of β - catenin observed in our LC-MS/MS data, we immunoprecipitated protein from infected neurons using anti-ubiquitin FK2 antibodies and immunoblotted for β – catenin. Primary adult sensory neurons were infected with HSV-1 n212 or HSV-1 KOS, treated with ACV, and allowed to establish latency for 7 days. Latent HSV-1 was reactivated by NTF deprivation and allowed to progress for 2 hrs before being collected in a non-denaturing buffer supplemented with MG132, PR619, and HALT protease phosphatase inhibitor cocktail. Equal amounts of total protein were loaded on Dynabeads conjugated to anti-ubiquitin FK2 antibodies and incubated overnight to immunoprecipitate proteins bound to K29, K48, K63, and mono-ubiquitin chains. Total protein (InP) and IP eluate were loaded into an 8% SDS page to assess the ubiquitination status of β - catenin 2 hrs post reactivation of HSV-1 (Fig. 4.4). We observed comparable levels of β – catenin ubiquitination in uninfected neurons (UI), neurons infected with HSV-1 n212, and those infected with HSV-1 KOS but total β - catenin was decreased in KOS-infected

neurons. This result implicates ICP0 in the reduction of β - catenin during HSV-1 latency prior to the reactivation stimulus.

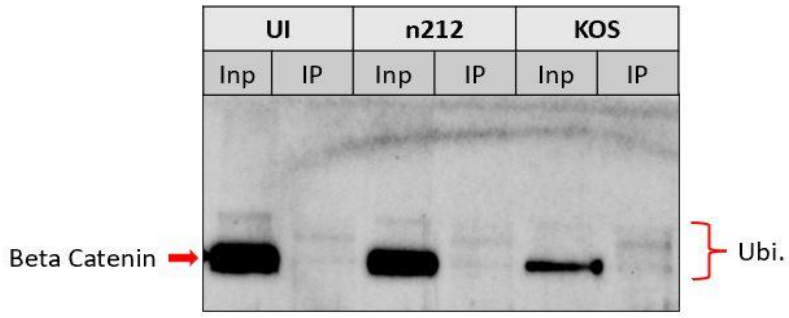

Figure 4.4. Ubiquitination status of β - catenin 2 hrs post neurotrophic factor deprivation.

Immunoprecipitation was conducted using Dynabeads conjugated to anti-ubiquitin FK2 antibodies targeting K29, K48, K63, and monoubiquitin chains. Blot was subsequently probed for β - catenin. Total protein (InP) and Immunoprecipitation eluate (IP) for uninfected (UI), HSV-1 n212 (n212), and HSV-1 KOS (KOS) are displayed.

β – catenin is reduced during HSV-1 latency and inversely correlates with ICP0

To investigate the reduction of β - catenin observed in our immunoprecipitation, we decided to analyze ICP0 and β - catenin protein expression over the 7 days necessary to establish latency. Primary adult sensory DRG neurons were infected with KOS or n212 and treated with ACV. Uninfected neurons were maintained in tandem for comparison. Independent samples were collected daily for 7 days to produce the protein profile of ICP0 and β – catenin expression (Fig. 4.5). ICP0 protein was observed at its highest on day 1 post-infection for KOS-infected neurons and steadily decreased over time. Although neurons were treated with ACV and allowed to establish latency, ICP0 protein was detected until 6 dpi (Fig. 4.5A). ICP0 mRNA was detected at its highest on Day 1 and, although it decreased, was still detected on 7 dpi (Fig. 4.5B). ICP0

protein was not detected in n212 infected neurons, as ICP0 is truncated and translated as a fragment that is rapidly degraded. For β – catenin, we observed a steady increase of protein in uninfected neurons as the primary adult neurons become more established and acclimated in culture. This steady increase in β - catenin was also observed in HSV-1 n212 infected neurons treated with ACV for 7 days as β – catenin reached levels comparable to uninfected controls. HSV-1 KOS infected neurons displayed an increase in β - catenin equivalent to UI and n212 until Day 3. After day 3, β - catenin in KOS-infected neurons plateaued and did not increase for the remainder of the 7-day latency establishment period. To confirm that these expression profiles occurred during periods of latency establishment, we also assessed LAT expression for 7 days (Fig. 4.5C). LAT transcripts were expressed at lower levels than ICP0 transcripts for the first 3 days but increased above ICP0 by day 4. Overall, RNA transcripts of ICP0 and LAT correlated over the course of the 7-day establishment of latency (Fig. 4.5B and C). These data suggests that β - catenin is not overtly ubiquitinated post-reactivation but decreases overall during the establishment latency potentially mediated by ICP0.

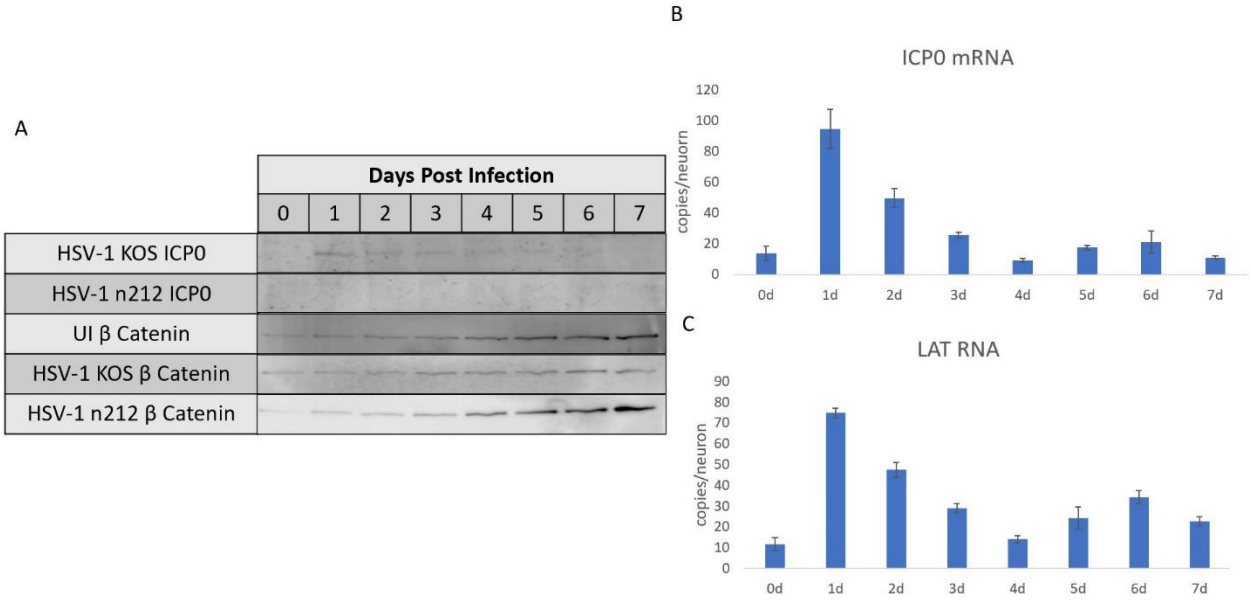

Figure 4.5. β - catenin and ICP0 during the establishment of latency.

(A) Immunoblots depicting the comparative protein profile of β - catenin and ICP0 during the establishment of viral latency for Uninfected (UI), HSV-1 KOS infected, and HSV-1 n212 infection primary adult sensory neurons. All neurons are treated with ACV. (B) Digital droplet PCR readings of ICP0 (B) and LAT (C) transcripts during the establishment of latency. Readings are displayed as copies per neuron.

HMG I/Y and ICP4 protein profiles positively correlate with β - catenin during the establishment of latency

ICP0, as an E3-ubiquitin ligase, catalyzes the transfer of ubiquitin to the target substrate but does not possess the ability to bind DNA or RNA. ICP0 can, however, form complex protein-protein interactions to influence gene expression and protein functionality (29, 30). The inverse relationship of β - catenin and ICP0 during the establishment of HSV latency indicates a more profound correlation that may impact the ability of HSV-1 to establish latency in adult sensory neurons. We searched through published literature to identify how ICP0 and β - catenin could collectively influence HSV-1 pathogenesis for establishing latency. β - catenin is known to be upregulated and stabilized during periods of cell stress (27). The increased stability of β - catenin allows it to localize to the nucleus and initiate transcription of host genes that aid in the mediation

of stress, in part, through interactions with various transcription factors such as TCF/lef and HMG proteins (31). Of the multiple HMG proteins, β - catenin has been reported to induce upregulation of HMG I/Y in some cancers, and together these proteins facilitate growth and development (32).

HMG I/Y has previously been linked to HSV-1 pathogenesis. As a master regulator of chromatin and architectural transcription factor, HMG I/Y was shown to bind three regions of the HSV-1 genome (TAATGARAT sequences, TATA Box, and ICP4 binding sites) and modulate the expression of the Latency Associated Transcript promoter 2 (LAP-2) (33). The binding of HMG I/Y to HSV-1 sequences is mediated by immediate early viral protein ICP4. ICP4 has nucleic acid binding capabilities and can be an activator of gene transcription or a repressor, depending on its cofactors (34, 35). ICP0 and ICP4 are reported to antagonize each other (36); ICP0 functions support productive infection by upregulating viral gene transcription, and ICP4 functions support a quiescent infection, suppressing viral gene expression (37-39). We hypothesized that HMG I/Y and ICP4 could be the link to clarify the inverse relationship observed between β - catenin and ICP0. To test this hypothesis, we infected adult sensory DRG neurons with HSV-1 KOS or n212 and treated them with ACV to establish latency. Over 7 Days, independent samples were collected and immunoblotted to establish the protein profile of ICP4 and HMG I/Y during the establishment of latency (Fig. 4.6A). In UI neurons, HMG I/Y expression was low and marginally fluctuated over 7 days. A low, nearly undetectable level of HMG I/Y is common among most differentiated cell types, as high levels of HMG I/Y expression occur during embryonic development and cancer. In KOS-infected neurons, HMG I/Y expression was low compared to UI neurons until day 3, when HMG I/Y started to increase, eventually reaching levels higher than UI neurons. In contrast, in HSV-1 n212 infected neurons, HMG I/Y protein expression increased, starting at day 3, with bold bands not previously observed for HMG I/Y in our primary adult sensory neurons.

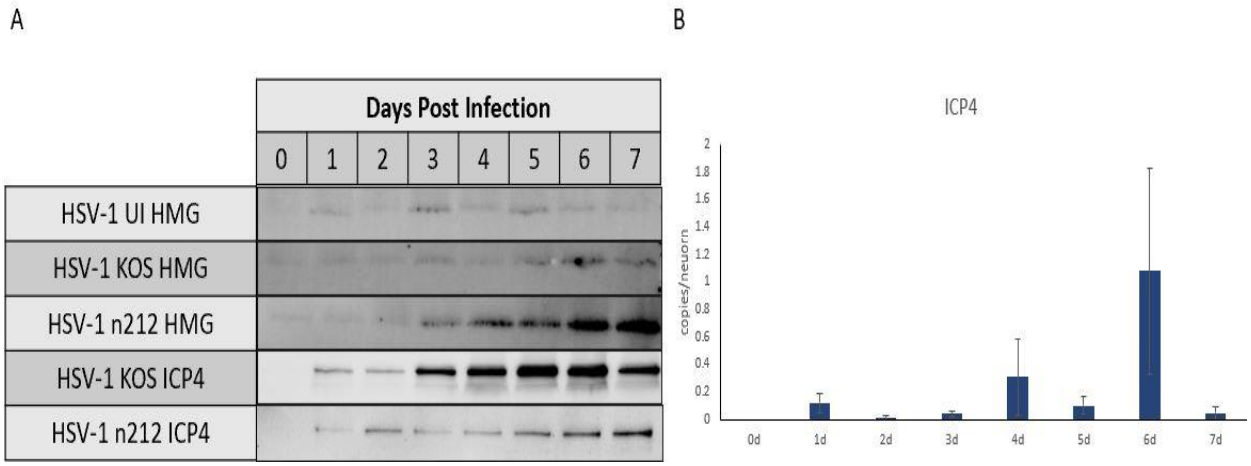

Figure 4.6. HMG I/Y and ICP4 during the establishment of latency.

(A) Immunoblots depicting the comparative protein profile of HMG I/Y and ICP4 during the establishment of viral latency for Uninfected (UI), HSV-1 KOS infected, and HSV-1 n212 infected primary adult sensory DRG neurons. All neurons are treated with ACV. (B) Digital droplet PCR readings of the mRNA transcripts for ICP4 during the establishment of latency. Expression is displayed as transcript copies per neuron.

In addition to the stark increases in HMG I/Y, over the 7 day establishment of latency ICP4 expression substantially increased in KOS-infected neurons. ICP4 first appeared at 1 dpi. On day 3, ICP4 protein expression increased, a trend that continued until day 7 (Fig. 4.6A), inversely correlating with the previous observation for ICP0 (Fig. 4.5A). ICP4 levels increased in HSV-1 n212 infected neurons but did not reach the levels observed for KOS-infected neurons. To further explain the sharp increases in ICP4 for KOS-infected neurons, we utilized digital droplet PCR (ddPCR) to assess the mRNA transcripts during the 7-day latency period (Fig. 4.6B). The mRNA transcripts for ICP4 remained relatively low (Fig. 4.6B) compared to what was observed for ICP0 (Fig. 4.5B), indicating that ICP4 translation is very efficient, and the protein potentially persists within the infected cells. Overall, HMG I/Y and ICP4 show a positive correlation with β - catenin and an inverse relationship with ICP0 during the establishment of viral latency in adult sensory neurons.

Conclusion and future directions

The key to HSV-1 success as a viral pathogen is its ability to establish latency in adult primary neurons. Latency is depicted as a passive process primarily dependent on host proteins, as viral proteins are hypothesized not to be readily expressed (13). Despite the commonality of infection and intense research, the mechanism in which latency is established, maintained and disrupted to cause reactivation is currently unclear. Through the assessment of trends in our LC-MS/MS data, we observed a decrease in β – catenin for infected neurons. Upon further evaluation of β – catenin and its ubiquitination status, we showed that β – catenin is not increasingly ubiquitinated but this protein is suppressed during latency in the presence of functional ICP0. The suppression of host proteins by ICP4 has been previously reported during viral infection (35), but the effects of this concerning the viral establishment of latency have not been explored.

While further exploring the repression of β – catenin during the establishment of latency in the presence of ICP0, we observed an inverse relationship between host and viral proteins, which has never been shown during the establishment of latency. However, we can detect ICP0 protein and mRNA presence for 7 days post-infection after treatment with ACV. This expression of ICP0 was also observed in the presence of LAT transcripts, a marker of HSV-1 latency. The inverse relationship prompted us to search for a link between β – catenin and ICP0. To our knowledge, a direct interaction between ICP0 and β – catenin has not been reported, and our immunoprecipitation experiments did not indicate signs of increased ubiquitination for HSV-1 KOS infected neurons. compared to HSV-1 infected neurons and controls.

Through research conducted in our lab with HMG I/Y and β – catenin and primary literature, we were able to show a dynamic relationship involving two viral and two host proteins. ICP0 protein expression steadily decreased during the establishment of latency, but β – catenin, HMG I/Y, and ICP4 showed an inverse relationship, with increased protein expression as latency was further established. This relationship indicates a distinct correlation, and a complex biological mechanism that could explain the latent-lytic switch of HSV-1. Using the data presented in these previous experiments, we speculate on a signaling cascade that could explain how HSV-1 is able to establish latency in primary adult neurons (Fig. 4.7). We

hypothesize the β – catenin induces upregulation of HMG I/Y in neurons infected with HSV-1 to resist the stress of viral infection and promote survival. The increase in HMG I/Y serves a dual purpose benefiting the neuron and HSV-1. Neurons that express high levels of HMG I/Y during stress can transcribe cell survival genes and increase the chance of survival. Neuron survival is essential to HSV-1 latency; therefore, with the increased presence of HMG I/Y, immediate early protein ICP4 functions as a repressor reducing the expression of viral and some host genes, i.e. β - catenin, promoting viral latency. ICP0 can influence the system by promoting productive infection by potentially ubiquitinating HMG I/Y. If ICP4 cannot associate with appropriate amounts of HMG I/Y to facilitate latency, it will function as an activator joining ICP0 in promoting productive infection. If ICP4 can interact with appropriate amounts of HMG I/Y, it will suppress viral transcription and establish latency. ICP4-mediated suppression has been reported on various host and viral promoters consisting of TATA Boxes, TAATGARAT sequences, and other ICP4 binding sites within the HSV genome (33).

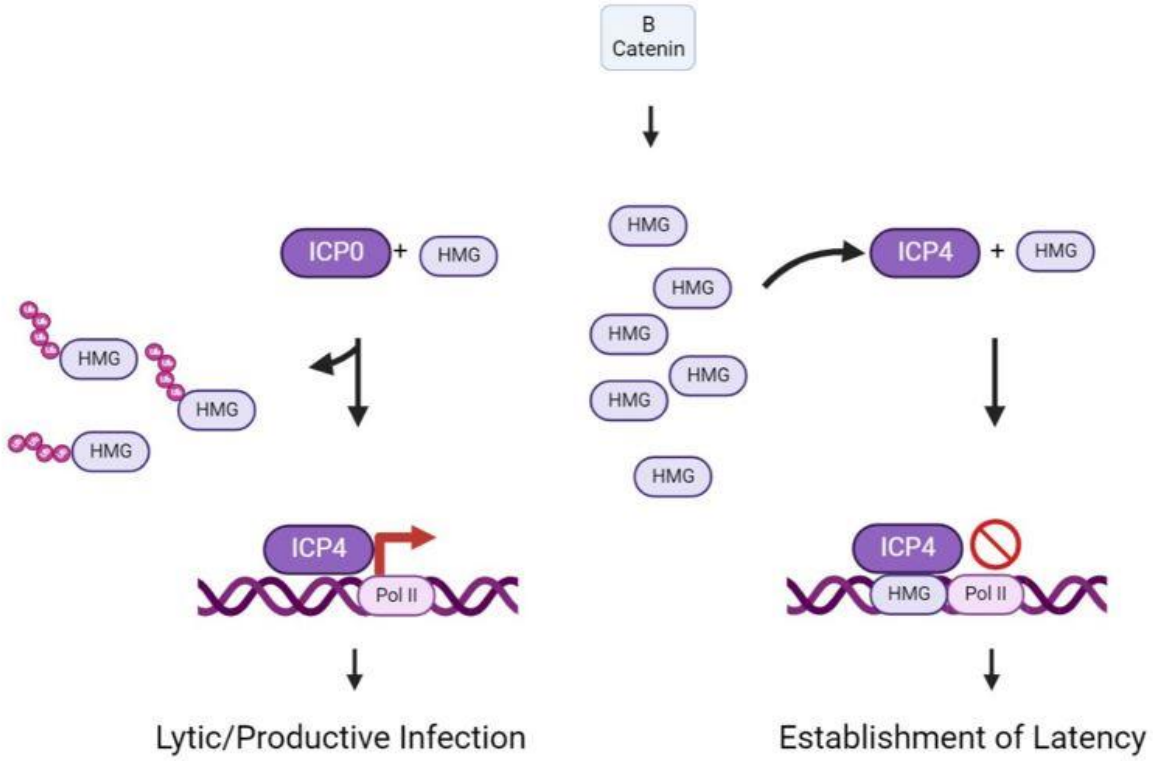

Figure 4.7. Hypothesized molecular mechanisms for the establishment of latency.

During cellular stress, the rapid turnover of β catenin ceases. The now stabilized β catenin can localize to the nucleus and induce cell survival genes such as HMG I/Y. The upregulation of HMG I/Y within the cells indicates cell growth/development and resistance to stress. Viral proteins utilize the presence of beta-catenin, and mostly HMG I/Y, as a determining factor to proceed to productive infection or establish latency. ICP4 is a crucial protein that can function as an activator or repressor, depending on its cofactors. ICP4, in combination with HMG I/Y, serves as a viral repressor and will repress viral transcription in support of latency. ICP4 without HMG I/Y serves as a viral activator and will increase viral transcription in support of a productive infection. ICP0 can influence the system in support of productive infection by ubiquitinating HMG I/Y to encourage ICP4 to function as an activator supporting lytic infection. If ICP0 can ubiquitinate enough, HMG I/Y HSV-1 will go lytic; if ICP4 associates with more HMG I/Y, HSV-1 will go latent. The determining factor of HSV-1 latency is the amount of HMG I/Y and its ability to resist stress.

Latency is thought to be a passive process during which lytic-associated proteins are not produced, and the only readily detectable transcript is the Latency Associated Transcript (13), which is used as an indicator of viral latency. We were able to show that ICP0 and ICP4 are detectable during the establishment of

latency, as both RNA transcripts and protein, and that they exhibit an inverse relationship. This inverse correlation presents an alternative view of HSV-1 latency, suggesting that latency is an active process in which HSV-1 represses its own gene expression by utilizing host proteins influenced by neuronal stress. In opposition to the accepted lytic-latent dogma of HSV-1, the period following infection of HSV-1 in adult sensory neurons should be further investigated to illuminate further the mechanisms that govern the establishment of latency.

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5) CONCLUSION

The research presented in this dissertation was the first investigation of proteins ubiquitinated by ICP0 during acute and reactivated infection of HSV-1 in primary sensory neurons, where HSV establishes latency throughout the host's life. Although challenging in many aspects, the multiple milestones and failures collectively provided insight into the neuron-specific HSV-1 viral mechanisms and reinforced that there is still much to be learned about HSV-1, especially in neurons.

Key findings

Study 1: Herpes simplex virus (HSV-1) infected cell protein 0 (ICP0) targets of ubiquitination during productive infection in primary adult sensory neurons

This study provided insight into the complex host-pathogen interactions during acute HSV-1 infection in adult sensory neurons. ICP0, HSV-1 E3-ubiquitin ligase, is an immediate-early protein that modulates the host cell environment to benefit viral infection. This study revealed that the ICP0 protein profile differs in adult sensory neurons compared to non-neuronal cells. In sensory neurons, ICP0 exhibits a biphasic protein profile over the first 24hrs of acute infection in neurons, reaching $1/5^{th}$ of the levels observed in non-neuronal cells. This biphasic pattern is unique to neurons and supports that ICP0 has various functions in neurons and different ubiquitin targets compared to non-neuronal cells.

To study ICP0's ubiquitination targets, we immunoprecipitated ubiquitinated proteins from neurons infected with HSV-1 KOS and HSV-1 n212. We were able to determine that ICP0's ubiquitination ability in sensory neurons is highly dynamic, targeting host and viral proteins that are time-dependent, emphasizing the exquisite control exerted by HSV-1 upon viral infection. Based on LC-MS/MS analyses of proteins ubiquitinated by ICP0 during acute infection, we selected Transactive Response DNA binding protein 34 (TDP43) and High mobility group protein I/Y (HMG I/Y) for further study. TDP43 and HMG I/Y proteins exhibited patterns characteristic of ubiquitination at 8 hrs and 10 hrs, respectively, in the presence of functional ICP0. TDP43 increased when ICP0 was non-functional and decreased below uninfected levels

in the presence of functional ICP0. HMG I/Y increased with functional ICP0 and was increasingly ubiquitinated and degraded sharply at 10 hpi. The changes observed for TDP43 and HMG I/Y collectively suggest viral-mediated repression and time-dependent degradation of host proteins during HSV-1 infection of sensory neurons.

This study provided insight into the complex host-pathogen interactions that ICP0 can exert in support of acute HSV-1 infection in adult sensory neurons. In addition to upregulating the expression of viral proteins, ICP0 can degrade viral proteins in seemingly counterproductive means of control. The host-pathogen interactions that occur during HSV-1 infection of sensory neurons are host anti-viral, viral anti-host, and viral anti-viral, suggesting that HSV-1 can use ICP0 to self-regulate, promoting dynamic regulatory mechanisms beyond the sole influence of host proteins.

Future studies

HMG I/Y is a diverse protein with multiple functions and potential binding partners. Our studies supported HMG I/Y as a ubiquitination target of ICP0 during HSV-1 infection of primary adult sensory neurons. Still, we did not specifically explore additional post-translational modifications or specific types of ubiquitin chains bound to HMG. Further studies should focus on HMG I/Y and its many post-translational modifications, as these modifications can alter its relationship with its binding partners, such as ICP0 and ICP4. HMG I/Y is a dynamic protein essential in malignancies and cancer. Still, more emphasis should be placed on HMG I/Y in the context of infectious diseases, specifically that of HSV-1 and its pathogenesis in sensory neurons.

TDP43 is a unique protein with multiple nucleic acid binding and modulating functions. In our study, ICP0 ubiquitinated TDP43, but we could not pursue a functional assay for TDP43 due to a lack of existing specific small molecular inhibitors. Additional studies to pursue TDP43's direct effect on HSV-1 beyond the correlation of protein profiles presented in this study would help to decipher TDP43's role in HSV-1 pathogenesis.

Study 2: Infected cell protein 0 (ICP0) modulates host cell homeostasis during latency in primary adult neurons

The study provided a unique insight into the mechanisms that occur during latency to permit HSV-1 to reactivate in adult sensory neurons. Unlike acute HSV-1 infection, where the virus has to become established in a naïve host cell, reactivation starts from latent HSV-1 genomes established with the host cell. The dogma of HSV-1 acute and latent pathogenesis is that lytic-associated proteins are absent when viral genomes are latent. During this study, we detected ICP0 at the protein level immediately post-reactivation stimulus, suggesting that ICP0 is present during latency in minuscule amounts.

To identify proteins ubiquitinated by ICP0, we infected neurons with HSV-1 KOS and HSV-1 n212, established latency for 7 days, and immunoprecipitated ubiquitinated proteins after a 2 hr reactivation. We analyzed the immunoprecipitated proteins of HSV-1 KOS and HSV-1 n212 infected neurons by LC-MS/MS. We identified β - catenin, a previously studied protein in our lab, and showed that it decreased in HSV-1-KOS infected neurons where ICP0 is functional but not in HSV-1 n212 where ICP0 is non-functional.

β - catenin had a consistent pattern of ubiquitination among HSV-1 KOS and HSV-1 n212 infected neurons, but an overall reduction of total β – catenin was specific to KOS-infected neurons. These results suggest β - catenin is repressed before reactivation, during the establishment of latency, in the presence of functional ICP0. We assessed the protein levels of ICP0 and β - catenin by immunoblot using protein samples collected during the 7 days allotted to establish latency in HSV-1 KOS and HSV-1 n212 infected neurons. We discovered an inverse relationship between ICP0 and β – catenin. These proteins have not been reported to interact directly, so we searched primary literature to identify a mechanism in which β – catenin could influence ICP0 and the establishment of latency. We expanded our 7-day latency assessment to include HMG I/Y, researched in Study #1, and ICP4, another HSV-1 immediate-early protein. We observed an inverse correlation with β – catenin, HMG I/Y, and ICP4 when compared to ICP0 during the establishment of latency. We hypothesized that β - catenin is intricately involved in establishing and maintaining HSV-1

latency in primary neurons by upregulation of cell survival genes to mitigate stress. HMG I/Y is a stress-induced gene upregulated by β – catenin but can also interact with ICP4 to function as a viral repressor. Utilizing a combination of our research data and published literature, we produced a hypothesized model depicting a molecular mechanism that determines if HSV-1, when infecting adult sensory neurons, will pursue a productive or latent infection.

This study revealed mechanisms that occur during the establishment of latency and reactivation of HSV-1 in sensory neurons. In opposition to the dogma of HSV-1 pathogenesis, HSV-1 lytic-associated proteins are present at varying amounts during latency, and their function is partially dictated by the presence of host proteins within the host cell. ICP0 and ICP4 are immediate-early proteins that can promote lytic infection but, in some instances, can promote latency, specifically when in combination with host proteins such as HMG I/Y and β - catenin. The preexisting state of a neuron that promotes HSV-1 latency is likely a complex interplay of these 4 proteins: ICP0, ICP4, HMG I/Y, and β – catenin. This alternative approach to latency suggests that it is an active process mediated by the virus. The conditions set forth during its establishment can potentially dictate if HSV-1 will reactivate when appropriate stimuli are encountered.

Future studies

Knowledge of β - catenin and ICP0 is limited in neurons, especially those of the peripheral ganglia. We hypothesize that β – catenin, ICP0, ICP4, and HMG I/Y may determine the viral outcome of infection in primary neurons. Further understanding of the interplay between these four proteins would help illuminate the viral mechanisms contributing to establishing and maintaining HSV-1 latency. Overall, the inverse relationship between ICP0 and β - catenin shows promise. Further exploring the interaction and interplay of β – catenin and HMG I/Y with HSV viral proteins could identify a targetable cascade that could be used to develop therapeutics that prevent reactivation of HSV-1 from neurons, the site of viral latency and persistence.

Contributions

The studies presented in this dissertation have challenged assumed notions and dogma of HSV-1 pathogenesis. We can explore the viral mechanisms in neuronal cells by utilizing more sensitive technologies and studying HSV-1 in primary adult neurons. Unlike the non-neuronal cells, HSV-1 can progress through a lytic infection or establish latency only in neurons, from which it can reactivate to cause recurrent disease symptoms. This is why we have specifically studied HSV-1 in primary adult sensory neurons. The perspectives of these experiments are unique, offering an alternative yet physiologically relevant view of HSV-1 in neurons. More emphasis should be placed on the neuronal aspects of HSV-1 to decipher the mechanisms that permit its persistence within a host.

Significant contributions and alternative perspectives of HSV-1 pathogenesis presented in this dissertation

1. During acute HSV-1 infection of adult sensory neurons, ICP0 modulates complex protein-protein interactions to promote an environment supportive of viral infection. As an E3 ubiquitin ligase, ICP0 can target host and viral proteins for degradation, alter their function, or reduce their protein amount within the cell.
2. Outline some of the dynamics of HSV-1 infection in adult sensory neurons, exemplifying that a combination of "host anti-viral", "viral anti-host", and "viral anti-viral" mechanisms contribute to HSV-1 pathogenesis.
3. HSV-1 lytic-associated proteins are present at varying amounts during latency. Contrary to the dogma of HSV-1 pathogenesis, HSV-1 latency is most likely an active process mediated by the virus but influenced by the host cell.
4. A new hypothesized model of the latent-lytic switch of HSV-1 involving β-catenin, HMG I/Y, ICP0, and ICP4
5. Incremental protein profile of ICP0 during the initial onset of productive infection (24hrs), reactivation from latency (32 hrs), and daily analysis of the establishment of latency (7 days) in primary adult sensory neurons.
6. Protocol for isolating and enriching ubiquitinated proteins from primary adult sensory neurons.

7. LC-MS/MS datasets for the identification of ICP0 ubiquitinated protein comparing, HSV-1 KOS, and HSV-1 n212 (Acute productive infection – 8hrs, Immediate reactivation – 2hrs)